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MESENCHYMAL STROMAL CELL: NEW APPLICATIONS FOR REGENERATIVE MEDICINE

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INTRODUCTION

1. Regenerative medicine

Bodily tissues are subjected daily to various injuries; however, each tissue has its own intrinsic ability to access and repair said damages. In instances where damages exceed the tissue's ability to repair said damages, scar tissue begins to form, an excess of which can compromise tissue function. Due to medical progress made within the last fifty years, public demand for innovative treatment has increased and patients now expect to understand the physical conditions that were present at the onset of the disease, in addition to the knowledge of the medical problem at stake. As a result, there is currently a large amount of public interest which exceeds solely the knowledge of how to promote tissue repair and prevent scar tissue formation. In the last two decades a new discipline called regenerative medicine has emerged. The objective of regenerative medicine is to develop products which can restore tissue function and prevent scar tissue formation.

Musculoskeletal tissues range from bone, with an elevated ability to regenerate, to cartilage, which has a limited ability to self-repair. In ninety nine percent of all simple fractures which occur in human subjects, the damaged bone will self-generate to exactly its original state in matter of months, given that mechanical stability is provided through a rigid cast. On the other hand, damaged cartilage will not heal fully and the formation of new fibrous cartilage in the damaged tissue will be observed – even if the subject is provided with all of the most advanced therapeutic approaches designed to aid cartilage in its self-regeneration (Huey, *et al.,* 2012). The reason such a different clinical outcome is observed in human cartilage is most likely due to the peculiar anatomy of each of these tissues. While bone is a highly vascularized tissue which undergoes constant remodeling, cartilage is the only avascular tissue in the human body and as a result has a quite limited capacity for remodeling through self-generation.

Although bone has a large capacity for self–regeneration, there are certain defects in bone, known as critical-size defects, which are difficult to repair. Critical-size defects are generally caused by trauma, bone diseases, prosthetic implants, or tumor excision. There are also clinical conditions, such as diabetes, age, osteoporosis, and vascular

necrosis which cause bone's capacity for self-regeneration to be compromised. In all these circumstances the endogenous process of self-regeneration is not adequate for tissue repair and it necessary to intervene clinically. Currently, the highest standard in the treatment of bone defects lies in orthopedic surgery which consists of the transplantation of autologous bone grafts which are used as osteogenic substitutes. The main disadvantage associated with this technique is the limited availability of both the autologous material and the number of acceptable cadavers from which said material can be harvested.

In an attempt to solve these problems, the scientific community has been focused on developing bone allograft substitutes through the development of new bio-compatible materials which improve cell invasion, resulting in the formation of new tissue. This method is highly desirable due to the fact that it avoids the use of autologous bone grafts.

Within the last ten years, there has been a dramatic increase in the number of publications which are concerned with the production of new biomaterials and with their applications in clinical surgery. These biomaterials are made using either synthetic or natural polymers which are polymerized *in vitro* in 3D structure (defined as scaffold), a structure which mimics normal human tissue structures. The main advantage obtained through the use of these biomaterials is the fact that they can be produced in large amounts; however, their drawback is that they lack the ability to induce specific cell differentiation. In order to overcome this limitation and to improve cell induction, an attempt to combine the use of biomaterials with molecules such as recombinant growth factors (bone morphogenic proteins) or differentiation factors (which induce cells to differentiate into specific lineages) was carried out by several researchers. However, elsewhere in scientific literature it has been reported that *in vivo* these factors may aid cancer development (Carragee, *et al.,* 2011) and/or neurotoxicity (Smith *et al.*, 2008).

Within the last twenty years, tissue engineering has emerged as an intriguing alternative in the field of tissue regeneration. Tissue engineering combines biomaterials and mesenchymal stromal cells (MSC) to obtain a graft *in vitro* which can improve tissue regeneration (Manfrini *et al.,* 2012). One advantage in tissue engineering is the possibility to use autologous cells, a strategy which avoids both possible tissue rejection and negative immune response. However, the drawbacks of said tissue engineering are:

1) the existence of low MSC numbers found in the bone marrow generally requires an additional laboratory step - namely of cell expansion in order to obtain a sufficient cell number and 2) said cell expansion is financially costly and in addition it has been reported that cell expansion *in vitro* can reduce the osteogenic capacity of the MSC *(*Banfi *et al.*, 2000; Jakob *et al.,* 2012). A possible approach which may ameliorate these problems would be to eliminate the cell expansion step described in step one above and to reduce the process to a single surgical procedure in which cells are isolated and concentrated in the same surgical session in which they are implanted.

Once the tissue with the highest number of progenitor cells has been identified, it is possible to surgically harvest said tissue and, using specific instruments, to concentrate cells during the same surgical procedure and to inject the product into the tissue defect or onto a pre-made scaffold (Di Bella *et al.,* 2010; Mehrkens *et al.,* 2012). This innovation could simplify the use of MSC in clinical application, thus eliminating the high costs associated with cell expansion.

In the past, several different clinical trials have been carried out in order to attempt to clarify the most beneficial use of MSC and to better understand their contribution to the field of tissue regeneration. The results obtained from said trials have been contradictory – therefore additional data results must be collected in order to confirm the beneficial effects of using MSC in the quest for full bone regeneration. If data which has been collected up until this point in time can be confirmed, a breakthrough may be on the horizon for the improvement of overall patient quality of life.

2. BONE

2.1 The anatomy

Excluding the sesamoids, the adult human skeleton contains 213 bones. Bone is a metabolically active connective tissue which is highly vascularized. By functioning as levers to which muscles attach, bone provides structural support as well as facilitation of movement in the human body. Bone, as an organ, is also an important factor in maintaining mineral homeostasis, acid-base balance, the reservoir of growth factor, cytokines and minerals, and the protection of vital organs. In addition, bone is also a site for hematopoiesis (Buck and Dumanian 2012a).

The adult human skeleton is composed 80% cortical bone and 20% trabecular bone; however, each bone has a different ratio of trabecular to cortical bone. The outer part of each bone, which is composed of a relatively dense mass with a low porosity (max 30%), is defined as **cortical bone.** The internal part of each bone is called **cancellous** (or **trabecular**) **bone** and is characterized by a higher porosity (30-90%) and consists of a network of trabecular plates and rods which contain blood vessels and bone marrow. A fundamental characteristic of cancellous bone is a higher rate of metabolic activity and a higher capacity for bone remodeling as compared to cortical bone. Moreover, cortical

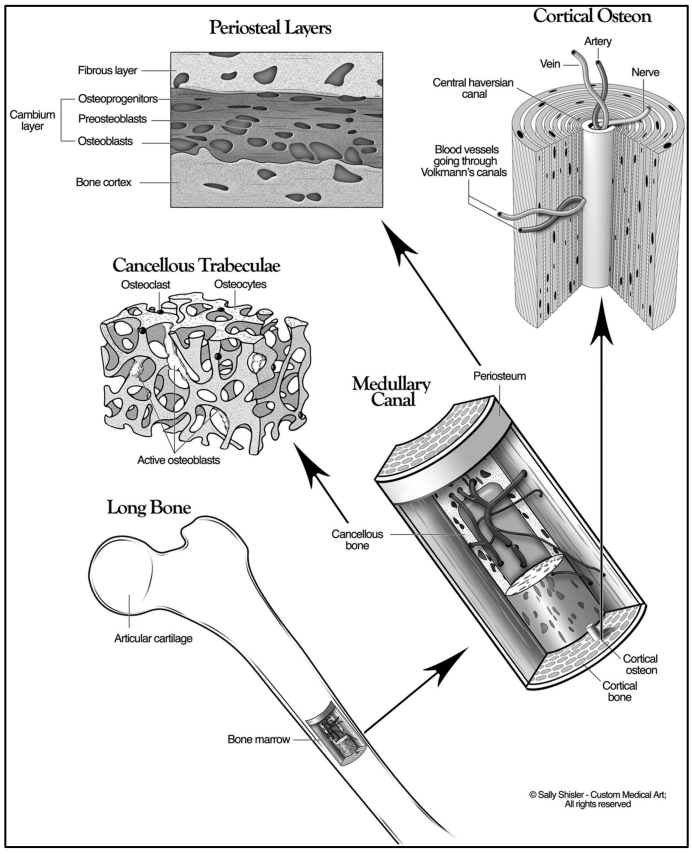

Figure 2.1 A representative image of bone structure (Buck and Dumanian 2012a)

bone response is higher after mechanical stimuli as compared to that of trabecular bone due to the fact that primary bone cells lie on the surface of bone and are in closer proximity to circulating growth factors and cytokines.

Both cortical and trabecular bone is composed of osteons. In the cortical bone these osteons are called Harversian systems, while in cancellous bone their name is packets. Harversian systems and packets differ in both shape and size.

Histologically, bone can be classified as either woven or lamellar. Primary/woven bone is found in the human embryonic skeleton and is characterized by a disorganized and irregular pattern of collagen fibrils and mineralization. Lamellar bone has a highly organized structure formed by tightly packed collagen fibrils organized into sheets with high mineralization and uniform distribution of osteocytes and bone matrix. This structure makes lamellar bone rigid and strong. During its growth, and in the later stage of fracture healing, lamellar bone completely replaces woven bone.

Externally, bone is covered by **periostium,** a fibrous connective tissue sheath that surrounds the outer cortical surface of bone. The only exception occurs at joints, which contain blood vessels, nerve fibers, osteoblasts, and osteoclasts. The periostium is tightly attached to the outer cortical surface of bone by thick collagenous fibers called Sharpey's fibers, which extend into underlying bone tissue. The **endosteum** is a membranous structure covering the inner surfaces of cortical bone, trabecular bone and blood vessel canals (Volkmann's canals). The endosteum is in contact with the bone marrow space, trabecular bone, and blood vessel canals and contains blood vessels, osteoblasts, and osteoclasts.

As previously mentioned, bone is vascularized by a complex network of inter-osseous canals and sinusoids. Tubular bones, or long bone, have a dual blood supply, the predominant of which is obtained via nutrient diaphyseal arteries that often enter the middle third of the diaphysis. Successively, they enter the medullary canals and divide into two branches. The second source of blood supply to tubular bones is obtained through a network of smaller vessels that supply the adjacent joint with blood (Buck and Dumanian 2012b).

Normal bone develops via two mechanisms: the first of which is called **intramembranous bone formation** and is mediated by the inner periosteal osteogenic layer of bone and is initially synthesized without the mediation of a cartilage phase. The

second mechanism, called **endochondral bone formation,** establishes the synthesis of bone on a mineralized cartilage scaffold after epiphyseal and physeal cartilage have shaped and elongated the developing organ. With intramembranous bone repair, mesenchymal cells differentiate along a pre- osteoblast to osteoblast line while endochondral bone formation is characterized by the initial synthesis of cartilage followed by the endochondral sequence of bone formation (Shapiro 2008). Deeper endochondral ossification is initiated by the proliferation of chondrocytes. As the chondrocytes proliferate (within the proliferative zone), they become hypertrophic and release alkaline phosphatase, which eventually results in chondrocytes apoptosis and the release of angiogenic factors which function as vascular endothelial growth factor. The zone of dead chondrocytes (calcification zone) creates a barren matrix which promotes capillary in-growth and migration of osteoprogenitor cells that then differentiate in osteoblasts and produce bone (Buck and Dumanian 2012a).

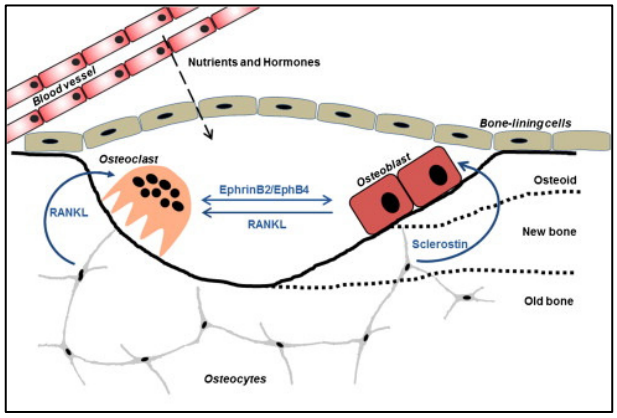

Figure.2.2 The BMU unit (Kular *et al.*, 2012)

The formation of new bone is essential during both childhood and adolescence; however, bone continues to be modeled and remodeled for the entirety of adult life which results in the gradual yet fundamental adjustment of the human skeleton to the forces that it encounters.

In order to maintain mineral homeostasis in the human body, bone is exposed to remodeling that begins before the birth and continues until death. Bone remodeling occurs through the concerted action of cells termed the basic multicellular unit (BMU). The BMU consists of osteoclasts which reabsorb bone into the blood stream, osteoblasts which form new bone, the osteocytes within the bone matrix, and the bone lining cells which cover the bone surface and the capillary blood supply (Figure. 2.2) (Kular *et al.,* 2012).

During the remodeling process old bone is replaced by new bone. Bone remodeling is essential for it avoids the accumulation of bone micro-damage. In the activation

phase, osteoclast precursors are recruited and once in place they undergo differentiation and mature. Once osteoclasts are activated they initiate bone matrix reabsorption by releasing vesicles containing lysosomal proteases and cathepsin K, which are responsible for the digestion of collagen I. Digested bone matrix proteins are then internalized by endocytosis, pass through osteoclast cytoplasm and are released into the extracellular space where they enter the blood stream (Boyce *et al.*, 2012). The activation phase last around two to four weeks in total, immediately after which there is a shift between the prominent osteoclast and osteoblast activity. This phase, called the reversal phase, is lengthy and last four to six months. During the reversal phase the osteoblasts, which are responsible for bone formation, secrete vesicles which accumulate both calcium and phosphate. These cells are then entrapped in the newly formed matrix and may have one of three different fates: they either undergo apoptosis, differentiate further into osteocytes and begin to form an extensive canalicular network which is essential for communication between cells in the bone, or become quiescent lining cells (Clarke 2008; Kular *et al.,* 2012).

Within the reversal phase, the balance between osteoclast and osteoblast activity is fundamental for the correct maintenance of bone structure. In fact, an increase in osteoclastic activity would result in weakened bones and would put the subject at an increased risk for bone fractures. On the contrary, if osteoclastic activity is drastically reduced, bones will become extremely dense, a condition which is defined as osteopetrosis.

2.2 Bone composition

As previously described, bone tissue is constituted by different cell type that they conduct a specific role in the physiology of the organ.

2.2.1 Osteoclasts

Osteoclasts are the cells responsible for the reabsorption of old bone.

Osteoclasts are formed from the fusion of mononuclear progenitors of the monocytes/macrophage family during osteoclastogenesis. Osteoclast cells are usually located close to endosteal surfaces within the Harvesian system as well as on the

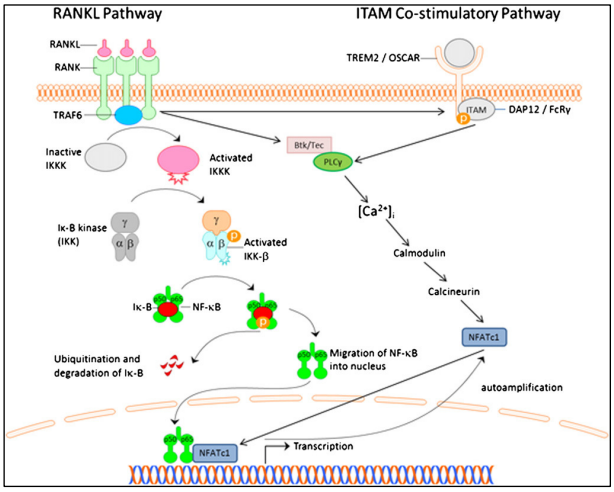

Figure.2.3. A schematic picture of the signaling pathway induced during the osteoclast differentiation

periosteal surface beneath the periosteum. Osteoclasts are rare cells in human bone and normally only two to three cells are present per 1 μm^3 of bone surface.

The event most essential to cell differentiation activation is the production of cytokines and growth factors by marrow stromal cells, osteoblasts, or T- lymphocytes.

In order for differentiation to begin, two factors are necessary: macrophage colony-stimulating factor (M-CSF) and the receptor responsible for the activation of the ligand nuclear factor kappa B (NF-kB) RANKL.

M-CSF is produced by both osteoblast and stromal cells. It functions by binding to its receptor, namely c-fms. The interaction between receptor c-fms and M-CSF is necessary for the maintenance of cell proliferation, macrophage maturation, as well as the survival and differentiation of osteoclast precursors.

The ligand RANKL, produced by osteoclasts, T cells, and endothelial cells is essential for osteoclast formation. It functions by binding to its receptor RANK which is present on the membrane of osteoclasts and their precursors and induces the trimerization of both molecules (Figure 2.3) (T. L. Burgess *et al.,* 1999; Lacey *et al.,* 1998). RANKL activity can be inhibited by the presence of soluble Osteoprogerin (OPG) which sequesters RANKL and prevents osteoclast differentiation (Mizuno *et al.,* 1998). Although the binding of RANKL has been shown to be the essential signal for osteoclast differentiation, co-stimulatory pathways are also required for this process. NFAT (nuclear factor of activated T cells) and CaMK (calmodulin kinase) are both examples of said co-simulatory pathways.

Once differentiated, osteoclasts interact with bone matrix via integrin receptors. Even if the integrins β1 subunit binds collagen, laminin and fibronectin, the main integrins subunit involved in resorbing bone is the dimer $\alpha_v\beta_3$ which binds osteopontin and sialoprotein, both of which are found in fully-formed human bone.

The subsequent step to this interaction is the polarization of the osteoclast membrane and the release of vesicles containing H^+ and Cl^- ions as well as many enzymes necessary for bone digestion. The H^+ ions acidify and dissolve the mineral component of bone matrix. Once osteoclasts have completed their work they undergo apoptosis within reabsorption lacunae. The exactly molecular signaling which causes this apoptosis is still unknown, but it has been shown that a high extracellular percentage of Ca^+ ions can initiate said molecular pathway.

2.2.2 Osteoblasts

Osteoblasts are the cells responsible for the deposition and formation of new bone. As described previously, osteoblasts are recruited in a second phase by factors secreted by osteoclasts.

Within bone marrow there is also a small percentage of stromal cells (known as mesenchymal stromal cell) that have the capacity to differentiate into different lineages such as bone, cartilage and fat. The commitment of mesenchymal stromal cells to the osteoblast lineage requires the activation of the Wnt-canonical pathway and but the TGF-β super-family also plays an important role in osteoblast differentiation. In fact, TGF-β is essential for the commitment to the osteogenic lineage by way of activation of Smad2/3 signaling such as the BMP pathway.

Mesenchymal stromal cell have a fibroblastic-like shape but once differentiated into osteoblasts they acquire a cuboidal phenotype and start to release alkaline phosphatase. One can recognize functionally active osteoblasts due to their large nuclei, enhanced Golgi structures, and extensive endoplasmic reticulum. These cells are involved in the secretion of type I collagen as well as other matrix proteins, all of which are fundamental for new bone formation. Once activated, osteoblastic cells can remain quiescent osteoblasts lining cells or can become osteocytes. Lining cells form the endosteum on both trabecular and endosteal surfaces and underlie the periosteum on the mineralized surface (Clarke 2008).

Osteoblastic cells express different gene repertoires which can be explained by the heterogeneity of trabecular micro-architecture at different skeletal sites, anatomic site-specific differences in disease states, and regional variation in the ability of osteoblasts to respond to agents used to treat bone disease.

2.2.3 Bone marrow

Bone marrow (Figure. 2.4) is located within the central cavities of both axial and long bones. It consists of hematopoietic tissue islands and adipose cells surrounded by vascular sinuses interspersed within a meshwork of trabecular bone. In addition, bone marrow accounts for approximately 5% of human body weight (Travlos 2006).

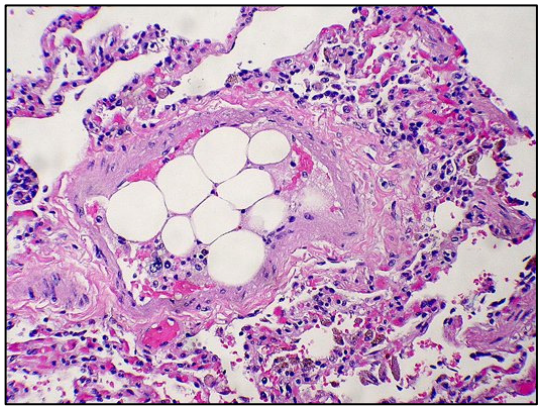

Figure. 2.4 A representation of bone marrow tissue.

Bone marrow is a major hematopoietic organ, a primary lymphoid tissue, and is responsible for the production of erythrocytes, granulocytes, monocytes, lymphocytes, and platelets.

Bone marrow is vascularized by a dense network of capillaries which transport nutrients into the center of the bone marrow. The hematopoietic tissue consists of a variety of cell types which include blood cells and their precursors, adventitial/ barrier cells, adipocytes, and macrophages. These cells have a particular and specific organization inside the hemapoietic tissue (Weiss and Geduldig 1991).

In order for hematopoiesis to occur, a supporting microenvironment must exist and said microenvironment must be able to recognize and retain hematopoietic stem cells and provide the factors (e.g., cytokines) required to support proliferation, differentiation, and maturation of stem cells along committed lineages. The hematopoietic microenvironment consists of adventitial reticular cells (e.g., barrier cells), endothelial cells, macrophages, adipocytes, and bone lining cells (e.g., osteoblasts) and elements of the extracellular matrix (Travlos 2006).

All these factors influence hemopoiesis and are equally important for the correct hemopoietic process to occur.

2.2.4 Bone matrix

Bone matrix constitutes 90 percent of overall human bone volume. There are four main components which make up the bone matrix, namely: inorganic and organic matrix (which are the two principal components of bone matrix), lipids, and water. The composition of bone matrix is fundamental for the maintenance of mineral homeostasis

(calcium, phosphorous, magnesium and calcium) within the human body. The inorganic matrix is mainly made up by hydroxyapatite and is responsible for bone stiffness and its resistance to compressive forces. The organic part of the bone matrix is composed mainly by type I Collagen and is secreted by fully differentiated osteoblasts, but also by type III, V and FACIT Collagen (type IX; XII, XIV, XIX, XX and XXI are included in this Collagen family). The organic part of the bone matrix is important for the stability of extracellular matrices. Traces of non-collagenous proteins such as serum albumin can be found in bone matrix, which help in mineralization and the α2-HS-glycoprotein, both of which are involved in cell proliferation. In addition, the proteins inteleukin-1 and 6, osteocalcin osteonectin, bone sialoprotein (BSP) and bone morphogenetic proteins (BMPs) are also all contained within the organic matrix. All these components are important for maintenance of a healthy human skeletal apparatus.

Bone represents one of the most metabolically active connective tissues in the human body. It is a complex organ and the balance and communication between the activity of all cells is fundamental. The sequential phases of osteoblast commitment and differentiation are regulated by a variety of complex activities, including hormones, growth factors, and mechanical stimuli. A deeper knowledge of bone biology is necessary in order to optimize the research connected to bone regenerative medicine.

3. Mesenchymal Stromal/Stem Cell (MSC)

3.1 A brief introduction

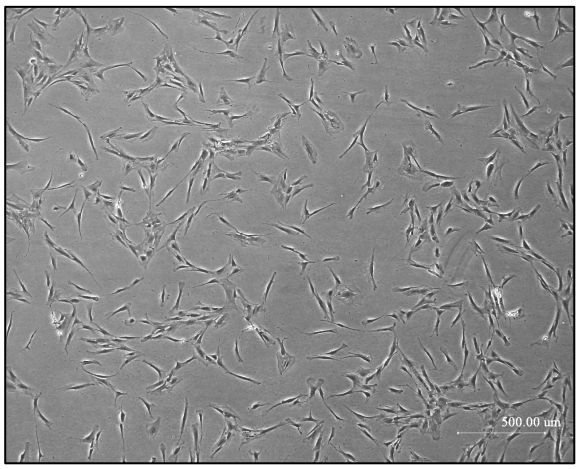

Figure 3.1 A representative picture of bone marrow derived MSC in culture.

The first evidence of the presence of non-hemapoietic stem cells in bone marrow was reported by Cohnheim in 1867 (Cohnheim 1867).

In the late 1960's Friedenstein and coworkers isolated and cultured for the first time this cell type from bone marrow (Friedenstein, *et al,* 1966). These cells (Figure. 3.1) were able to form colonies derived from single cells and after a few days these adherent cells of heterogeneous appearance, began to proliferate and to differentiate into mature cells of mesenchymal lineages such as osteoblast (A. Friedenstein, *et al.,* 1970; Friedenstein 1976). The initial clones of adherent cells expanded into round-shaped colonies composed of fibroblastoid cells, thus the term 'Colony Forming Unit - fibroblasts' (CFU-f) was coined (Augello, *et al,* 2010).

Successively, MSC are found in different adult tissues. In 2001, Zuk published the isolation of MSC from adipose tissue from a population of cells with biological characteristic similar to bone marrow derived MSC (Zuk *et al.*, 2001). In the last ten years, MSC have been identified in tissues including peripheral blood (Zvaifler *et al.,* 2000), cord blood (Erices, *et al.,* 2000), menstrual blood, amniotic fluid (in't Anker *et al.,* 2003), synovial membrane (De Bari *et al.,* 2003), and placenta (Koo *et al.,* 2012)

Mesenchymal stromal cell are not a homogenous population but rather are, most likely, heterogeous cell cultures which are comprised of a varying amount of committed cells. To address the inconsistency between the nomenclature and biological properties of this heterogeneous population, the International Society for Cellular therapy has suggested that these plastic adherent cells, regardless of the tissue from which they are

isolated, be termed 'multipotent mesenchymal stromal cell', and that the term 'mesenchymal stem cell' should be used only for the subset that meets specified stem cell criteria (Horwitz *et al.,* 2005; Baer and Geiger 2012).

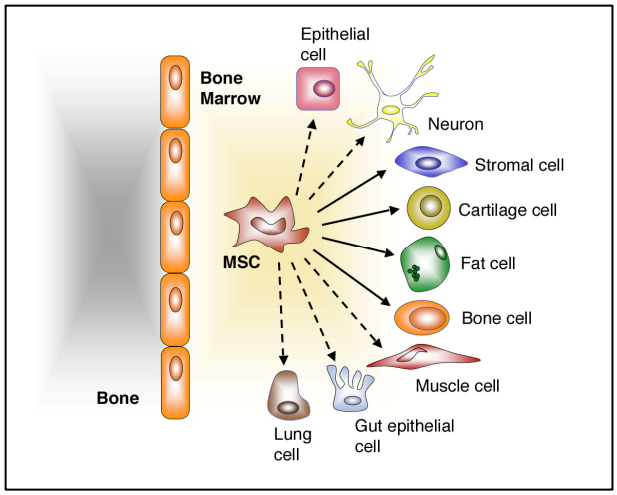

Figure 3.2 Flowchart elucidating possible commitment of mesenchymal stromal cell.

Mesenchymal stromal cell are interesting cells because, when induced, they are able to differentiate into many different lineages (Figure 3.2). The ability of MSC to differentiate in mesodermal lineages, such as the osteogenic, adipogenic, and chondrogenic lineages, was discovered early in the research process; however, new evidence reveals that these cells are even able to differentiate into cardiac (Gnecchi, *et al.,* 2012; Makino *et al.,* 1999), neurogenic (Bae *et al.*, 2011; Montzka *et al*, 2009), and epidermal (Paunescu *et al,* 2007) lineages. These results are intriguing due to their possible application in regenerative medicine and tissue engineering which could improve the natural process of healing of tissues.

3.2 Mesenchymal stromal cell characterization

To classify MSC, The Mesenchymal And Tissue Stem Cell Committee Of The International Society For Cellular Therapy proposed, in 2006, four criteria for the identification of human mesenchymal stem cell. There criteria are: 1) plastic-adherence when maintained under standard culture conditions; 2) ability for osteogenic, adipogenic and chondrogenic differentiation; 3) expression of CD73, CD90 and CD105 and 4) lack of expression of hemapoietic markers (CD14, CD11b, CD34, CD45, CD19 and CD79) (Dominici *et al.*, 2006;Witkowska-Zimny and Walenko 2011).

As of today, even the most focused research has yet to discover the key marker for the identification of mesenchymal stromal cell. The discovery of this marker could reduce problems linked to the isolation and identification of MSC because all of the markers described above are also expressed by other cell populations within the human body.

3.3 Mesenchymal stromal cell differentiation

As previously described, mesenchymal stromal cell have the ability to differentiate into several mesodermal lineages.

When induced into osteogenic differentiation (using dexamethasone, β-glycerophosphate and ascorbic acid), MSC begin to express genes and proteins associated with the osteoblastic phenotypic as such as Collagen I, osteopontin, osteocalcin, Runx2 and BSP , and acquire a morphology comparable to osteoblasts (round cells with large nuclei, enhanced Golgi structures and an extensive endoplasmic reticulum). Moreover, they begin to deposit extracellular matrix enriched with deposits of hydroxyl-epatite, which is characteristic of bone tissue. The evaluation of differentiation can be done using alizarin red S (Figure. 3.3 on the left) which stains

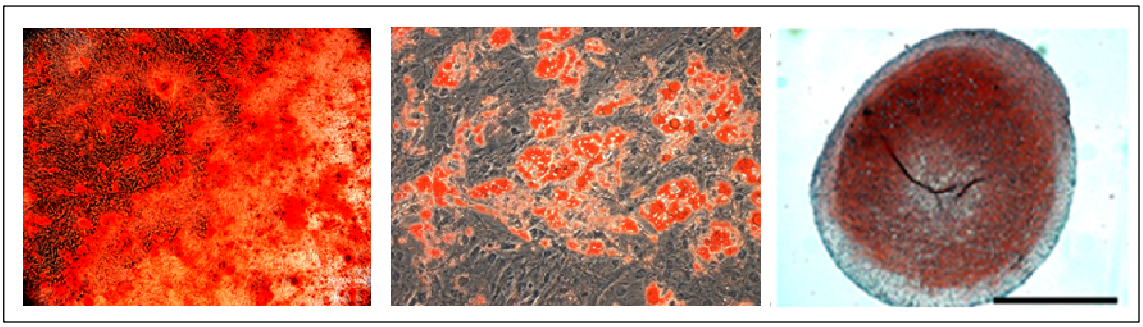

Figure.3.3 On the left. Representative micrographs of hMSC after osteogenic differentiation stained with Alizarin Red solution. On the center. hMSC after adipogenic differentiation. The lipid droplets are stained with OIL red O solution. On the right. hMSC after chondrogenic differentiation. Sections are stained with safranin O solution (Deehan *et al*., 2012).

calcium deposits present in the extracellular matrix or by evaluating alkaline phosphatase activity in cells post-differentiation. Mesenchymal stromal cells are able to differentiate into an osteogenic lineage not only *in vitro* but also *in vivo*. Moreover, MSC can adhere to and proliferate on specific scaffolds and begin to differentiate. The combined use of MSC and scaffold have clinical applications in the repair of bone tissue defect which results following a trauma to the body or a cancer excision.

In the last couple of years there has been a growth in the level of interest in reconstructive surgery, especially with regard to the application of soft tissue repair. In this field the ability of MSC to differentiate into adipogenic lineages has great relevance. Adipogenic differentiation is induced by insulin, isobutylmethylxanthine, dexamethasone and indomethacin. However, current literature suggests that there is a clear lack of standardization of said induction (Scott *et al.,* 2011). MSC induced into

adipogenic differentiation begin to express genes such as PPARγ, GLUT4, C/EBPβ and GPDH. Morphologically, it is possible to observe the presence of lipid vacuoles in differentiated cells which can meld to one other (Figure. 3.3 on the center).

One of the reasons why there is great interest surrounding MSC is due to their application in the regeneration of cartilage, the tissue with the lowest ability to self-regenerate in the human body. Current literature suggests that MSC can differentiate into chondrogenic lineages if cultured using a pellet system and in a medium of a serum un-supplemented with dexamethasone, TGF-β1 and ascorbic acid. Under these conditions, MSC begin to produce an extracellular matrix formed by collagen X and test positive during Safranin O staining (Figure. 3.3 on the right), Alcian Blue and Toluinine Blue.

In addition to their ability to differentiate into mesodermal lineages, MSC have been shown by recent literature to contain the ability to differentiate into both neurogenic (Cardozo, *et al.,* 2012; Safford K. M. *et al.,* 2002) and cardiogenic (Carvalho *et al.,* 2012) lineages. Overall, these results are able to improve the main application of mesenchymal stromal cell, namely their role within the field of tissue engineering.

3.4 Adipose derived stromal cell

Adipose tissue in the human body is involved in energy homeostasis since it acts as a large scale endocrine organ capable of secreting a wide variety of cytokines, chemokines, and adipokines. These molecules are involved in several different biological processes such as inflammation, immunity, metabolism (Alexander 2012). In 2001, the adipose tissue was recognized not only as energy reservoir but also as a rich source of multipotent stem cells. Zuk and coworkers were the first to identify multipotent stromal cells in adipose tissue (Zuk *et al.,* 2001).

Stromal Vascular Fraction (SVF) is a mixture of cells such as: MSC, HSC, Treg cells, pericyte-EC, mast cells, dendritic cells, and fibroblasts (Tang *et al.*, 2008), all of which are isolated using enzymatic dissociation of adipose tissue and several different centrifugation steps in order to remove differentiated adipocytes.

In the past, much research has been focused solely on the use of adipose derived stromal cell (ASC) (Katz *et al.,* 2005; Hiroshi *et al.,* 2012) due to the advantages which exist in the use of adipose derived stromal cells (Ad-MSC or ASC) as opposed to bone

marrow derived mesenchymal stromal cells (BM-MSC). One of these advantages is that lipoaspirate is readily available as a discarded by-product of liposuction procedures. Compared to that of bone marrow, the harvest of lipoaspirate is simpler and less invasive. Moreover, the volume of lipoaspirate which can be harvested is significant higher than that of bone marrow and the frequency of mesenchymal stromal cell in liposuction is larger by 2500 fold - an essential pre-requisite for stem cell based therapies (Baer and Geiger 2012b; Fraser *et al.*, 2008; Mizuno *et al.,* 2012; Yarak 2010; Gimble *et al.,* 2011). In 2001, Gronthos found that BM- and Ad-MSC cultures are very similar with regard to properties such as proliferation, clonogenicity, and expression of the stromal markers CD105, CD90 and CD166 (Gronthos *et al.,* 2001). However, recently Dmitrieva and coworkers observed that these two cultures differ in cytokine secretion prolife. In fact, ASC secrete more VEGF, SDF1, MCP1, and TGFβ1. Dmitrieva et al also noted that during *in vitro* expansion, BM-MSC were detected during the early onset of senescence while ASC were not expressed at the same point (Dmitrieva *et al.,* 2012).

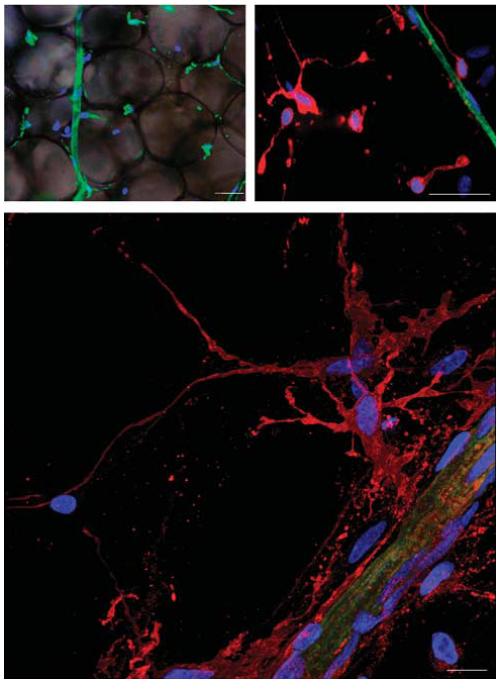

Figure. 3.4 IF ASC localication in adipose tissue. (Green lectin; CD34: CD34) (Maumus *et al.,* 2011).

Despite the large interest in and dedication to adipose derived stromal cells, the *in vivo* localization of these cells is still not fully clarified. It was not until 2009 that Da Silva Meirelles observed, using an equine model, that the frequency of ASC in adipose tissue is proportional to blood vessel densities (Da Silva Meirelles *et al.,* 2009). Recently, Maumus observed that ASC *in vivo* express sialomucin CD34 yet lack this expression during *in vitro* expansion (Braun *et al.,* 2012; Maumus *et al.,* 2011). Immunofluorescence of CD34 within a section of human adipose tissue showed that CD34/NG2 positive cells are located close to blood vessels. The results of Maumus et al were confirmed by Zimmerlin and coworkers (Maumus *et al.*, 2011; Zimmerlin *et al.,* 2013).

3.5 Mesenchymal stromal cell niche

In 1978, Schofield proposed the niche concept to describe the physiological microenvironment that support stem cells *in vivo* (Shofield 1978). Many co-culture experiments have supported Shofield's hypothesis which was the starting point for an area of new research. A stem cell niche is defined as the microenvironment in which the adult stem cells resides and also includes the surrounding cell (Figure. 3.5). Both low oxygen content and growth factor gradients provide signals which allow for the maintenance of quiescent stem cells and for the modulation of their activation (Kaewsuwan *et al.,* 2012). The mesenchymal stromal cell niche has been identified in several different adult tissues as such as intestinal, neural and epidermal tissue (Kolf, *et al.,* 2007). This niche is able to maintain stem cells primarily in the quiescent, un-differentiated state but can also cause the stem stems to become committed to a certain lineage. The difference between the un-differentiated and the committed state is the result of a delicate balance between self-renewal and differentiation (Nwajei and Konopleva 2013).

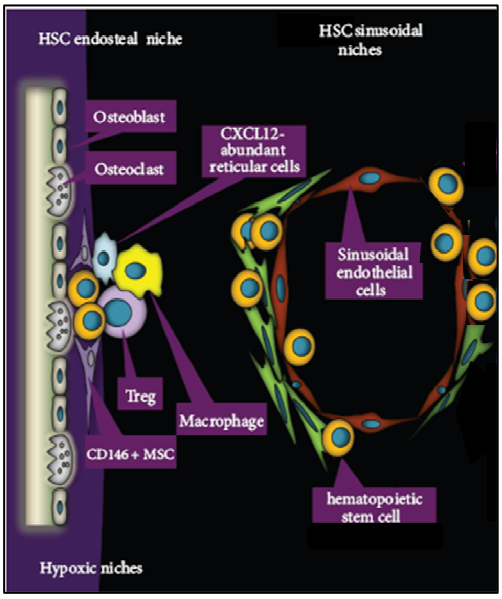

Figure. 3.5 Cartoon depicting bone niche. Adapted from Nwajei and Konopleva 2013.

The regulation of the fate of a stem cell with the mesenchymal stromal cell niche is the result of the combination of both intrinsic programs and external signals. The different cells which are included within bone marrow niche secrete many different molecules into the extracellular space. Some of said molecules include sonic hedgehog, Wingless proteins, bone morphogenic proteins, fibroblastic growth factors and Notch which orchestrates the appropriate fate of all stem cells and their respective lineage commitments.

The data obtained from the study of the composition and the regulation of the mesenchymal stromal cell niche *in vivo* is an important step in improving the system of culturing adult stem cells in general and, more specifically, in culturing mesenchymal stromal cell. The knowledge of the molecular mechanisms involved in the processes

regarding the preservation of stem cells could be applied during the expansion *in vitro* of the culture of MSC culture for future clinical applications.

4. CD34

The CD34 family is constituted by cell-surface transmembrane proteins and consists of CD34, podocalixin, and endoglycan.

Although the biological function of CD34 has not yet been fully clarified, several roles have been attributed to the proteins of CD34 family. CD34 is widely accepted as marker for the identification and isolation of hemapoietic stem cells (HSCs) even though it was proposed as a promoter of the proliferation involved in the blocking of cell differentiation and the trafficking and migration of HSCs (Nielsen and McNagny 2008; Scherberich, *et al.,* 2013).

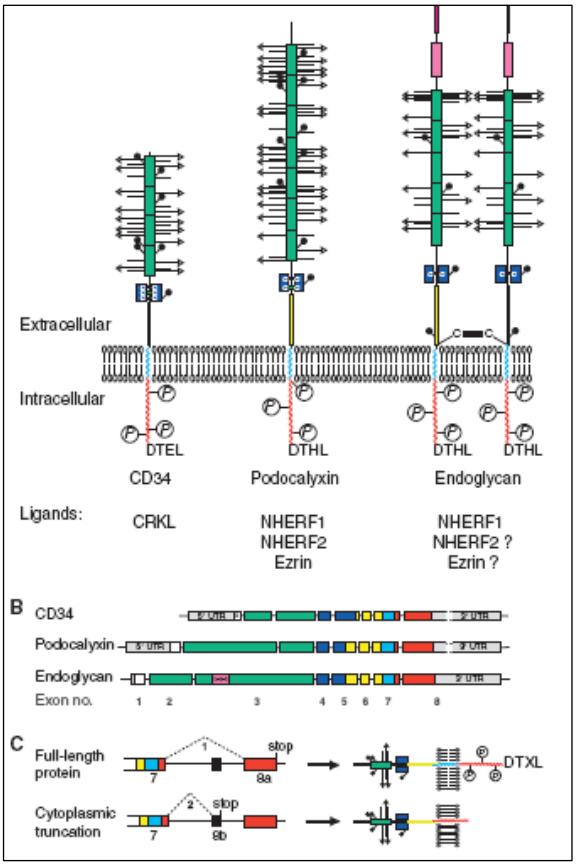

Figure. 4.1. In the panel are represented the CD34 family members. (Nielsen 2008)

The components of the CD34 family are not expressed ubiquitously in all tissues but rather certain components are expressed in certain tissues. In fact, while CD34 is expressed in vascular progenitor cells and podocalyxin, it is first and foremost defined as a marker of kidney glomerular epithelial cells.

Genomic studies have claimed that the genes which code for the three proteins CD34, podocalyxin, and endoglycan are evolutionarily related.

Each protein is encoded by eight exons, and, across the family, individual exons code for equivalent protein motifs which are consistent in length (Nielsen and McNagny 2008). Another similarity between these three proteins is the intronic distances and the alternative splicing found in each which generates a protein lacking much of the cytoplasmatic tail.

Distribution	CD34	Podocalyxin	Endoglycan
Multipotent precursors	+	+	+
T cells	–	–	+ (thymocytes)
B cells	–	–	+ (TLR activated)
Mast cells	+	–	–
Eosinophils	+	–	–
Macrophages	–	–	–
Erythrocytes	–	+ (anemic)	–
Platelets	–	+	–
Muscle satellite cells	+	–	–
Hair follicle stem cells	+	–	–
Vascular endothelia	+	+	+
Smooth muscle	–	–	+
Podocytes	–	+	±
Neurons	±	+	+ (30%)
Fibrocytes	+	–	–
Mesothelia	–	+	–

Table 4.1. expression of CD34 family member in the different human tissues.
± weak expression; + expressed and – not expressed.

4.1 The structure

As described in Figure. 4.1, the extracellular domain of CD34, podocalyxin and endoglycan includes both a serine-, threonine- and proline-region that is extensively O-glycosylated and sialylated, a cysteine-bonded globular domain as well as a juxtamembrane stalk in the extracellular domain. This high level of modification influences the molecular weight of the protein which is is estimated to fall between 90-170 KDa.

The transmembrane domain consists of a single helix which is highly conserved between the three proteins as is the cytoplasmic domain which contains phosphorilation sites and a PSD- 95- Dlg- ZO-1 (PDZ)-binding domain.

Even if their domain structures seem quite similar to one another, CD34, podocalyxin and endoglycan differ in several notable respects. First of all, the extracellular domain is quite different in length amongst the three proteins. For example, endoglycan has a couple of cysteine residues in justamembrane domain which are most likely involved in homodimerization. CD34 and podocalyxin both lack these cysteine residues.

Cristallografic results suggest that the proteins endoglycan and podocalyxin both contain a similar cytoplasmic tail while in the protein CD34 its motif is slightly altered – a result which can be linked to a different intracellular ligand binding which exists in the protein CD34.

4.2 CD34 family function

Table 4.1 depicts an overview of the expression of CD34 family members in all human cells. It is important to note that the expression patterns of these proteins are all unique but at the same time often overlap with one another.

In some tissues, such as vascular progenitor cells, all three proteins (namely CD34, Podocalyxin, and Endoglycan) are present which suggests that their expression is a

necessity which later prevents serious consequences from occurring. In other cases, such as with mast cells (CD34) or with kidney podocytes (podocalyxin), only one protein is expressed.

The sequence connected with the cytoplasmic tail is conserved between the three members of the CD34 family, however there are several differences which can be explained by referencing specific interactions with intracellular ligands.

Firstly, a protein related to PDZ-family, namely NHERF, and a Na^+/H^+ exchange regulator co-factor, namely NHE-RF2, were both found to interact with podocalyxin proteins.

NHERF is a intracellular protein with a ezrin-radixin-moesin (ERM)-binding domain and two tandem PDZ domains. The ERM-domain facilitates the interaction between NHERF and other ERF family members.

It is known that ezrin can mediate the interaction between cell surface proteins and the actin cytoskeleton (Orlando *et al.,* 2001) and therefore it was hypothesized that both podocalyxin and endoglyn were involved in cellular processes such as trafficking, transport, and signaling.

Even though CD34 has a cytoplasmic tail quite similar to the other two family members, it does not interact with NHERF-1 and -2. Within progenitor cells it was found that CD34 interacts with the adapter protein CRK like (CRK-L). This protein has two different domains (SH2 and SH3) which interact with membrane proteins, membrane proteins which lack an intrinsic kinase capacity which would enable them to activate intracellular pathways directly. Although the role and function of the CD34 protein was not initially fully understood, the various proteins which bind CD34 suggest that the protein CD34 explains various biological roles – roles which are dependent on the specific tissues in which the protein is expressed. CD34 used to be described as an identification marker of multipotent hemapoietic progenitors cells and it has been hypothesized that it is a protein involved in the enhancement of cell proliferation and in inhibiting cell differentiation. A scientific paper published in 1996 suggests that after stem cells maturation there is a reduction in the level of CD34 expression (Krause *et al.,* 1996).

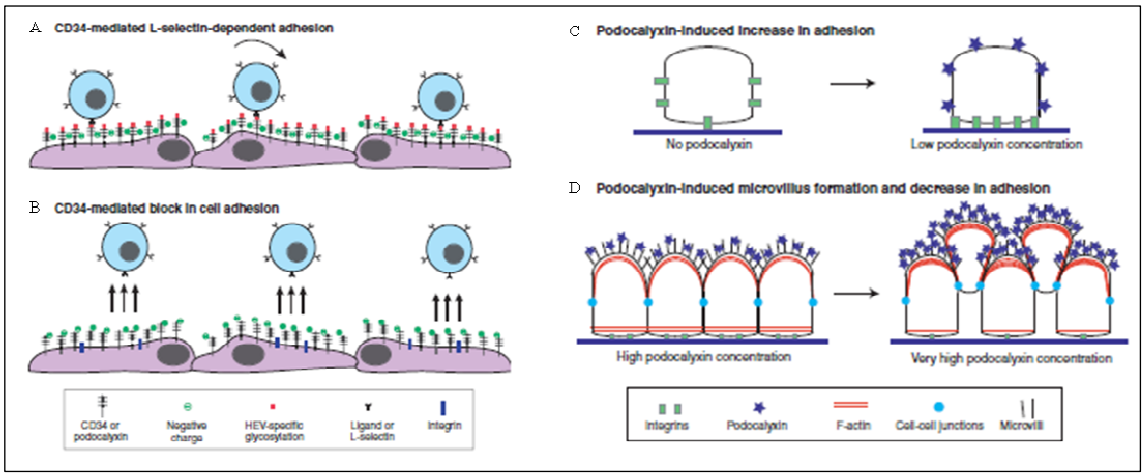

Figure. 4.2. Proposed functions of CD34-family proteins. A). Adhesion in lymphocites is mediated by L-Selectin and a specific glycosilation of CD34 extracellular domain. B) If this post trasductional modification is not present, the CD34 itself inhibits the adhesion. "balance mechanism" of podocalyxin. C) When the expression of podocalyxin on the cellular membrane is low, said adhesion is mediated by integrins, but D) when podocalyxin expression is too high, adhesion inhibition results (Nielsen & McNagny, 2008).

Furthermore, CD34-knockout mice have less progenitor hemapoietic cells. In addition, a proliferation defect of adult-derived progenitor cells was observed in adult mice (Cheng *et al*., 1996)

Several studies have focused instead on the homing of hemapoietic stem cells. When hemapoietic stem cells are infused intravenously, they arrive in the bone marrow where they travel between endothelial cells, enter the bone marrow parenchyma, and chemotax towards a specific subendosteal niche for the maintenance of said niche (Nielsen and McNagny 2008). Experiments using mice have shown that when hemapoietic cells are infused with $CD34^{-/-}$, a lesser number of cells were found in the bone marrow as compared to the number of cells found in the bone marrow when the cells were infused with $CD34^{+/+}$. Moreover, the infusion of $CD34^{-/-}$ into hemapoetic cells (again during experiments carried out in mice) created a significant reduction of bone marrow repopulation compared to normal cells.

These results support the hypothesis that CD34 is involved in the migration of HSC from endothelial cells to hemapoietic stem cells using niches within the bone marrow.

Several different papers report data which suggest the involvement of the CD34 family in many biological processes. For example, there is evidence which shows the correlation of CD34 protein and lymphocyte adhesion. Lymphocytes adhesion is mediated by L-selectin which recognizes specific glycosilated residues of the extracellular CD34 domain (sulfated form of sialyl Lewis X, a tetrasaccharide

carbohydrate). This post-translational modification is carried out in very few HEV. even if most cells express both CD34 and podocalyxin. The discrepancy between the number of cells which express CD34 family members and the number of cells which have the ability to modify these proteins post-traslationally represents an exception and not a general rule regarding the mechanism of this action.

As was previously notes, podocytes express podocalyxin in the human kidney. In order for the kidney to function correctly, a small degree of space must exist between the podocyte cells. It was discovered, in the early 1980's, that the high glycosilation of the extracellular domain of podocalyxin induces the correct spacing between said podocytes.

During the early years of the twentieth century, several researchers hypothesized that the mechanism associated with podocalyzin is correlated to with its level of expression (Nielsen and McNagny 2008). Podocalyxin is usually expressed in apical domain and, in this position is able to induce adhesion. On the contrary, when the expression of podocalyxin is too high it can move from the apical position to basolateral face – an action which reduces the adhesion surface (Figure 4.2).

Another aspect of the mechanism which was investigated was the role of podocalyxin in cell morphogenesis. Evidence supporting this role was found in 1984 when Kerjaschki and coworkers identified podocalyxin on the membrane of kidney podocytes and successively associated this discovery with the presence of extensions of the body of the cells (Kerjaschki, *et al.,* 1984). Later, the expression of podocalyxin was investigated on other cell types and it was found that podocalyxin is frequently expressed on the surface of cells (neurons, megakaryocytes) with complex membrane extensions, as previously described with regard to kidney podocytes. This new found data delineated the general role that podicalyxin plays in cell shape.

Currently, many research groups are focused on the study of CD34 function in order to increase general knowledge on this protein and in order to try to link the expression of this protein with cell function. Some evidence suggests that the protein CD34 plays a role in cell division in HSC fate determination but this hypothesis has yet to be confirmed.

5. Integrins

Integrins are adhesion molecules which play an important role in many biological processes such as cell-cell contact, cell-extracellular matrix interaction, immune response, and are also involved with cancer. The name "integrins" originates from the capacity of these proteins to establish transmembrane connections which connect the cell with the cytoskeleton as well as their capacity to activate many intracellular signaling pathways. Functioning as adhesion molecules, integrins are unique due to their ability to dynamically regulate their adhesive properties using a process termed 'inside-out signaling' or 'priming'. During this process, stimuli received by cell surface receptors for chemokines, cytokines, and foreign antigens initiate intracellular signals which in turn impinge on cytoplasmic domains and alter the adhesive properties of many extracellular ligands. In addition, ligand binding causes the transduction of signals from the extracellular domain to the cell cytoplasm in the classical inside-out direction. All of these dynamic properties of integrins are central to their correct functioning in the immune system (Luo, *et al.,* 2007).

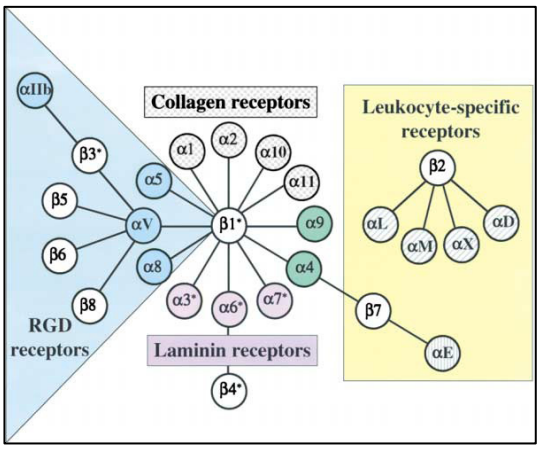

Figure. 5.1 A representative table of integrin subunits.

In addition to their role in adhesion, integrins are fundamental to the transmission of mechanical stresses in the microenvironment. Deformation of the cell membrane, be it caused by fluid flow, pressure variations, vibration dynamic strain, or other factors, is transmitted to the cytoskeleton via activated integrins (Thompson, *et al.,* 2012).

Integrins have been identified in metazoa yet have evolved differently in different phylia. In vertebrates it is possible to distinguish between eighteen α subunits and eight β subunits, which together are able to interact with one other in order to form until twenty four different heterodimers (Figure. 5.1) which all are able to bind, each with its own specific affinity, a wide range of ligands (Barczyk, *et al.,* 2010). All integrins

connect to microfilaments within the cytoskeleton except for the specific integrans α_6 and β_4, which interact with intermediate filaments instead.

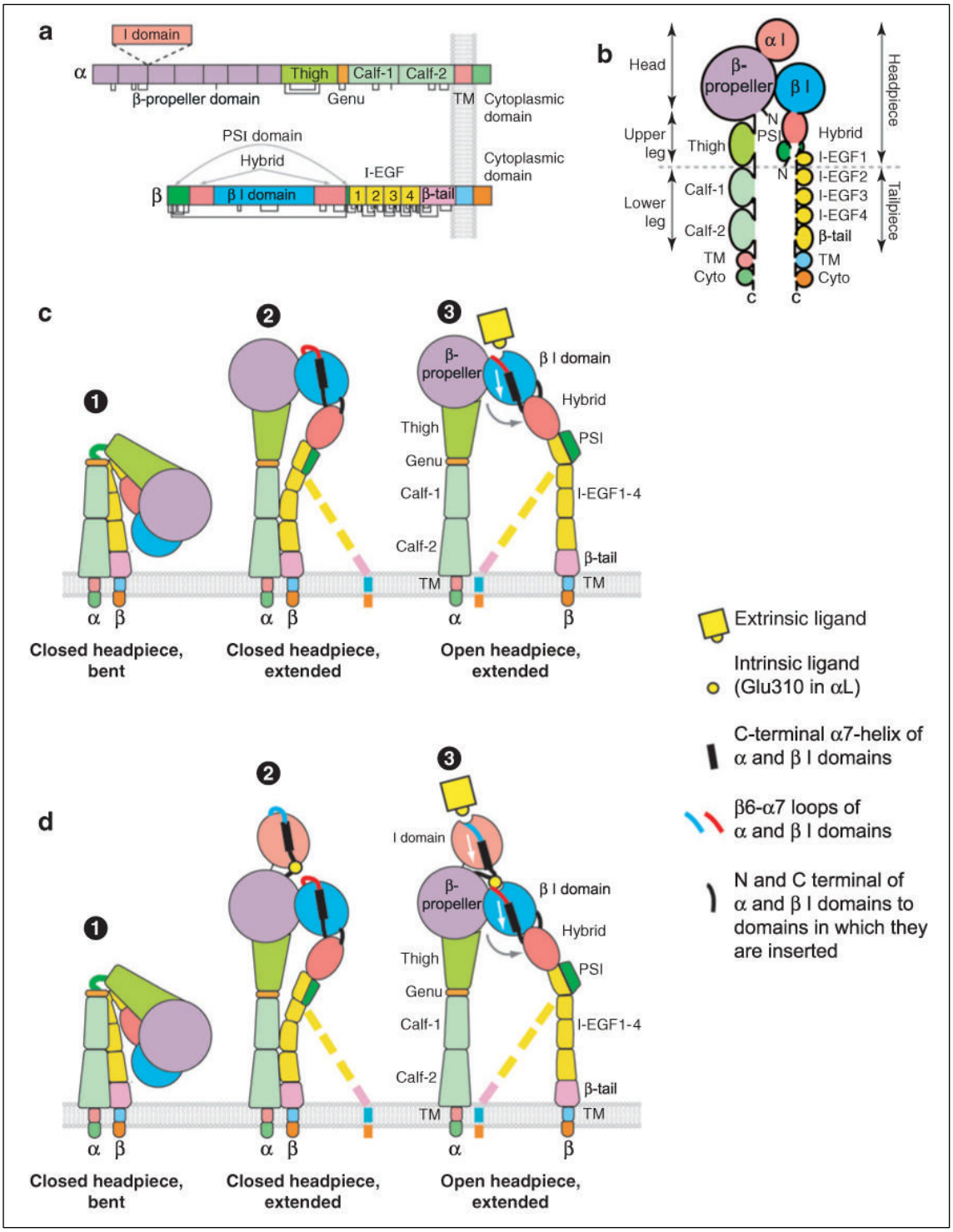

Figure. 5.2. A representative image of integrins subunits. a) synthetic representation of the different domain presents in α- and β- subunit; b) 3D representation of integrin subunits; c) and d) Rearrangement of domains during activation of integrins that lack c) or contain d) an α I domain (Luo *et al.*, 2007)

5.1 The integrin structure

Both α- and β-integrin subunits are type I transmembrane (TM) glycoproteins with large extracellular domains, single spanning TM domains, and, with the exception of β4, are also characterized by short cytoplasmic domains (Figure. 5.2 part a) (Fu, *et al.,* 2012).

5.1.1 The α- subunit

The extracellular part of the α- subunit is composed by a domain of around 200 amino acids known as the I domain or Von Willebrand factor A domain which is the major ligand-binding site on integrins. It is important to note that not all α- subunits have the I domain; however, when it is present it is located between second and third β sheets of the β-propeller.

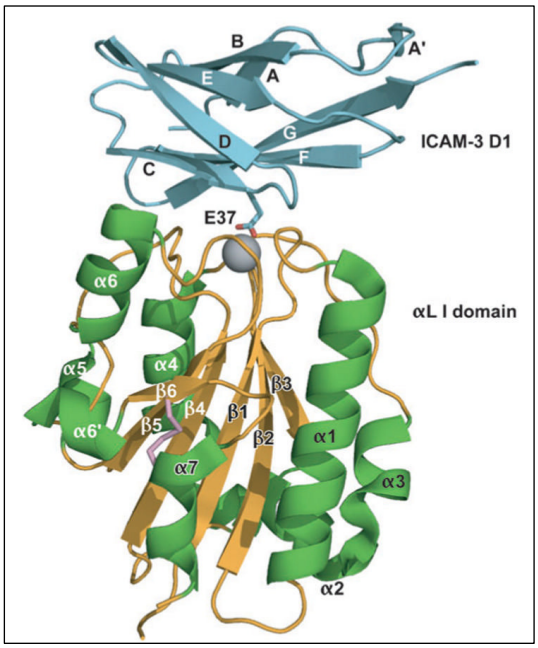

Figure. 5.3 Crystalized structure of α I domain (Luo *et al.,* 2007).

The α I domain adopts the dinucleotide-binding or Rossmann fold, which is characterized by α- helices surrounding a central β- sheet. There are seven major α- helices which exist, and several short α- helices that differ between I domain through various α subunits. The β- sheet contains five parallel β-strands as well as an antiparallel one. β-strands and α- helices tend to alternate in the secondary structure, with the α- helices wrapping around the domain in counterclockwise order when viewed from the top (Figure 5.3) (Fu, *et al.,* 2012; Lacey *et al.,* 1998). The α I domain contains a ion binding site known as Metal Ion- Dependent Adhesion Site (MIDAS) and the amino acids responsible for the coordination of the metal ions are important for ligand binding.

The N- terminal domain is composed by seven repetitions of around sixty amino acids with high homology to one another. In the secondary structure, these repeats are organized into a seven bladed β-propeller domain.

The main role of this domain is its interaction with β-subunit and, when the α I domain is not present, it is also responsible of the ligand binding.

In summary, we can distinguish the α- subunit as the 'head part' (β-propeller domain and α I domain) and the C terminal region as the 'leg part'. The 'leg' is composed of three different β- sandwich domains called thigh, calf-1 and calf-2. The 'knee' is located between the first and second domain and is characterized by a short sequence of amino acids responsible for the conformational variation during the different states of integrin activation.

5.1.2 The β- subunit

Analogous to the α subunit, the β- subunit looks like a head because it is formed by a β I domain inserted in the β- sandwich hybrid domain, which is itself in turn comprised within a plexin/ semaphorin/integrin (PSI) domain. The second main domain, while shorter compared to the "head" of beta integrin, includes four integrin epidermal growth factor- like domains (I- EGF) and a short β-tail. The crystallized structure of αVβ3 has established that the 'knee', which functions in the movement of the subunit, is located between domain 1 and 2 of I-EGF.

The integrin β I domain is a highly conserved domain of about 240 residues and is analogous in structure to the α I domain since it contains a MIDAS domain for the binding of Mg^{2+}. In the β I domain two additional metal ion-binding sites are present: the first one is called the "synergistic metal binding site"(SyMBS) and the second one is called the "adjacent to metal ion-dependent adhesion site" (ADMIDAS). Both sites bind Ca^{2+} and aid in the coordination of Mg^{2+} within the MIDAS site. There are two additional segments within the β I domain: one is known as the specificity-determining loop due to its role in ligand binding and the other helps form a critical interface with α-subunit β-propeller (Fu *et al.,* 2012). In integrin dimers where the α subunit does not have the I domain, the β I domain is fundamental for ligand interaction and is activated; however, when the α I domain is present the function of β I domain seems to regulate ligand binding within the α I domain.

5.1.3 Between inactivated to activated state

Integrin receptors are able to bind a wide variety of ligands and conversely many extracellular matrix (ECM) and cell surface adhesions proteins bind to multiple integrin receptors. One possible explanation for this added complexity lies in the evolutionary selection of common acidic peptide motifs in ECM proteins which mediate integrin binding via coordination to a divalent cation-containing binding pocket (Campbell and Humphries 2011). It is possible to define four classes of integrin-ligand combinations due to the fact that specific residues of ligands fit into specific sites in either α- or β- I domain or in β-propeller domain.

As described previously, both α- and β-subunits have a transmembrane domain as well as a short cytoplasmic domain which are both devoid of enzymatic activity. As a result, inside-out signaling by integrins depends largely on the existing interactions with neighboring receptors, adaptors, and signaling proteins.

Within crystallized structures several different research groups observed that integrins are able to assume three different conformations known as bent, intermediate and open (Figure 5.2).

Many researchers have hypothesized that integrins do not rest constantly in their activated state. Rather, there is evidence which suggests that integrins in the bent conformation are indeed inactive.

Moreover, when the integrin molecules are in the bent conformation, the ligand-binding pocket may be oriented toward the plasma membrane, thereby impeding ligand engagement. However, flexibility at the juxtamembrane domain could enable a "breathing" movement for the conversion of bent/inactivated conformation to the extended/activated conformation of the integrin. Currently ,the molecular mechanism responsible for the change between bent and open conformation is not well understood; however, new evidence suggests that the movement of the hybrid domain within the β-subunit could be a key passage and a consequence of the conformational change of the I domain. Indeed, a swing-out of the hybrid domain away from the α-subunit pulls downward on the α7 helix of the β-I domain and favors the upward movement of the α1 helix (Xiao *et al.,* 2004). The end result of the movement of these two α-helices is a change within the β-I domain from an inactivated to an activated state.

Besides the translation between the inactivate and the active state of integrin molecules, the TM and cytoplasmic domains of both subunits have also been studied.

In the activation phase, the TM domains seem to separate from each other instead of rearranging- an event which may prove to be an important factor in the intracellular activation of integrin.

Recent research has noted that in the inactivated phase, the integrin molecule's α- and β- subunit cytoplasmic domains are close to one another, yet then undergo significant spatial reorientation upon inside-out activation – an activation process which is induced by phorbol ester or talin head domain. Significant spatial reorientation is also observed during outside-in signaling – a process which is induced by ligand binding.

There is currently a significant amount of evidence available which suggests the order of the essential steps involved in the interaction between the integrin molecule's subunits and the specific ligand to which they are binding. There is still much to discover, however since the year 1986, when integrins were first discovered, there has been a significant increase in the level of interest in said integrin molecules an in the investigation of the molecular mechanisms involved in the signaling pathways activated by integrins.

5.1.4 Integrin and signaling pathways

Integrin signaling is critically important in regulation of signal transduction pathways through different mechanisms. It is known that most adherent cells come into contact with specific substrate for the purpose of their own **survival** and that the loss of these cell-matrix interactions induces cellular apoptosis. The mechanism responsible for cellular apoptosis is essential for it causes the avoidance of excessive cell growth in inappropriate tissues and/or sites. This survival signaling involves phosphatidylinositol-3-kinase (PI3K)-mediated protein kinase B (PKB/AKT) activity. When α6β4 integrin binds specific ligands, it activates NFkB-mediated survival signals in specific cultures. We know that integrins which are not ligand bound have the ability to trigger cellular apoptosis of fully adherent cells through the use of recruitment and activation of caspase-8 – a fact which indicates that a given integrin profile is uniquely specific to its ECM environment and that this specificity is essential for the cells survival (Huveneers *et al.,* 2007).

This cell-extracellular matrix interaction is fundamental for **cell proliferation** and **differentiation**. First of all, recent literature cites several different pathways through which integrin molecules are connected to the cell cycle progression. In fact, integrins which bind ECM residues stimulate a FAK/Src signaling complex at said sites of adhesion. In other cases, some α-subunits are coupled to the Src family Fyn kinase. Once activated, Fyn kinases recruit and activate Shc which creates a link to the ERK pathway. The last 'pathway' is the activation of PKC or PAK as mediated by integrin adhesion which in turn induces the activation of MEK. In differentiation processes, adhesion to specific substrates enables the modification of specific genes which are involved in differentiation. The inhibition of the formation of contracting myotubes by an embryonic myoblast by way of integrin-blocking antibodies is an example of the specificity of intercellular modification which is possible (Menko and Boettiger 1987). In osteoblastic differentiation, ECM-integrin-activated signals are responsible for the activation of Mitogen-Activated Protein Kinase (MAPK). Fibronectin and Collagen promote an increase in the activation of MAPK pathway, while the interaction of Vitronectin and integrin causes an osteogenic effect which must be associated with another mechanism (Hidalgo-bastida, *et al.,* 2010).

5.1.5 Integrins and their role in regenerative medicine

As was previously described, the integrin-ECM interaction is an important factor in the differentiation of mesenchymal stromal cells into mesodermal lineages.

In order to understand its possible application in regenerative medicine, it is fundamental to understand this mechanism fully.

Many clinical applications combine the use of bioengineered materials and mesenchymal stromal cells to help damaged tissues regenerate. The use of biomaterials together with specific peptides can help in the differentiation process of mesenchymal stromal cells – a process which has been fully investigated in certain specific tissues.

In 2004, Salasznyk published a research paper in which he described how the adhesion of vitronectin to collagen can induce the osteogenic differentiation of bone marrow derived mesenchymal stromal/stem cells. Salasznyk et al observed that only the seeding of BM-MSC on coating plates alongside these two molecules induces said osteogenic differentiation (Salasznyk, *et al.,* 2004).

Seeing as integrins are the primary link between cells and their extracellular matrix, the composition of the ECM is also a key aspect in the determination of the optimal conditions for hMSC differentiation. Most of the cell adhesion studies which have been conducted for MSC agree that fibronectin is the most effective compared to other ECM components. This increased attachment can be explained by fibronectin's ability to recognize twenty different binding receptors on integrin molecules (Hidalgo-bastida, *et al.,* 2010).

Mesenchymal stromal cells either modulate integrin expression in order to respond to cell culture conditions or they express specific integrins as a consequence of differentiation. The investigation of the molecular pathways involved in the regulation process described above is fundamental if we are to further clinical applications in the future.

In a paper published in 2001, Frith and coauthors hypothesized that through a clearer understanding of the changes made to both hMSC integrin expression and ECM composition during differentiation, biomaterials could be tailored to match such changes and thereby differentiation could be optimized (Frith *et al.,* 2012). Frith and coworkers observed that during osteogenic differentiation there is an increase in the expression of collagen -I and –IV as well as a reduction of fibronectin. Moreover, during the adipogenic differentiation process they observed a similar increase of Collagen –I and –IV production however as accompanied by an additional secretion of laminin. In this paper Frith also analyzed the expression of all integrin subunits at different time point during both the differentiation processes. In this way he was able to observe that after seven days of osteogenic induction, there was a significant increase in the expression of the α5 subunit which is involved with fibronectin even though during the differentiation process, MSC expressed collagen and not fibronectin. The hypothesis presented by Frith is that the α5 subunit is essential in the initiation of the induction process and that then other mechanisms were implicated in the modulation of integrin expression (Frith *et al.,* 2012).

Other reports have investigated mesenchymal stromal cells and the effects of low oxygen levels in the expression of integrins. It has been reported that MSC *in vivo* reside in the bone marrow as well as in other tissues where the oxygen concentration is lower compared to in vitro conditions. This notable difference in oxygen concentration

could be cause for an altered phenotype and characteristic. In a paper published in 2012, Saller observed an upregulation of all alpha integrins during hypoxia conditions compared to normal oxygen concentration conditions. HMSCs favor hypoxic condition in terms of stemness and migration (Saller *et al.,* 2012).

All the results from this study suggest that integrins are highly regulated by many different external factors yet also play an important role in the cell biology of mesenchymal stromal cells.

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PART I

Identification of key regulators of adipose-derived mesenchymal stem cell progenitor properties

Aim of the work

In the late of 1960s, Friedenstein identified in the bone marrow a population of cells able to adhere to plastic and to differentiate in the main mesenchymal lineages (Friedenstein, *et al*, 1966). These cells were later defined as mesenchymal stromal cell (MSC) and they have been intensively investigated. In the last decade different groups have identified mesenchymal stromal cells in other tissues, like fat (Zuk *et al.,* 2001b), dental pump (Gronthos, *et al.,* 2000) and other adult tissues (Fukuchi *et al.,* 2004; Patki, *et al.*, 2010).

The wide range of differentiation potential of MSC, the possibility of their engraftment, their immunosuppressive effect, and ability to extensively proliferate in culture led to an increasing clinical interest in the use of MSC in numerous pathologic situations through either intravenous infusion or site-directed administration alone or associated with specific biomaterials.

It is known that not all human tissues have the same ability to regenerate themselves after an injury or after a trauma. Some tissues, like skin and bone, for example, display a high ability to regenerate, but others, like cartilage, are not able to repair damages. In order to use MSC for clinical applications, it is fundamental to expand them *ex vivo.* In fact, MSC frequency *in vivo* is very low: in the bone marrow only 0,001% of cells are MSCs while this percentage increase to 3% in the fat tissue (Fraser, *et al.*, 2008).

In according to Good Manufacturing Practice guidelines, *ex vivo* expansion of MSC consists of cyclic phases where cells are seeded in culture dishes at a fixed density and are left to growth until they reach a 60-70% of confluency. Cells are passaged using enzymes like collagenase or trypsin, and either replaced up to obtain a enough number of MSC for clinical applications. The safety for the patient is the main concern associated to expanded *in vitro* cells.

Whether this procedure affects the genomic and/or chromosomal instability of cells is controversial and conflicting data have been published about this topic (for a review see Ferreira *et al.,* 2012). Most likely transitory alteration of the karyotype appears during culture. At the moment there are not enough data to fully understand all the possible implications that these transitory alteration have on the safety of the cells.

However, it must be noticed that so far none was able to demonstrate that cells with altered karyotype cause tumour formation in experimental animal models (Choumerianou *et al.,* 2008; Izadpanah *et al.,* 2008; Safwani, *et al.,* 2012) nor tumour formation in patients that have been implanted with MSC has ever been published (Lalu *et al.,* 2012). For this reason several groups are focused to define in details all parameters, including cell culture medium, cell density, passage and so on, which can be critical during expansion phase.

In the last 10 years, there is an increase of interest in the study of the *in vivo* environment where MSC are located. The most accepted hypothesis is that the environment where MSC are, may contribute to maintain their progenitor properties properties and their capacity to differentiate in different lineages (Kaewsuwan, *et al.*, 2012)

In this study, we aimed at establishing an *in vitro* niche for adipose-derived MSC (Ad-MSC or ASC) in order to maintain their progenitor properties during expansion. Furthermore, we identified potential regulators of the maintenance of ASC progenitor properties. Long-term cultures of ASC, without passaging after reaching cell confluence in order to allow cell-extracellular matrix and cell-cell interactions, were established and passaged ASC were used as control.

This type of culture could be applied model to determine the molecular pathways involved to preserve the characteristics of ASC *in vivo*, which would be useful for their application in regenerative medicine approaches. Another interesting aspect would be the standardization of the cell expansion procedure, which would allow to obtain cells with similar properties for each patient.

Materials and methods

Adipose derived mesenchymal stromal cell (ASC) isolation

Adipose tissue, in the form of liposuction or excision samples, was obtained from 11 donors after informed consent from the patient and following protocol approval by the local ethical committee (ref nr. EK 78/07 universitatsspital Basel). The tissue was digested for 60 minutes at 37°C in 0.15 % (W/V) Collagenase NB 6 GMP Grade from C. histolyticum (0.12 U/mg collagenase, PZ activity at 25°C, SERVA Electrophoresis GmbH, Germany) diluted in Phosphate Buffered Saline (PBS, Gibco). After centrifugation at 190 g for 10 min, the lipid-rich layer was discarded and the cellular pellet was washed once with PBS and it was filtered through 100 µm nylon-mesh strainer (BD Falcon; BD Biosciences, San Diego, http://www.bdbiosciences. com) to remove fibrous debris. For analysis, red blood cells were lysed by incubation for 2 min in a solution of 0.15 M ammonium chloride, 1 mM potassium hydrogen carbonate (both Merck, Darmstadt, Germany, www.merck-chemicals.com) and 0.1 mM EDTA (Fluka Analytical, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). The resulting SVF (Stroma Vascular Fraction) cells were then re-suspended in complete medium (CM), consisting of α-MEM supplemented with 10% of foetal bovine serum (FBS), 1% HEPES, 1% Sodium pyruvate and 1% of Penicillin-Streptomycin-Glutamin (100x) solution (all from Gibco), nucleated cells were stained with Crystal Violet (diluted 1:50 with phosphate buffer solution Sigma) and counted by using a Neubauer chamber (Güven *et al.,* 2012).

ASC Cell Culture

After isolation, SVF is seeded at a density of 2000 cells/cm^2. At a subconfluent density, cells are washed once with PBS, detached using trypsin 0,05% (Gibco) for 5min at 37% and its activity is blocked by complete medium. The cellular suspension is centrifuged at 1500rpm for 3 min. The pellet is re-suspended in CM and counted using a Neubauer chamber. The ASC are seeded at passage 1 (P1) at a density of 1700 cells/cm^2 until Passage 5 (PASS condition) in CM conditioned with FGF-2.

For the Unpass condition, ASC are seeded at a density of 2000 cells/cm^2 in a T150cm^2 flask (TPP, Techno Plastic Product, Switzerland) and left in the same flask for 28 days changing medium twice per week.

To value the ability of our cells to create a niche *in vitro* we seeded Pass/Unpass condition in Petri dishes at a density of 1700 cells/cm^2 and we left to reach the confluency (approximately one week) (Ctrl). One Petri per condition are left for 2 weeks more (Treated) and cells are characterized by FACS analysis.

Colony Forming Unit –fibroblast (CFU-f) assay

To value the clonogenic capacity of ASC we have plated them at low density (5 cells/cm^2) in CM with FGF 5ng/mL, after SVF isolation and every passage. We changed medium twice for week for two weeks. At the end we washed once with PBS, fix for 10min in 4% formalin and stained with crystal violet (Sigma) for 5 min. CFU-f frequency of freshly isolated ASC determines the initial number of ASC present in SVF.

Flow citometry analysis and flow cytometry sorting assay

Single cell suspensions ($\geq 10^5$ cells) were washed once with PBS 5% FBS (FACS buffer) and re-suspended in FACS buffer with saturating concentration of fluorochrome-conjugated antibodies against the indicated proteins or an isotype control (dilution 1:100) listed in the table below and incubated for 30min at 4°C. At the end of the incubation we washed cells twice with FACS buffer and analyzed using FACSCalibur flow cytometer (Becton, Dickinson and Company). For each sample, at least 30000 list mode events were collected. The results are analyzed using Flow Jo software.

In the case of FACS sorting cells are washed with FACS buffer supplemented with 2mM EDTA (Sigma) and stained for the specific antibody for 30min 4°C. The ASC are sorted according to the specific expression of the selected markers (CD34, CD49e and CD73).

Antibody	Provided by	Antibody	Produced by
CD29-PE	BD Pharmingen	CD49f	AbD Serotec
CD34-APC	BD Pharmingen	CD51/61-APC	BD Pharmingen
CD49a-PE	BD Pharmingen	CD73-PE	BD Pharmingen
CD49b-FITC	BD Pharmingen	CD90-FITC	BD Pharmingen
CD49c-PE	BD Pharmingen	CD105-FITC	AbD Serotec
CD49d-PE	BD Pharmingen	CD146-PE	BD Pharmingen
CD49e-PE	BD Pharmingen	IgG1 PE	BD Pharmingen
IgG1 FITC	BD Pharmingen	IgG1 APC	BD Pharmingen

Table 1. List of fluorochrome-conjugated antibody used for Flow cytometry analysis.

Adhesion assay

Untreated 60 well plate (Nunclon Surface, Nunc) were coated for 1 hour at room temperature with human laminin (L-4544, Sigma Aldrich), rat tail Collagen 1 (354249, BD Bioscences), human fibronectin (F-0895, Sigma Aldrich) and human plasma vitronectin purified protein (CC080, Millipore). All proteins are diluted at final concentration of 40µg/mL in Phosphate Buffered saline (PBS, Gibco) 0,01% Tween-20 (P-1379, Sigma Aldrich). Washed the wells with PBS and blocked unspecific sites using 5% dried milk in PBS 0,2% Tween-20 for 30min at room temperature. Discarded the solution and washed twice with PBS (Salasznyk et al., 2005).

Cells are collected after tripsinization, blocked by serum-containing medium and counted using a Neubauer chamber. We transferred $1x10^5$ cells in a 1,5mL tube, centrifuged at 1500rpm for 3min, discarded the medium and washed twice in PBS. The cell pellet is re-suspended in Dulbecco's Modified Eagle Medium (DMEM, Gibco), to a final concentration of 100cells/µL and plated 10µL of cellular suspension in the wells and left to adhere for 30 min at 37°C 5% CO_2. To remove unbound cells, wells were then filled with PBS and inverted the plate and plotted it dry with clean paper. The wells were then washed twice this PBS. The bound cells were fixed by 4% formalin for 10min at room temperature, discarded the fixation solution and washed twice this PBS, stained with 0,1% Crystal Violet for 15min and washed twice with tap water. The bound cells are then acquired using a light microscopy and counted using ImageJ software.

Osteogenic differentiation 2D

Osteogenic differentiation was induced in 2D cultures as previously described (Jaiswal, *et al*, 1997). Briefly Pass and Unpass cells were seeded in 96-well plate (TPP, Switzerland) at a density of 3000 cells/cm^2 in α-MEM supplemented with 10%FBS until the confluency, then we started the osteoinduction using medium composed by α-MEM supplemented with 10% FBS, 10mM β-glycerophosphate (Sigma), 10nM dexamethasone (D-2915, Sigma) and 0,1mM L- Ascorbic acid-2-phosphate (Sigma). We induced cells for three weeks changing medium twice per week.

Readout: Cells layers cultured in osteogenic medium were analized using OsteoImage Bone mineralization Assay (Lonza, Switzerland) as described in the datasheet. Briefly, cells are washed with PBS and fixed with 4% formalin for 20min at room temperature. After fixation, we washed twice the wells with diluted wash buffer (1X). We Added 100µL to each well of diluted staining reagent and incubated 30min protected from light. At the end of the incubation, discard the reagent buffer and wash the wells three times with wash buffer leaving wash buffer in the well for 5min.

Adipogenic differentiation

Adipogenic differentiation was induced in 2D cultures as previously described (Barbero, *et al*, 2003). Briefly, cells were seeded at a density of 3000 cells/cm^2 and cultured in complete medium until the confluence. The medium was then supplemented with 10µg/mL Insulin (provided by the Hospital), 1µM Dexamethasone, 100µM indomethacin (I-7378, Sigma) and 500µM 3-isobutyl-1-methyl xanthine (Sigma) (Adipogenic induction medium) for 72 hours and subsequently with 10µg/mL insulin (adipogenic maintenance medium) for 24 hours. The 96-hour treatment cycle was repeated four times.

Readout: Adipogenic differentiation was evaluated using Oil Red O staining (Sigma), which shows the presence of triglyceride deposits. In brief at 21 days from the adipogenic induction, ASC cultures were washed with PBS, fixed in paraformaldehyde 4% for 10 min and stained with three volumes of Oil Red O (0,3% in isopropanol) and two volumes of H2O for 15 min at room temperature. Adipogenic-differentiated cells

were recognized by their characteristics being round shaped and containing lipid droplets and we acquired representative fields using contrast microscope (Olympus IX50 camera Color View Olympus). To perform the quantification of triglyceride accumulation, Oil Red O was solubilized with 100% isopropanol and the optical density (Perkin Elmer Elisa reader) was measured with a spectrophotometer at 500 nm. Experiments for the quantitative assessment of adipogenic differentiation were performed in triplicate in cells from different donors (Donzelli *et al*., 2011).

Chondrogenic differentiation

The chondrogenic differentiation capacity of ASC cells was investigated in pellet culture by using a chemically defined, serum-free medium, consisting of DMEM containing 4.5mg/ mL D-glucose, 1mM sodium pyruvate, 10mM HEPES buffer, 100U/mL penicillin, 100mg/ mL streptomycin, and 0.29mg/mL L-glutamine (all from Gibco) further supplemented with ITS^{+1} (10mg/mL insulin, 5.5mg/mL transferrin, 5ng/mL selenium, 0.5mg/mL bovine serum albumin, and 4.7mg/mL linoleic acid), 1.25mg/mL human serum albumin, 0.1mM ascorbic acid 2-phosphate, and 10^{-7} M dexamethasone (all from Sigma), and 10ng/mL TGF- b1(R&D). Aliquots of $5x10^5$ cells were centrifuged at 250 g for 5min in 1.5mL polypropylene conical tubes (Saarstedt) to form spherical pellets and were cultured for 3 weeks at 37°C 5% CO2.

Readout: Some pellets were fixed and paraffin embedded, and then 7µm sections were serially stained with hematoxylin and safranin-O and we acquired representative fields using microscope (Leitz Dialux 20).

Osteo-induced adipose derived mesenchymal stromal/stem cells for implantation with actifuse.

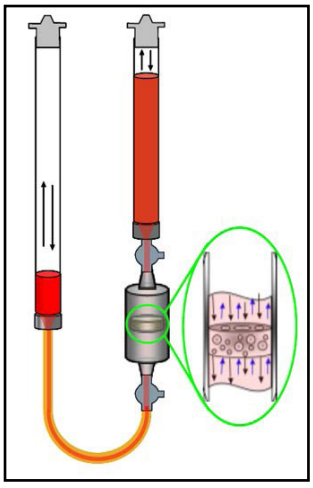
To test the ability of adipose derived mesenchymal stromal cell (ASC) to produce bone *in vivo* we cultured the cells for two weeks in a dish with osteogenic medium. At the end of the pre-induction, we discarded the medium and washed once with PBS. To detach cells from the plastic cells were treated for 15min at 37°C with 0,3% collagenase type II and we collected detached cells in a tube. After the treatment with collagenase, ASC were exposed to trypsin 0,5% for 5 min at 37°C and the trypsin activity was

blocked with complete medium. The cells obtained were passed by a 100 µm strainer, counted and aliquoted 10^6 cells for each tube and then centrifuged at 1500 rpm for 5 min. Meantime, we prepared tripsin (40mg/ml) and fibrinogen solution (12U/mL).

In a well of a 96-well plate we added a "fixed" volume of actifuse granules (Baxtersurgery, Apatech LDH), we re-equilibrated the granules with medium and then added cells and the two solution and wait for 10min at 37°C 5%CO_2. At the end of the incubation, we detached the obtained constructs from the surface and we implanted them in nude mice as described below (*in vivo* experiments in nude mice).

Three- dimensional perfusion cultures

For the direct perfusion of a cell suspension through the pores of 3D scaffolds we used a bioreactor system developed by Cellec (Cellec, Basel, Switzerland). As shown in the picture below, scaffolds were placed in silicon chambers (one scaffold per chamber) that were positioned at the bottoms of two silicon columns and connected through a U-shaped tube at their base. The silicon columns allow oxygen exchange. Flow of the cell suspension was induced with the use of a vacuum pump and the flow rate regulated with a flow meter. The direction of flow was reversed when the fluid level in one column reached an optical sensor placed near the top of each column. The sensor detected the cell suspension, actuating a pair of solenoid valves, switching the vacuum to the opposite column, and therefore reversing the direction of fluid flow. Because scaffolds were press-fit into the chamber, the cell suspension could not deviate around the scaffold and was therefore forced to flow through its pores. The bioreactor was oriented vertically to avoid cells from settling onto the glass columns as would occur if in horizontal oriented (Wendt, *et al.,* 2003).



SVF, Pass and Unpass cells were perfused at a velocity of 3 ml/minute through porous hydroxyapatite ceramic scaffolds (ENGIpore; Fin-Ceramica Faenza, Faenza, Italy, http://www.finceramica.it) with an average porosity of 83% ± 3% and the size of 8-mm diameter, 4-mm-thick disks ($2{,}5x10^6$ cells per disk when we seeded SVF, while 10^6 cells per disk in PASS and Unpass condition) in DAF medium (10^{-8} dexamethason,

5ng/mL FGF-2 and 0,1mM ascorbic acid). The day after the seeding we changed the intensity of perfusion from 3ml/min to 1mL/min. After 3 days, the initial cell suspension was totally removed and replaced by fresh DAF medium. After two additional days of perfusion with cell-free medium, the resulting constructs were implanted in nude mice as described below (Scherberich, *et al*, 2007).

In vivo experiments in nude mice

ENGIpore ceramic scaffolds were cutted in half, rinsed in PBS, placed in a 12-well plate, and incubated at 37°C for 2 h with 3 mL of 0.12 mM MTT [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, MO] to assess the spatial distribution of cells. MTT is converted by the mitochondria from a soluble yellow salt into an insoluble purple formazan salt and can therefore give a qualitative assessment of the location of cells within the scaffold (Wendt *et al.,* 2003). Some other constructs were implanted in the subcutaneous tissue of nude mice (CD1 nu/nu, Charles River, www.criver.com).

Assessment of bone formation following *in vivo* implantation

Twelve weeks after implantation, the mice were sacrificed by inhalation of CO_2. The maintenance, surgical treatment and sacrifice of animals were performed in strict application of the guidelines from the local veterinary agency (Kantonales Veterinäramt Basel-Stadt, permission #1797). The constructs were harvested, fixed in a 4% formalin buffer overnight and subjected to slow decalcification in 7 % w/v EDTA and 10% w/v sucrose (both from Sigma-Aldrich) at 37°C on an orbital shaker for 7-10 days, paraffin-embedded and sectioned at different levels (7-µm-thick sections).

Readout:

Sections were stained with **Hematoxilin and Eosin (H&E)** and observed microscopically to detect the formation of bone tissue for qualitative analysis (Leitz Dialux 20). Briefly, to re-hydrate the fixed tissue are necessary 3 steps in ultraclear to deparaffinate the slides, two steps in ethanol 100%, one in ethanol 96%, one step in ethanol 70% and one in ethanol 50% (90sec/each). After these steps, wash with tap water and then 3 stain with hematoxilin mayer (Medite) for three times and a final step

of washing. The second phase is to bath slides with HCl-alcohol 0,2% and water with NH_4 and then to stain them with eosin 0,2% (J. T. Baker) three times. Three final washing steps in ethanol 96%, 96% and 100% (respectively) and three step in ultraclear.

Trichrome de Masson – aniline blue variation (reactifs RAL). Trichromic staining combines three stains: a nuclear stain (Haemalun), a cytoplasmic stain (a mix of acid stains: ponceau fuchsin) and a specific stain for collagen (another acid stain: aniline Blue). Briefly, the slides are dewax and then re-hydrate (as described in of hematoxilin and eosin staining); stain with mayer Haemalum for 10 minutes, rinse with water and then stain with Ponceau funchsin solution for and incubate for 5 minutes; successively rinse in two baths of 1% acetic water, fix the stain in a bath of Phosphomolybdic acid for 3 minutes and stain in Aniline blue solution for 5 minutes. At the end of staining differentiate the slides in alcohols and dip in a bath of toluene or xylene and micrographs are captured of representative fields (Leitz Dialux 20).

RNA extraction and cDNA retrotrascription

Total RNA was extract from cell pellet stored at -80ºC according to NucleoSpin RNA II protocol (Macherey-Nagel, http://www.mn-net.com/). Briefly, sample is homogenized and the cells are lysed and RNases are inactivated. The lysate is then filtrate and acid nucleics (RNA and DNA) are captured in the silica membrane. The next step is digested DNA and finally dilute highly pure RNA which is quantify using NanoDrop (Thermo scientific,).

For cDNA retrotrascription we used Omniscript enzyme (Qiagen) and the protocol is described in the table below and the final volume is 20 µL.

Component	**µL (x1 sample)**
Random primers (Promega)	0,8µL
dNTPs (10X)	2µL
Buffer (10X)	µL
RNA (0,5 µg)+ H_2O DEPC treated	14.2µL
enzyme	1µL

Incubate the reaction for 60min at 37ºC and then store at -20ºC.

Rq- PCR

The cDNA obtained from the retrotrascription, was used as template for Rq-PCR. For a single reaction the final reaction volume is 20 µL and it is composed by: cDNA (4µL of the entire volume of retrotrascribed product diluted 1:4 with H_2O DEPC), Master mix (12,5 µL), assay on demand (or primer FW, RV and probe9 and H_2O DEPC to 20 µL.

Primers for OCT4/POU5F1 (octamer-binding transcription factor 4), KLF4 (Krueppel-like factor 4), Nanog, Sox2 are provided by Applied biosystem (Assay on demand); primers and probe for GADPH. The amplification reaction was carried out in 96 well plate in a ABI prism 7300 (Applied byosistem, Paisley, UK). The results obtained are normalized to that of GADPH.

Immuno Fluorescence (IF)

Immunofluorescence staining was performed on 50 µm-thick frozen section of human fat tissue. Sections are fixed in cold acetone at -20°C for 10 min. Successively they are washed twice with PBS and let's to dry at RT. The slides are incubated for 1 hr in a blocking solution (PBS 0,3% Triton and 2% goat serum) and then stained for 1 hr at the dark for the following antibodies and dilutions : CD49e (BD Pharmingen) at 1:50 and fibronectin (Abcam) at 1:100. At the end of the incubation, sections are washed with PBS+ 0,3% triton and then incubated for 1hr with secondary antibody diluted 1:200. Three washing steps and then sections mounted.

Fluorescence images were taken with 40x and 60x objectives on Nikon A1 laser

Results

Clonogenic ability

Adipose derived mesenchymal stromal cell (ASC) display the ability to form colonies when plated at low density on tissue culture plastic. These colonies are the so called colony forming unit-fibroblast (CFU-f).

The standard protocol for expansion of ASC for research and clinical applications, dictate that when cells arrived at a subconfluent density (70%) they were detached from plastic and expanded in new flasks (Pass cells). It has been described that ASC lose their pluripotency during in vitro expansion (Muraglia, *et al.,* 2000). In our hypothesis, 3D culture and cell-cell and cell-matrix interactions are fundamental for maintenance of ASCs properties. To allow these interactions, we decided to culture ASC for 28 days in the same dishes without passaging, reaching confluence and producing extracellular matrix (Unpass cells).

Unpass and Pass cells, were first analyzed for their clonogenic capacity. As described in the Figure I.1, Unpass cells displayed a significant increase of clonogenicity compared to Pass cells.

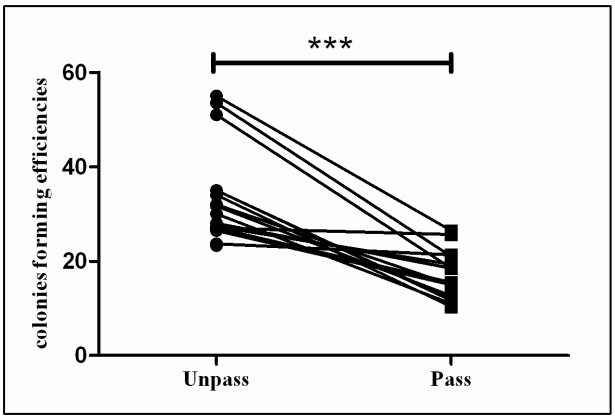


Figure I.1 The graph represents the results of "colony- forming unit efficiency" (CFU-e) of 6 donors in triplicate. Data are analyzed using one way ANOVA followed by unpaired t test (p<0,0001).

To investigate whether the increased number of colonies was due to a lower number of doublings performed by Unpass cells as compared to the control condition, cumulative doubling number and clonogenicity was determined.

Unpass cells divided less in 28 days (9,47±1,29) compared to Pass cells (25,79±3,25) (Figure I.2a). As shown on Figure I.2b at a comparable number of doublings (P0), Pass cells displayed already a decreased amount of colonies, indicating that Unpass were able to better preserve their clonogenic capacity despite expansion.

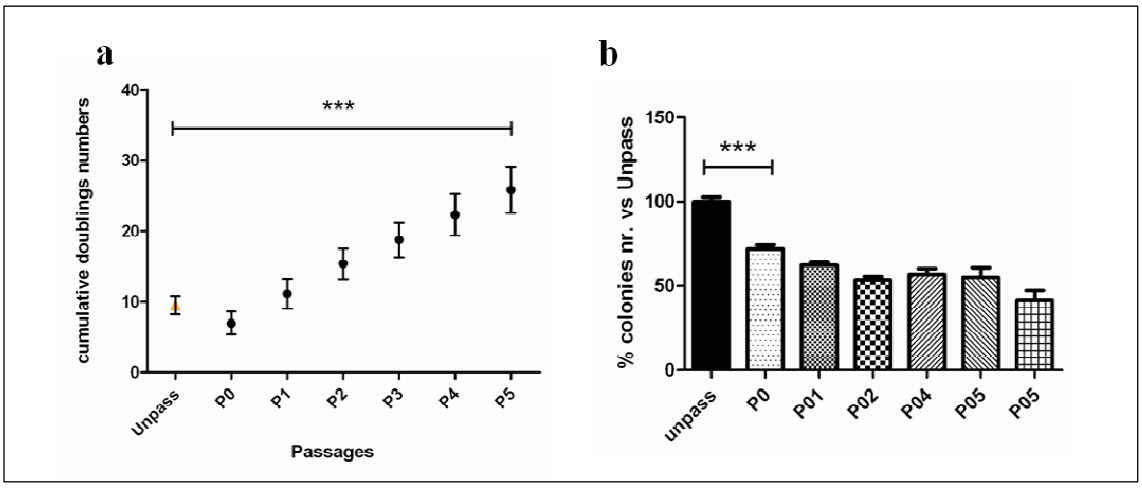


Figure I.2. a) The graph represents the cumulative doublings numbers of 11 donors in Unpass condition and during the normal culture. Data are analyzed using1way ANOVA followed by Bonferroni's multiple comparison test (p<0,01); b) Colony forming unit efficiency of 6 donors. Data were analyzed using 1way ANOVA followed by Bonferroni's multiple comparison test (p<0,01).

Unpass cells have higher level of expression of stemness genes

It has been previously described that stem cells express high levels of specific genes related to the maintenance of their pluripotency. NanOg, Sox2 , KLF4 and Oct4. NanOg, Sox2 and Oct4 (knows as POU5F1) are transcription factors involved in the maintenance of undifferentiated embryonic stem cells and somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by their transient ectopic overexpression (Bernhardt, *et al.,* 2012).

Different papers have described an upregulation of these genes in MSC obtained from different tissues (Liu *et al.,* 2009). For this reason we investigated the expression of NanOg, Sox2, KLF4 and Oct4 in Unpass and Pass cells.

The results, shown in Figure I.3, indicate that, compared to the Pass condition, Unpass cells expressed higher mRNA levels of the analyzed genes.

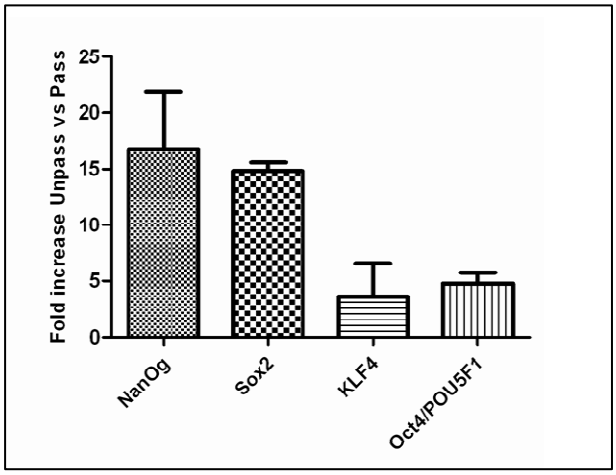


Figure I.3 The graph represents data obtained from real time PCR for three different donors. The values are normalized as a ratio between Unpass and Pass condition.

Unpass cells have an increased osteogenic, adipogenic potential.

ASC, after expansion *in vitro*, preserve their property to differentiate in different mesodermal lineages and recently it has been described that they can differentiate also in non-mesodermal lineages as for example cardiac (Vunjak-Novakovic *et al.,* 2010) and neurogenic cells (Safford *et al.,* 2002). To investigate whether the expression of the stemness genes described above in Unpass cells correlated with increased functional properties, differentiation potential towards the adipogenic, chondrogenic and osteogenic lineage was assessed. As control Pass cells and P0 cells (to compare the same number of doublings) were used. After 4 cycles of adipogenic induction, differentiation was evaluated using morphological techniques described at materials and methods section. For qualitative assessment, we stained the intracellular lipid droplets using OIL RED O, which was then quantified. As shown in Figure I.4, in the Unpass condition, ASC were able to form a significant higher number of adipocytes compared to Pass or P0 ASC.

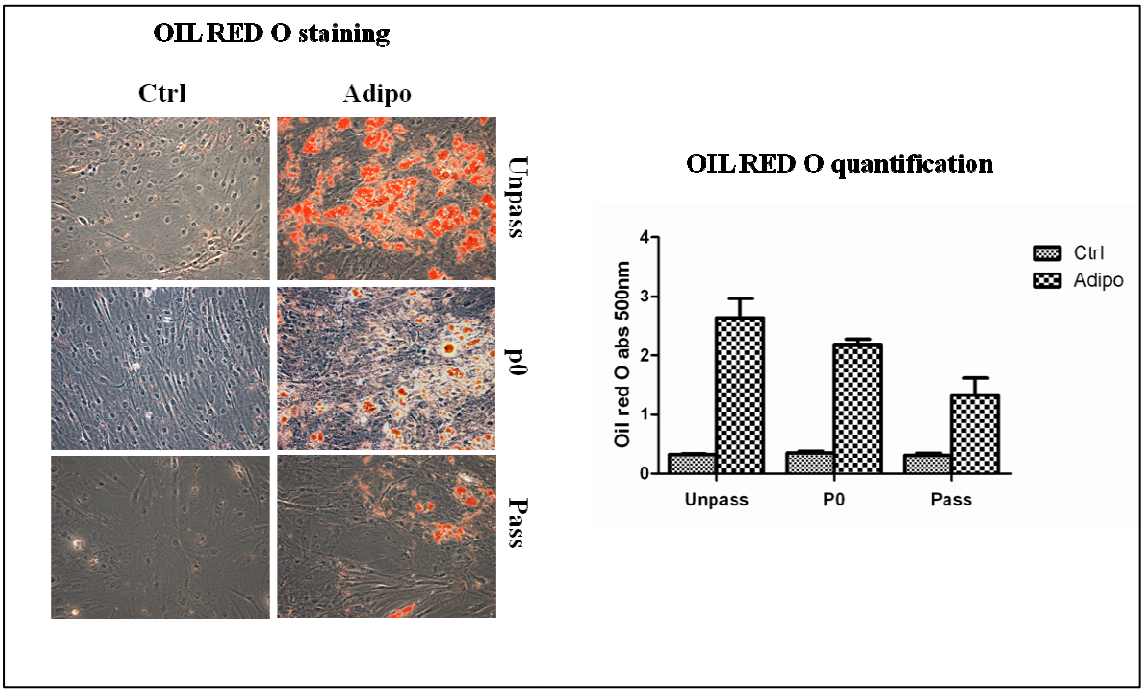


Figure. I.4. Adipogenic differentiation of hASC. a)Control untreated hASC (CTRL) and adipogenic induced ASC (ADIPO) were stained with Oil Red O after 4 cycles of induction. Micrographs are representative of different experiments performed with cells from three donors. b) Oil Red O was solubilized and optical density read at 500 nm to obtain a quantitative assessment of adipogenesis. The experiment was performed in triplicate with cells from three different donors and data are expressed as mean+standard deviation.

For osteogenic differentiation, ASC were induced for three weeks with osteogenic growth factors and then hydroxyapatite deposit s were quantified.

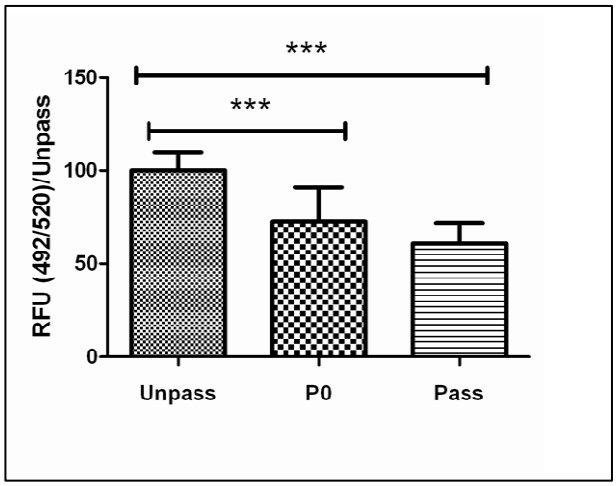


Figure. I.5. OsteoImage quantification after 3 weeks of osteogenic differentiation. Data are obtained from three donors and are analyzed using 1way ANOVA followed by Bonferroni's multiple comparison test (p<0,05).

The quantification of the hydroxyapatite deposits confirmed that Unpass Cells have an increased capacity to differentiate towards osteogenic lineage in vitro. ASC, which were expanded for one passage (P0 cells) had a reduced capacity to differentiate, similarly to Pass cells.

Figure. I.6 Micrographs of cells pellets after the staining with safranine solution. The absence of staining suggests that during three weeks of differentiation ASC does not differentiate. Pictures are representative of three different donors.

To determine chondrogenic differentiation capacity, ASC were cultured in a pellet system in serum free medium and in presence of TGF-beta1. The results show that were not able to produce cartilaginous matrix since no Safranin O staining could be observed in any of the conditions (Figure I.6). This could be explained with some reports present in the literature according to which this medium does not activate specific molecular pathway fundamental for the chondrogenic differentiation in ASC.

Osteogenic differentiation *in vivo.*

ASC, as bone marrow-derived MSC, have been investigated for their possible application in bone tissue engineering. For this reason it is fundamental to determine ASC bone forming capacity *in vivo*. For this purpose two different models were used. In the first approach, we pre-differentiated Unpass and Pass cells in the osteogenic lineage *in vitro* for two weeks, then we mixed them with silicated tricalcium phosphate granules (actifuse granules) and the constructs were implanted subcutaneously in atimic mice for 12 weeks.

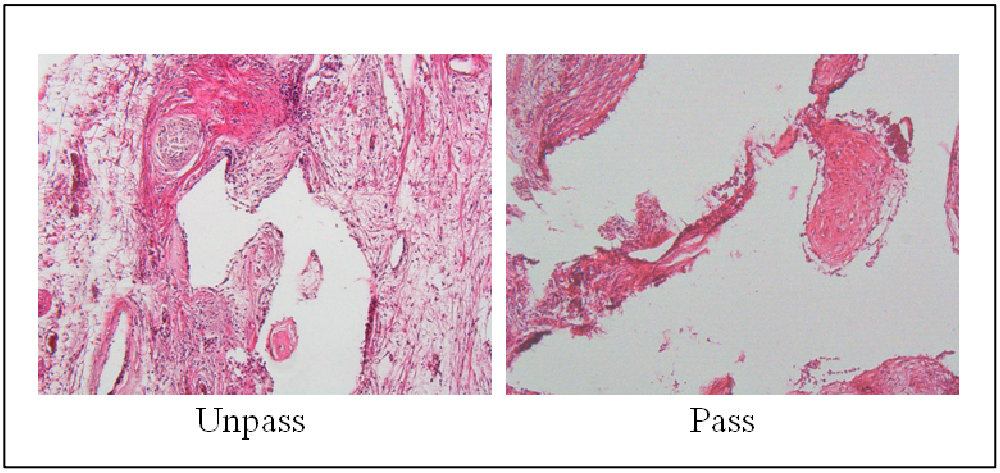


Figure I.7 Hematoxylin and eosin staining of 7µm sections of construct after explantation (10X objective).

Histological analysis showed that Unpass ASC have produced slack collagen fibers which were not visible in Pass condition. Furthermore, we did not observe the presence of osteoblastic lacunae (Figure I.7) in any of the conditions.

Observing these results our hypothesis was that the biomaterial and the process was not ideal for our cells. Based on a paper published in 2007 (Scherberich *et al.*, 2007) we decided to change model and to use ceramic scaffold and not granules of actifuse and to replace the static culture with a perfusion system (described in material and methods section).

MTT assay was performed after after 5 days of seeding on half of the construct to evaluate the efficacy and homogeneity of the seeding (data not shown).

Half scaffold was implanted subcutaneously in atimic mice for 10 weeks. As control in these experiments we used stromal vascular fraction (SVF) to reproduce the results obtained in the paper from Scherberich et al (Scherberich et al., 2007).

After 10 weeks, SVF and Unpass cells were able to form dense collagenous matrix characterized by the presence of osteocyte lacunae, whereas Pass cells only generated fibrous tissue within the scaffold pores (Figure I.8). These results taken together indicate that Unpass cells were able to maintain their *in vivo* bone forming capacity despite the expansion on plastic.

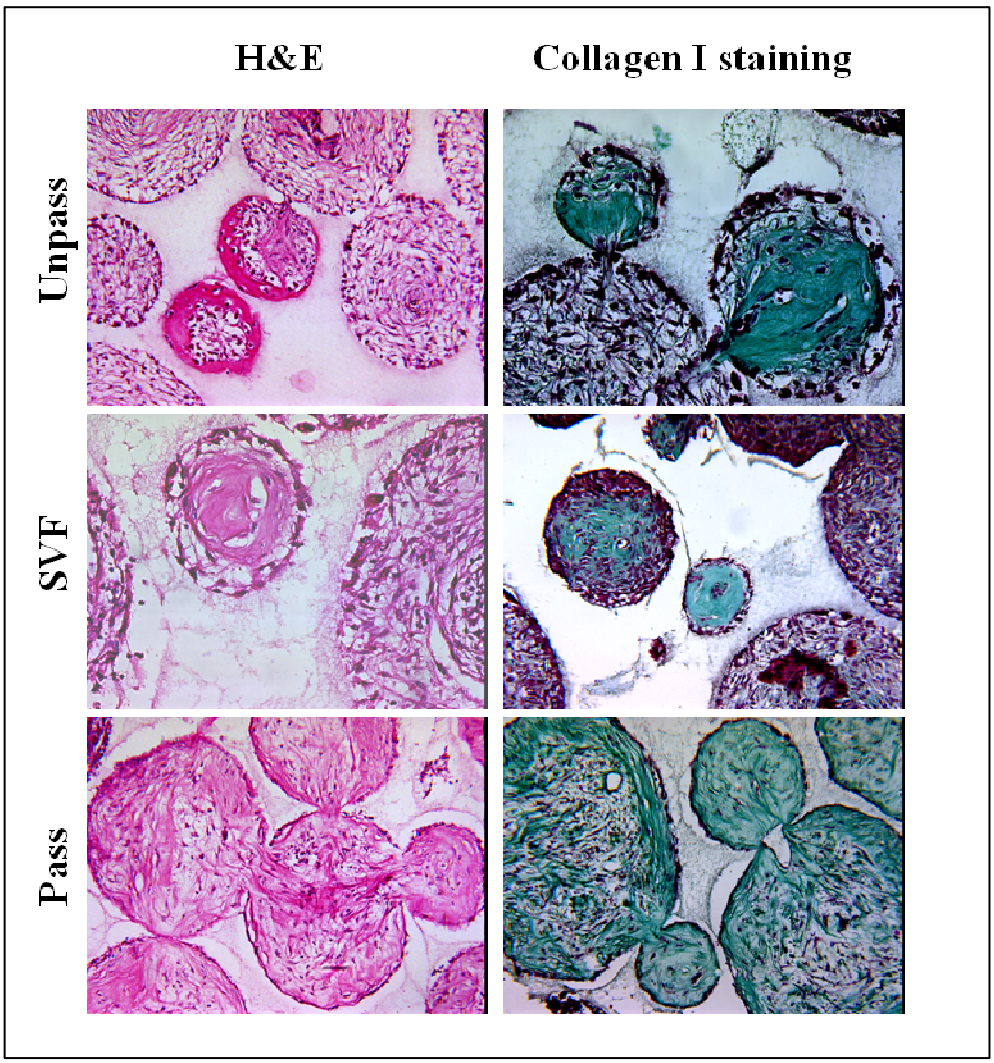


Figure I.8. Mibrographs of scaffold sections after *in vivo* implantation. On the left there are a representative image of tissue after Hematoxylin & eosin staining. On the right, sections were stained with Masson trichrome, specific for Collagen I. The micrographs are representative of two donors.(10X objective in all images accept for the image on the top on the right and in the middle on the left(20X objective)).

Phenotipic characterization

To investigate whether the increased osteogenic capacity was related to a specific phenotype, Unpass cells were characterized for expression of different surface markers (Table I.1). We observed that 20% of Unpass cells expressed CD34, which was instead not detectable in Pass cells. CD34 belongs to the sialomucin family and it has been used as a marker of endothelial cells and hematopoietic stem cells (Nielsen & McNagny, 2008). More recently, Maumus and coworkers (Maumus *et al*., 2011) claimed that ASC *in vivo* are characterized by the expression of CD34, which is lost upon expansion in

vitro. We hypothesized that the presence of a subpopulation CD34+ cells could explain the differences in stemness gene expression, clonogenicity and differentiation capacity in Unpass cells.

Markers	Unpass	Pass
CD29	+	+
CD34	20%+	-
CD49a	-	+
CD49b	-	+
CD49c	-	+
CD49d	-	+
CD49e	+	++
CD49f	-	-
CD41/61	+	+
CD73	+	+
CD90	+	+
CD105	-	+
CD146	-	-

Table I.1 The list of markers tested in ASC .

CD34+ subpopulation characterization

To test this hypothesis, Unpass ASC were sorted according to the expression of CD34. CD34+ and CD34- were then analyzed for their clonogenic capacity and for the expression of stemeness genes.

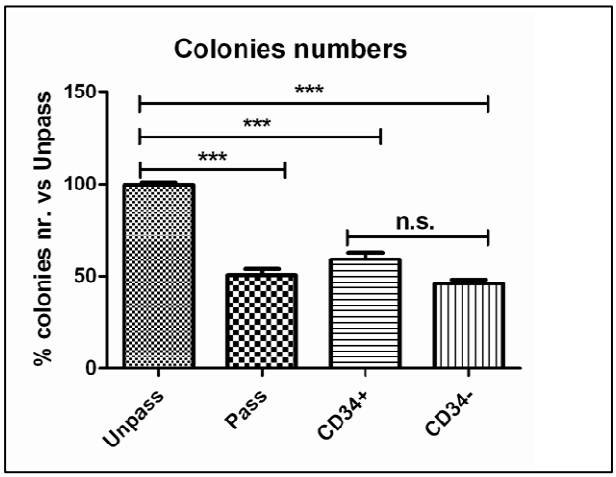


Figure I.9 The graph represents the data of colonies-forming unit-efficiency in Unpass, Pass and the two subpopulation (CD34+ and -.) The data are results of 5 different donors and are analyzed using 1way ANOVA followed by Bonferroni's comparable multiple test (p<0,01).

As shown in Figure I.9, CD34+ and CD34- displayed the same clonogenicity and no difference was observed for the expression of the transcription factors NanOg, Sox2, Oct4 and KLF4 (Figure I.10)

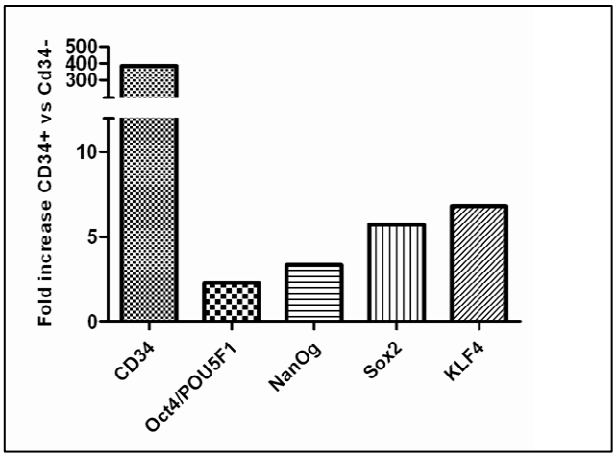


Figure I.10 The graph represents the levels of gene expression normalized to CD34- cells.

To assess the capacity to differentiate in vitro, CD34+ and CD34- cells were cultured in presence of osteogenic growth factiors. The results indicate that amount of hydroxyapatite deposits was comparable between the two conditions (Figure I.11), suggesting that CD34 expression does not identify a population of early progenitors with Unpass ASC.

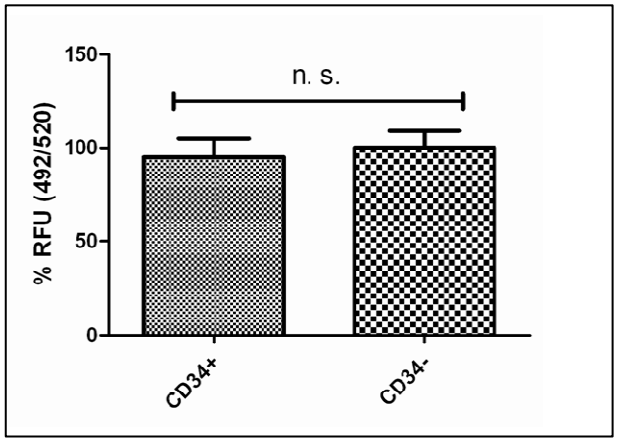


Figure I.11. Quantification of hydroxyapatite deposits with OsteoImage Assay kit. The data are obtained from three different donors.

After osteogenic differentiation *in vitro* we investigated also their ability to make bone *in viv*o. However, no difference was observed in the capacity CD34+ and CD34- cells to form bone tissue *in vivo*. In fact, as shown in the pictures in Figure I.12, only fibrous tissue was found in both conditions.

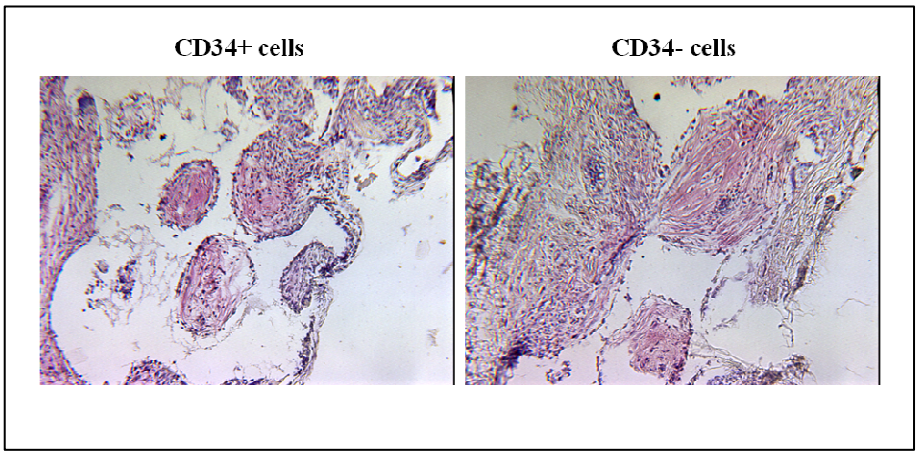


Figure I.12 Hematoxylin and eosin staining of paraffin embedding sections after *in vivo* implantation. The tissue is fully cellularized and we can distinguish few collagen fibers. Micrographs are representative of three different donors.

Many membrane markers are not stable expressed during life of a cells but they can be modulated in response to a specific environment or an external stimuli. The results obtained from the characterization of CD34 population have suggested that CD34 is not stably expressed by Unpass cells but rather modulated.

To confirm this hypothesis we investigated the expression of CD34+ and CD34- populations after sorting. Interestingly, after 5 days of culture, positive cells no longer expressed CD34, which was instead upregulated after 14 days of culture (Figure I.13). Negative cells also expressed CD34 when they reached confluency (14 days).

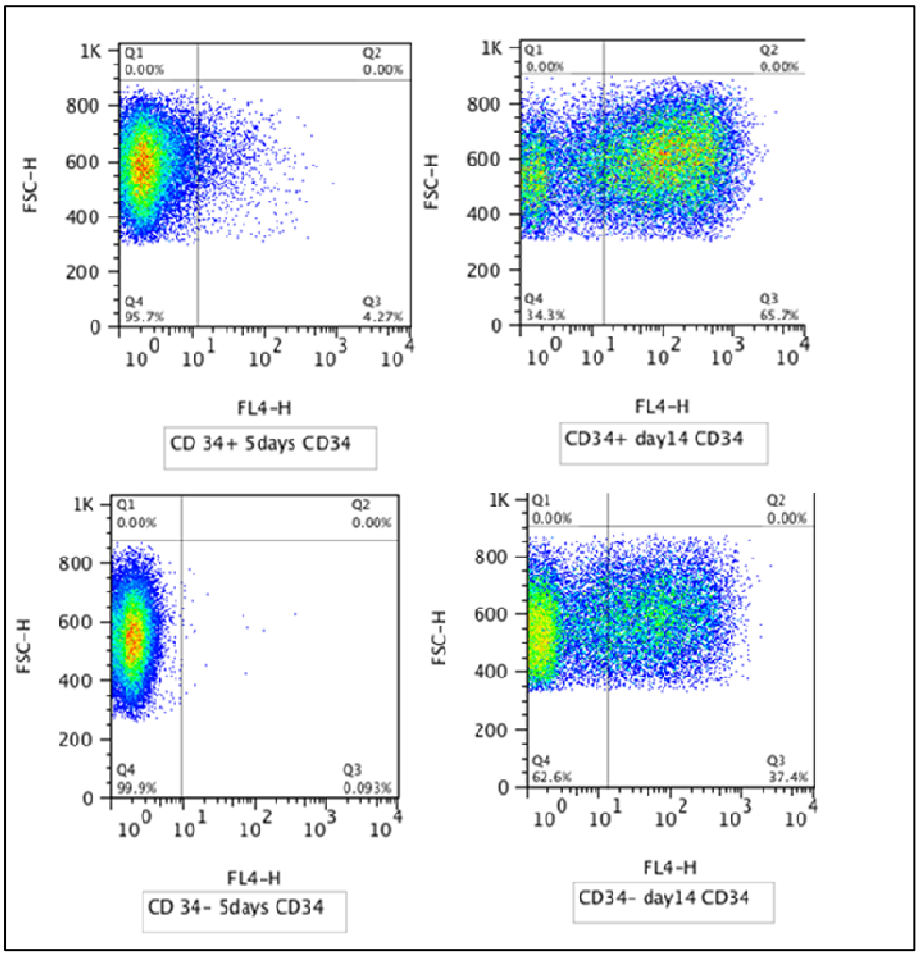


Figure I.13. FACS data of a representative experiment. On the top there are two plots of CD34 + population: on the left after 5 days from the FACS sorting and on the right after 14 days of culture. At the bottom, two plots are the characterization of CD34- populationafter 5 days and 14 days from the sorting respectively of the left and on the right.

In summary, our results show that CD34 expression does not identify a early progenitor population with Unpass ASC, but that it can be modulated according to culture conditions.

Different adhesion properties

Phenotypical characterization showed that Unpass e Pass cells differ in the expression of integrins, which have been involved in the interaction with the extracellular matrix.

In particular, Unpass cells only express low levels of α5 (Fibronectin receptor) and β1, whereas Pass cells express all integrins, including collagen receptors, and especially higher levels of α5. Surface marker analysis were confirmed by adhesion assays (Figure I.14).

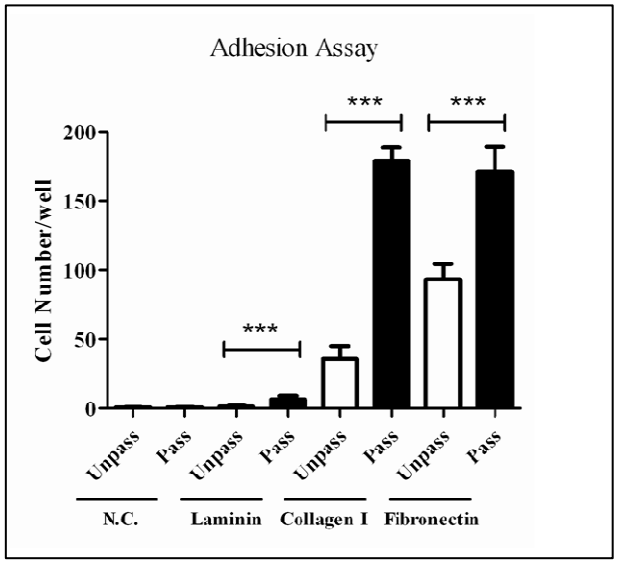


Figure. I.14. The graph represents the adhesion assay data obtained from three different donors. It highlights the difference in the adhesion between Unpass and Pass cells. Analysis was done using 1way ANOVA followed by Bonferroni's comparative multiple test (p<0,001).

To investigated whether Unpass ASC displayed a similar phenotype compared to freshly isolated ASC, SVF cells were analyzed for the expression of integrins. Interestingly, similarly to Unpass cells, Stroma Vascular Fraction cells only express integrin α5 (involved in fibronectin interaction) and β1 (involved in the heterodimerization and activation of integrins) and both at low levels,. Also in this case we correlated FACS results with adhesion functional assay. (Figure I.15b).

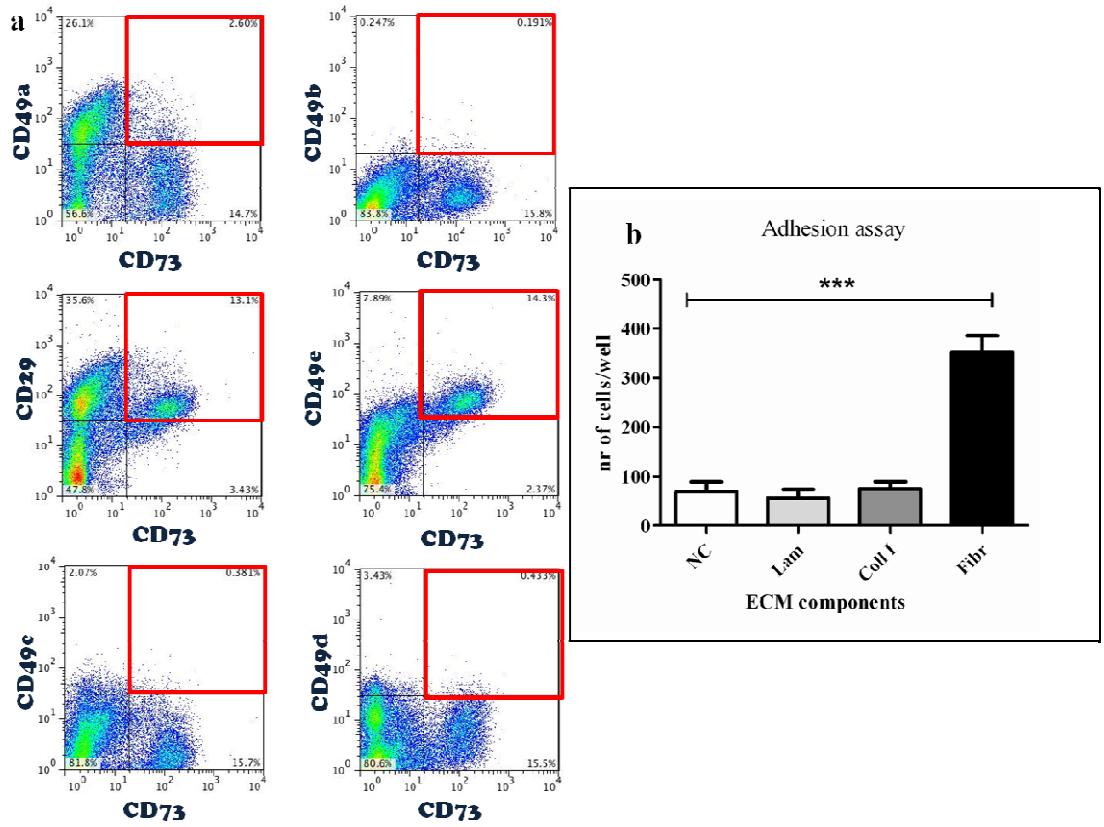


Fig. I.15 a) FACS data plots of StromaVascular Fraction (SVF). The double staining with CD73 and integrin described the level of expression in ASC just isolated. b) Functional experiment of adhesion assay which confirmed FACS data. As negative control we coated plate with 3% light milk. Data are analysed using 1way ANOVA followed by Bonferroni's comparative multiple test (p<0,0001). The results are representative of three different donors.

The expression of CD49e (integrin α5) in SVF cells suggests that *in vivo ASC* might be in contact with a matrix rich in fibronectin. Vessel lumens are rich in fibronectin and recently ASC have been localized in a perivascular position (Maumus *et al*., 2011). To show CD49e expression *in vivo*, human fat tissue was stained for α5 integrin and for fibronectin. As shown in Figure I.16 cells positive for α5 (red cells) were localized close to vessels (fibronectin coating, green), confirming a perivascular position of ASC *in vivo*.

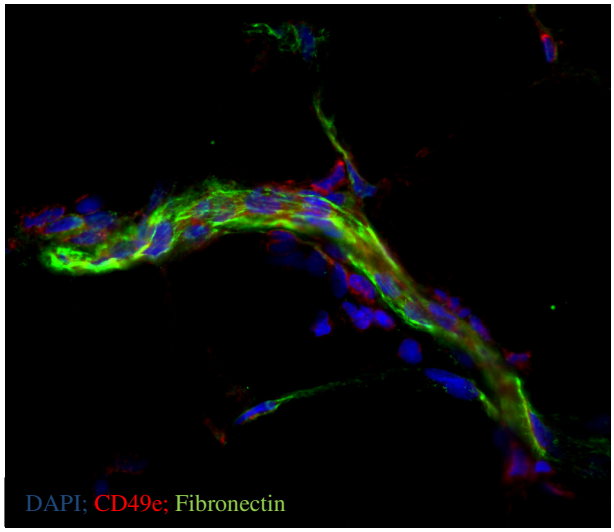


Figure. I.16. Micrograph represents a vessel in human adipose tissue. In green is stained fibronectin fibers which cover the blood vessel, while in red are stained cells positive for the expression of α5. (Blue DAPI for nuclei).The image was acquire with 60X objective.

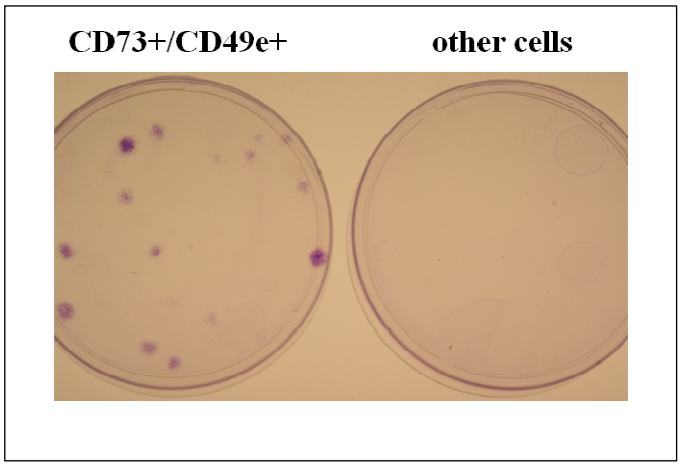


Figure I.17. Images of colonies-forming unit-efficiency after the sorting of stroma vascular fraction according to their expression of CD73 and CD49e. The images are representative of two different donors.

These results taken together indicate that Unpass ASC preserve their progenitor properties, such as clonogenicity, gene expression and differentiation ability. Moreover, Unpass ASC display a similar phenotype as compared to freshly isolated ASC. Furthermore, we investigated α5 role in the adhesion in SVF cells and we showed that fibronectin receptor is involved in the first adhesion to plastic (data not shown).

In order to determine whether α5 integrin could be used to isolate ASC from SVF cells, we performed a sorting according to the double expression of CD49e (fibronectin receptor) and CD73 (mesenchymal marker). We found that only CD49e+/CD73+ cells are the clonogenic cells (Figure I.17)

Discussion

MSC have raised the interest of the scientific community for their capacity to extensively proliferate *ex-vivo* and to differentiate into the main mesodermal lineages.

Although MSC were firstly identified in the bone marrow, more recently adipose tissue has been described as a promising source of pluripotent progenitors because of its abundance, easy accessibility and minimal donor site morbidity In bone marrow only the 0,01-0,001 % are mesenchymal stromal cells while in liposuction this percentage is increased up to 3% (Fraser *et al.,* 2008). Adipose derived MSC (ASC) are isolated from the stromal vascular fraction (SVF), which is the result of enzymatic dissociation of adipose tissue obtained from liposuction procedure. The SVF contains different cell type: leukocytes, erythrocytes, vascular endothelial, pericytic cells but also multipotent mesenchymal cells, which are referred to as adipose derived stem/stromal/progenitor cell (ASC) (Scherberich, *et al*, 2013). These cells showed similar biological features as compared to bone marrow derived MSC.

In vivo ASC are localized close to blood vessels (Maumus *et al*., 2011; Zimmerlin, *et al*, 2013), but it still remains unclear whether the microenvironment plays a role in the maintenance of their progenitor properties.

During the expansion phase *in vitro,* ASC are seeded on plastic until they reach a sub-confluent density (around 60-70%). They are then enzymatically detached from the flasks and re-seeded at a specific cell density in new flasks. This process does not allow the formation of the extracellular matrix which is continually disrupted and does not reproduce the *in vivo* situation. The aim of the present study was to establish a more physiological environment allowing cell-to-cell and cell-to-matrix interactions. For this purpose ASC were cultured for 28 days without passaging after reaching confluency. Our results show that Unpass ASC could better preserve their clonogenic and differention capacity compare to ASC which underwent standard *in vitro* expansion. In our project was also important to define whether Unpass ASC display a similar phenotype compared to freshly isolated ASC. Several reports have described phenotypic characteristics of ASC after expansion *in vitro* (Braun *et al.*, 2012; Goessler *et al.*,

2008) and controversial results have been described for ASC phenotype *in vivo.* Two recent papers have described that ASC *in vivo*, are located close to blood vessels (Da Silva Meirelles, *et al*, 2009; Maumus *et al*., 2011; Zimmerlin *et al*., 2013). Maumus firstly identified the expression of CD34 in ASC *in vivo.* This sialomucin was identified as marker of vascular endothelial progenitor cells (Andrews*, et al*, 1989; Nielsen & McNagny, 2008) but very little is known about its possible role in adipose derived cells. It was observed that CD34 is expressed only *in vivo* while adipose stromal cell lose to express it during expansion and proliferation *in vitro.* Interestingly, in Unpass ASC 20% of cells expressed this marker, suggesting that the maintenance of *in vivo* properties like higher clonogenicity, stemness gene expression, and differentiation could be linked to the expression of this glycoprotein. However, the results obtained have refuted this hypothesis: CD34+ cells did not display increased clonogenic capacity, nor a higher ability to differentiate in mesodermal lineage.

From the analysis of phenotypic characteristics of ASC freshly isolated from adipose tissue, we observed that they express only integrin α5, which recognize the fibronectin - RGD sequence. In adipose tissue we found cells positive for this integrin (Figure I.16) close to blood vessel suggesting that these cells are ASC.

Integrins are cell surface receptors involved in adhesion of the cell and play a major role in the connection between extracellular matrix and cell. Cellular function and phenotype are influenced by external stimuli, like fluid flow, pressure variations, vibration dynamic strain or changing in the microenvironment, and they are transmitted to the cells by the activated integrins. Different manuscripts described that integrins are differently regulated in mesenchymal stromal cell as response to various conditions, like differentiation, oxygen concentration, and so on (Hidalgo-bastida, *et al.*, 2010; Salasznyk,*et al.*, 2004; Saller *et al.,* 2012).

Surprisingly, Unpass cells, like freshly isolated ASC , only express integrin α5. On the contrary, the expression of the α1-α6 integrins, which are involved in the binding to collagen, laminin, and vitronectin, was observed in Pass cells. Moreover, Unpass cells and ASC from SVF express integrin α5 at lower level compared to the Pass condition. In literature it has been reported that non adherent BM-MSC express low level of integrin α5 and they correlated this low expression with an higher osteogenic potential (Baksh, *et al*, 2007). Furthermore, Yu and coworkers demonstrated in a recent paper

that the integrin α6 regulates proliferation and differentiation capacities through PI3K/AKT/p53 activity. They revealed, also, the crosstalk between the pluripotency genes OCT4 and SOX2 and CD49f in hMSCs and hESCs. (Yu *et al.*, 2012). Some preliminary results obtained in our study (data not shown) show that when Unpass ASC are induced in osteogenic differentiation they start to express more integrin α5, which is in accordance with data from literature (Hamidouche, *et al.*, 2010; Fromique *et al.,* 2012). These results can strengthen our hypothesis that, when ASC are cultured in Unpass conditions, they display a less committed committed phenotype. The data of colony formation of SVF cells after double sorting for CD73 (marker of mesenchymal stromal cells) and CD49e (integrin α5) confirmed that ASCs positive for CD49e are clonogenic.

To support our work another interest aspect is that Unpass ASC like ASC *in vivo* do not express CD105, which is instead upregulated during *in vitro* culture on plastic (Data not shown) (Braun et al., 2012b). This membrane protein is known as co-activator of TGF-β1 pathway and it seems to correlate with a more committed phenotype. Taken together, these aspects suggest that culture of ASC without disruption of the extracellular matrix, but promoting cell-to-cell and cell-to-matrix interactions, can preserve their stemness and phenotypic properties.

These results are important from a biological point of view because they suggest a fundamental role of the *in vivo* microenvironment in the maintenance of ASC progenitor features. From a clinical point of view, instead, our data suggest alternative culture conditions to expand ASC *ex vivo* and increase the performance of these cells in regenerative medicine applications.

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PART II

Labeling of Human Mesenchymal Stromal Cell with a new generation of magnetic nano-particles: effect on cell biology.

Aim of the project

In the last twenty years there has been an increase of interest in mesenchymal stromal cell (MSC) and their possible application in tissue engineering and regenerative medicine. Adult stem cell transplantation provides a new strategy for the treatment of a wide range human diseases and organ failure, indeed. The plasticity of MSC has been fully investigated and their ability to differentiate in different lineages, such as osteoblasts, adipocytes, or chondrocytes, is essential to regenerate musculoskeletal tissues.

Mesenchymal stromal cell can be isolated from different tissues such as bone marrow or adipose tissue and they have been described to repair damaged tissues *in vivo.* Moreover, MSC display immunosuppressive activity, being able to: inhibit T cell proliferation *in vitro*, inhibit the function of both naïve and memory T cells, suppress the development of monocyte-derived dendritic cells in an *in vitro* system (Zhang *et al.*, 2004). Furthermore, several studies have proved that allogeneic MSC have been transplanted without graft rejection or major toxicities (Reinders *et al*., 2013; Ryan, *et al.*, 2005).

At the moment, many studies aim to determine the fate of mesenchymal stromal cells after transplantation. *In vivo* tracking of stem cells is crucial for assessing their homing, migrational dynamic, differentiation processes and regeneration potential. Super-paramagnetic iron oxide nanoparticles (SPION) have been frequently used to track MSC *in vivo* because of their biocompatibility and their traceability with non-invasive magnetic resonance (Guo *et al.,* 2012). The goal of this project, is to label human mesenchymal cell with magnetic nano-particles in order to load them on magnetic scaffolds upon application of a magnetic field. For this reason prof. Matteo Santin and coworkers have developed a new generation of magnetic nano-particles. The novelty in this case is the envelope. Indeed, the Fe^{+} central core of magnetic nano-particles is clad by a layer of hyperbranched poly(epsilon-lysine) dendrons (G3CB) (Galli *et al.*, 2012). G3CB interact with the glycocalix of the cellular membrane avoiding the internalization of the beads. The hypothesis is that if the beads are not internalized by the cells they

cannot have cytototoxic effect or modify the abilities of mesenchymal stromal cell to proliferate and differentiate.

We first focused on the optimization of the labeling protocol. Then we investigated the possible cytotoxic effects of G3CB MNP on proliferation and differentiation potential of human MSC.

Materials and Methods

Bone marrow derived mesenchymal stromal cell (MSC) culture

MSC are obtained from bone marrow aspirates of three different donors. Briefly, bone marrow aspirate was diluted one to one using phosphate buffer (PBS) solution and centrifuged for 5 min at 2000 rpm. After the centrifugation, it was discarded the same volume of PBS previously added and the cells are counted after staining with crystal violet solution using a Neubauer chamber. Mononuclear cells are then seeded in plastic dishes at a density of 2,5 $*10^6$ cells/60mm dish and mesenchymal stromal cell are obtained according to their characteristic to adhere to plastic.

MSC are expanded complete medium (CM), consisting of α-MEM supplemented with 10% of foetal bovine serum (FBS), 1% HEPES, 1% Sodium pyruvate and 1% of Penicillin-Streptomycin-Glutamin (100x) solution (all from Gibco), and conditioned with FGF-2. At a sub-confluent density, cells are washed once with PBS, detached using trypsin 0,05% (Gibco) for 5min at 37% and its activity is blocked by complete medium. The cellular suspension is centrifuged at 1500 rpm for 3 min. The pellet is re-suspended in CM and counted using a Neubauer chamber. The MSC are seeded at 1700 cells/ cm^2.

Labeling of hMSC with G3CB-MNP nano-particles

To re-suspend the magnetic nano-particles (G3CB, MNP), we added 0,5mL of Ethanol 100% in eppendorf containing 3,5 mg of beads and we placed the tube in an ultrasonicating bath and we left to allow an homogenous solution. Occasional vortexing during the ultrasonication accelerated the re-suspension.

Meantime, we detached MSC, as previously described, and counted them. Cells were resuspended in a 50mL tube with an appropriate volume of medium (CM) to reach a final concentration of $4*10^4$ cells/mL.

The following step was to add to each tube the amount of MSC to reach the appropriate quantity for the number of cells. We tested three different concentrations of

beads: LOW (21,6 μg/mL for $3*10^4$ cells), MEDIUM (43,2 μg/mL for $3*10^4$ cells) and HIGH (86,4 μg/mL for $3*10^4$ cells).

The cells were incubated at 37 °C, 5% CO_2 on an orbital shaker (100 rpm) for three different time points: 5 min, 15 min and 30 min.

At the end of the incubation it was applied a magnetic field for 10 min to separate magnetized from non-magnetized cells and after we collected in a new tube the medium containing non-magnetized cells.

Magnetic and non magnetic cells were centrifuged for 5 min at 1500 rpm and then both cells were counted to identify the percentage of magnetized cells.

We re-suspended the magnetized cells in CM supplemented with FGF-2 and seeded in tissue culture dishes.

Evaluation of cytotoxicity

To value the cytotoxic effect of the labeling on MSC, after the labeling we seeded approximately 6 $*10^3$ cells/well in a 6 well plate. After 24hr, 48hr and 72hrs from the labeling we discarded the medium and incubated the cells for 20 min at 37 °C 5% CO_2 with Propidium Iodide solution (Sigma Aldrich) diluted 1:20 with CM-FGF-2. At the end of the incubation the medium was changed and we observed cells using microscope (Olympus IX50 con camera Color View Olympus) at bright light and using UV lamp.

Next step was count live and dead cells for each field and quantify the viability percentage .

Proliferation

After the labeling magnetized-MSC were seeded in tissue culture dishes at the density of 3000 cell/cm^2.

To value a possible effect of the labeling on the cell proliferation we allowed the proliferation rate for later two passages.

To calculate the proliferation rate we used the following rule:

$\mathbf{Log_2 (N/ N_0)/t}$

Where **N** is the number of cells obtained after a fixed time,

$\mathbf{N_0}$ is the number of cells seeded at time 0,

And **t** is the time between two passages.

Osteogenic Differentiation

Osteogenic differentiation was induced in 2D cultures as previously described (Jaiswal, *et al.*, 1997). Briefly were seeded in 6-well plate (TPP, Switzerland) at a density of 3000 cells/cm^2 in α-MEM supplemented with 10% FBS to reach the confluency, then we started the osteo-induction using medium composed by α-MEM supplemented with 10% FBS, 10mM β-glycerophosphate (Sigma), 10nM dexamethasone (D-2915, Sigma) and 0,1mM L- Ascorbic acid-2-phosphate (Sigma). We induced cells for three weeks changing medium twice per week.

Readout: Osteogenic differentiation was evaluated using Alizarin Red staining (Sigma Aldrich) which show extracellular hydroxyl-apatite deposits. Briefly, after 14 days of differentiation osteogenic medium was discarded and cells layer wash once with PBS and fixed with 4% formalin for 10 min at room temperature. At the end of the fixation step, cell layer was rinsed extensively with distill H_2O. Meantime, 2% alizarin red solution is made, dissolving the power in distill H_2O filter the solution with paper filter. Before to use it, we checked the pH of the solution would be 4.1-4.3.

Cells layer was incubated with Alizarin red solution for 10 min at room temperature and when the solution was removed we washed twice with ethanol 100% and we acquired representative fields using contrast microscope (Olympus IX50, Color View Olympus)

Read out: The quantification of the total calcium present in the matrix produced by MSC during osteogenic differentiation was evaluated using a dispensable kit (Calcium quantification kit CA-590, RANDOX). Briefly, the monolayer of cells after two weeks of induction was washed twice with PBS and HCl 0,5N in each well was added and cells were scratched using a tip. The cell lysate obtained was transferred in a eppendorf and they were shaken for 3 hours at 4°C on a orbital shaker. At the end of the incubation the solution was spun at 1000 rpm for 5 min. The supernatant was collected in a new tube and stored at -20°C until the analysis. We prepared a working solution with the equal part of two component: component R1 (calcium buffer) and component R2 (Calcium chromogen). In a 96 well plate we distributed 5µL of cell lysate and then added 195µL of the working solution and then the absorbance was read at 575 nm. The

standard curve was prepared using Calcium/Phosphate- CaP at 5 different concentration 0-100 μg/mL and it was treated as a sample.

Adipogenic Differentiation

Adipogenic differentiation was induced in 2D cultures as previously described (Barbero, *et al.*, 2003). Briefly, cells were seeded at a density of 3000 cells/cm^2 and cultured in complete medium until the confluence. The medium was then supplemented with 10μg/mL Insulin (provided by the Hospital), 1μM Dexamethasone, 100μM indomethacin (I-7378, Sigma) and 500μM 3-isobutyl-1-methyl-xanthine (Adipogenic induction medium) for 72 hours and subsequently with 10μg/mL insulin (adipogenic maintenance medium) for 24 hours. The 96-hour treatment cycle was repeated four times.

Readout: Adipogenic differentiation was evaluated using Oil Red O staining (Sigma), which shows the presence of triglyceride deposits. In brief at 21 days from the adipogenic induction, MSC cultures were washed with PBS, fixed in formalin 4% for 10 min and stained with three volumes of Oil Red O (0.3% in isopropanol) and two volumes of H_2O for 15 min at room temperature. Adipogenic differentiated cells were recognized by their characteristics being round shaped and containing lipid droplets and we acquired representative fields using contrast microscope (Olympus IX50 Color View Olympus) To perform the quantification of triglyceride accumulation, Oil Red O was solubilized with 100% isopropanol and the optical density was measured with a spectrophotometer at 500 nm. Experiments for the quantitative assessment of adipogenic differentiation were performed in triplicate in cells from different donors (Donzelli *et al*., 2011).

Results

Optimization of cell labeling

The labeling of mesenchymal stromal cell with G3CB -functionalized magnetic nano-particles is a very important step to be optimized. In a recent publication, the labeling with MNP was performed on adherent cells at different time points, up to 24h of exposure (Jasmin *et al.,* 2011).

Our hypothesis, instead, was that the G3BC functionalization could shorten the labeling time and that the attachment of the particles to the membrane would avoid their internalization. As control, non-functionalized MNP (only Fe^{+} core) were used. Moreover, the labeling was performed on cell suspension to test its feasibility in a potential intra-operative approach. To evaluate the efficiency of the labeling and the possible differences between G3CB-MNP and non-functionalized MNP, we tested three different time points, 5, 15 e 30 minutes.

After 5 min of exposure more than 60% of MSC were labeled with the G3CB-MNP and this percentage increase up to 80% after 15 min, remaining stable over time (Figure II.1). On the contrary, only 20% of cells were labeled with non-functionalized MNP and this percentage did not increase even after 30 min of labeling (Figure II.1).

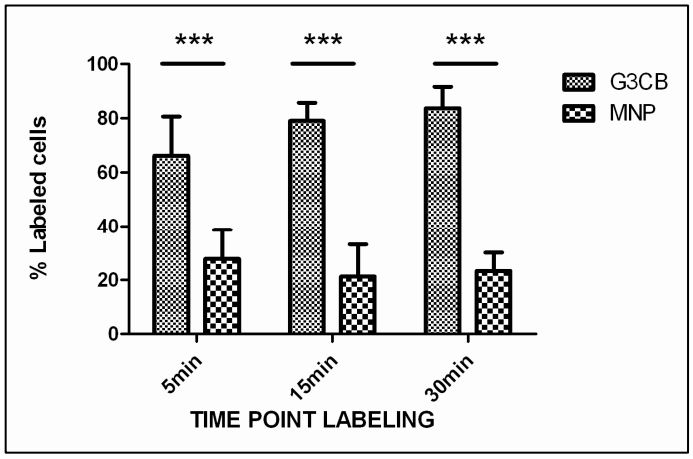

Figure II.1 Labeling of human mesenchymal stromal cell after 5, 10 and 15 minutes of exposure to G3CB or MNP. Data were analyzed using 2way ANOVA test followed by Bonferroni post test (p<0,01).

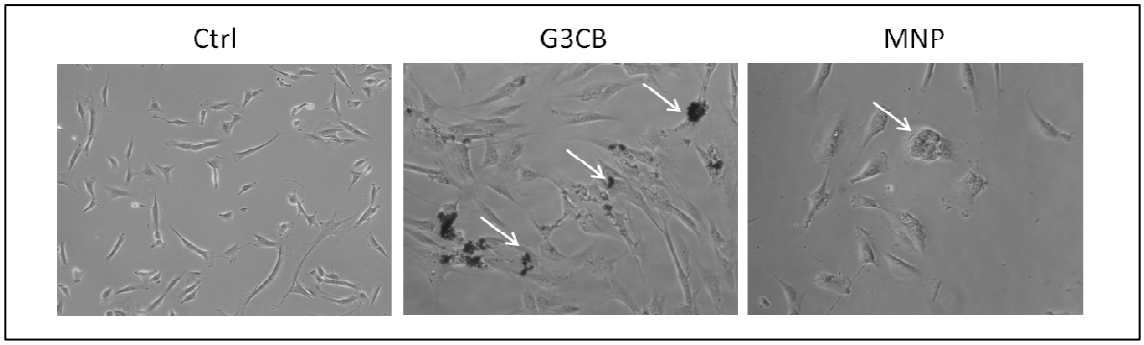

Figure II.2. Representative micrographs of mesenchymal stromal cell after the labeling with G3CB-MNP and not functionalized MNP. As control we took a picture of not labeled MSC. The arrows show in particular the magnetic nano-particles in both cases.

As shown in Figure II.2, G3CB-functionalized beads were able to aggregate on the cell membrane, due to the presence of poly-lisine dendrons. Cell morphology did not change compared to control (not labeled) cells. In the presence of non-functionalized MNP no aggregates were observed, but the MNP were internalized affecting cell morphology.

To assess whether the concentration of MNP could affect the labeling process, we tested three different concentrations of G3CB (defined as low, medium and high). As shown in Figure II.3, after 30 min of exposure more than 80% of MSC were labeled with all of the three concentrations of G3CB-MNP tested, indicating saturation of the process even at the lower concentration.

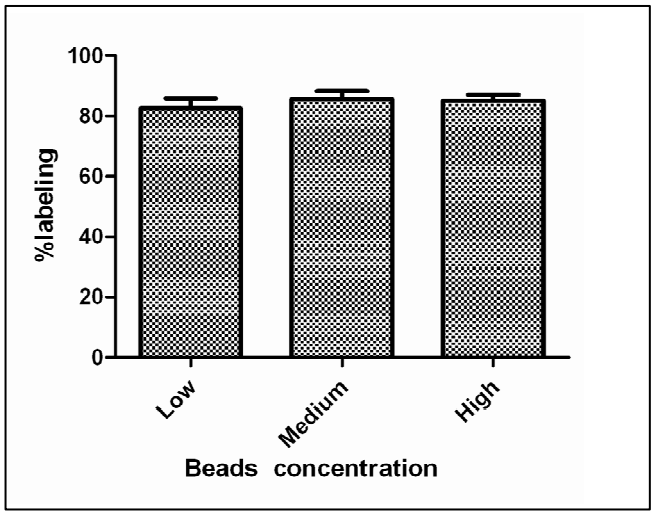

Figure II.3. The graph represents the percentage of labeled cells after 30min of exposure to three different G3CB-MNP concentrations.

Labeling of MSC with G3CB-MNP has not a cytotoxic effect

To investigate whether exposure of MSC to G3CB-MNP was cytotoxic, MSC viability was investigated from day 1 to day 3 after the labeling.

As shown in Figure II.4, MSC viability was around 100% , indicating that G3CB-functionalized MNP did not affect MSC survival.

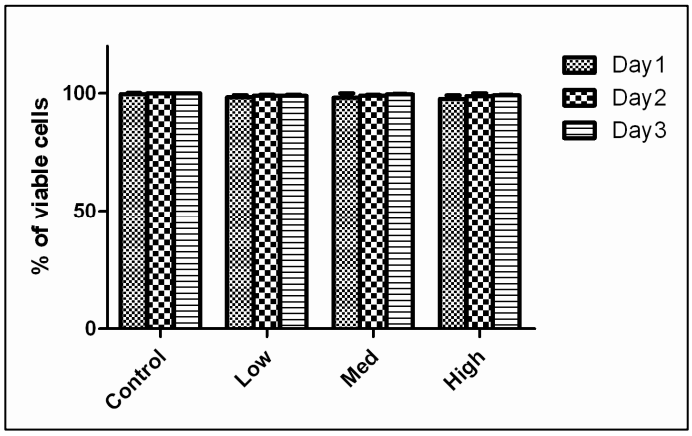

Figure. II.4. The graph represents the results the % cell viability of MSC exposed to different concentrations of G3CB three days after the labeling.

G3CB-MNP labeling does not affect the MSC proliferation

With the next experiment we evaluated if MNP-labeling of MSC can alter their ability to proliferate.

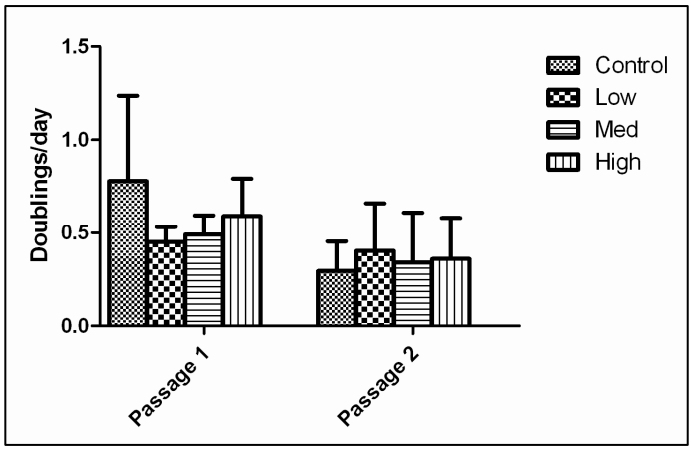

Figure II.5. A schematic representation of the MSC proliferation. Data are representative of three indipendent experiments.

After labeling MSC were cultured for two passages and the proliferation rate was determined. The results obtained from three donors, confirmed that G3CB-functionalized MNP did not affect MSC proliferation (Figure II.5).

G3CB-MNP labeling does not influence differentiation potential of human mesenchymal stromal cell

MSC can be induced to differentiate toward different mesodermal lineages, such as osteoblasts, adipocytes, chondrocytes as well as non-mesodermal lineages (cardiac cells, neurogenic cells, and so on).The maintenance of their multipotency during *ex vivo* manipulation is fundamental for their application in regenerative medicine. Therefore, we investigated the effect of G3CB labeling also on the maintenance of the differentiation capacity. For this purpose, labeled MSC were exposed to osteogenic and adipogenic differentiation media.

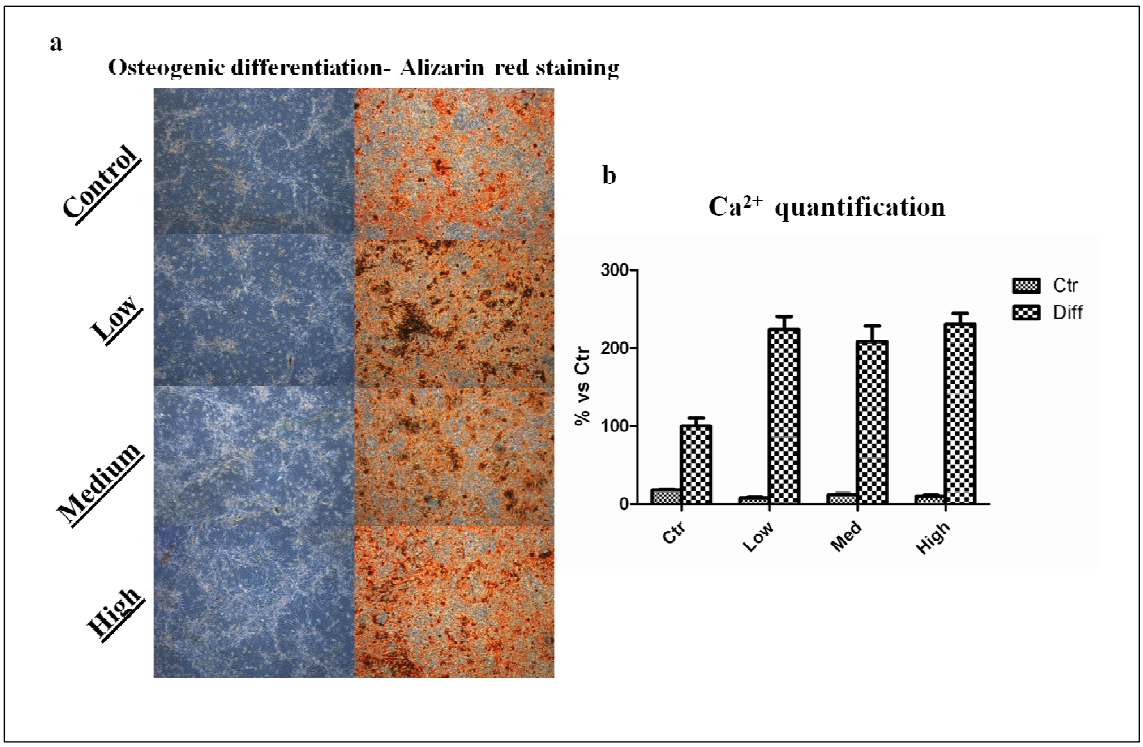

Figure II:6. a) Arizarin red staining of G3CB-functionalized mesenchymal stromal cell after two weeks of differentiation. (4X objective) Micrographs are representative of different experiments performed with cells from three donors. b) Quantification of the calcium deposited by MSC in the extracellular matrix during osteogenic differentiation.

Labeled MSC were able to differentiate towards to osteogenic lineage *in vitro*, as evidenced by calcium deposits stained by Alizarin Red (Figure II.6a) Differentiation was also assessed by the quantification of the calcium deposits in the matrix (Figure II.6b).

Under adipogenic conditions, MSC labeled with the G3CB MNP were also able to differentiate into adipocytes, at the same extent of control cells (Figure II.7).

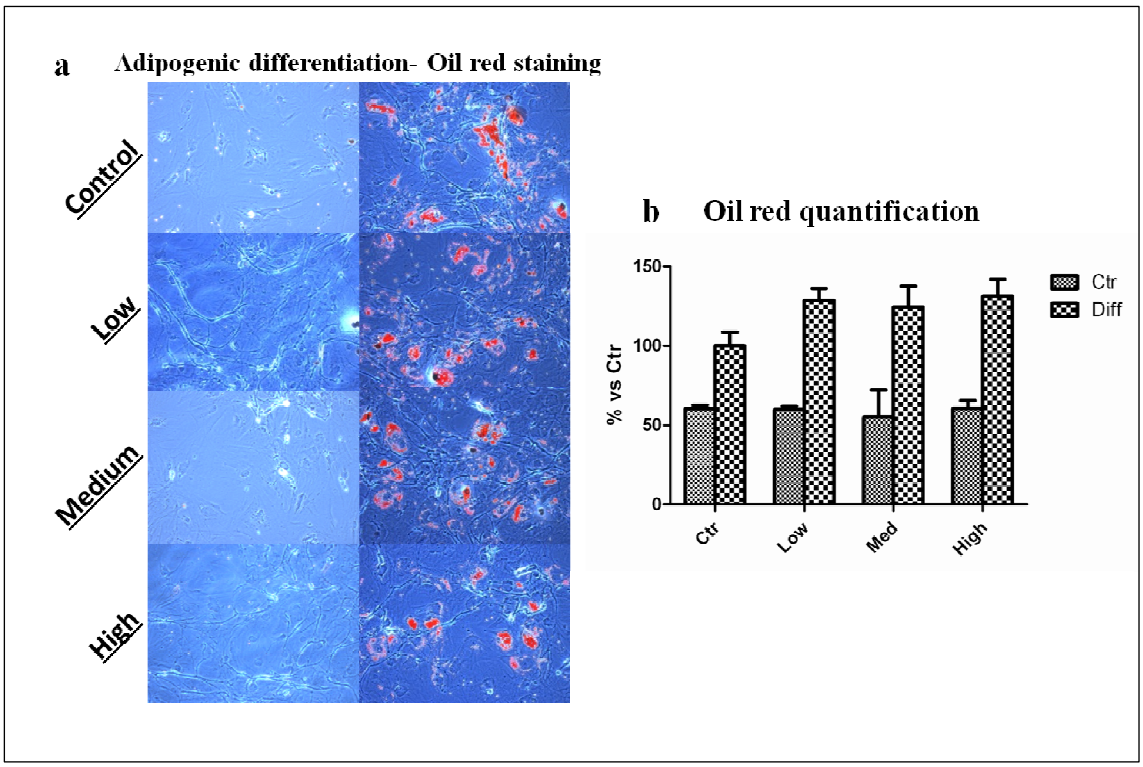

Figure II.7 a) Oil RED O staining of lipid droplets after four cycles of adipogenic diffentiation (10X objective). Micrographs are representative of different experiments performed with cells from three donors. b) Oil Red O was solubilized and optical density was read at 500 nm to obtain a quantitative assessment of adipogenesis. The experiment was performed in triplicate with cells from three different donors and data are expressed as mean+standard deviation.

These results together confirmed that the labeling of MSC with G3CB-functionalized MNP, does not affect their ability to differentiate toward the osteogenic and adipogenic lineage.

Discussion

Mesenchymal stromal cell are a promising candidate for regenerative medicine approaches. Critical-sized bone defects are generally caused by trauma, bone diseases, prosthetic implant revision or tumor excision. The consequent bone tissue loss cannot be repaired by physiological regenerative processes and mesenchymal stromal cell have been applied in clinical trials to improve tissue regeneration (Calori, *et al.,* 2011; Panseri, *et al.,* 2012).

The success of stem cell therapies in patients requires methods to assess their bio-distribution, their fate after infusion and their contribution to regenerate tissues. Magnetic Resonance Imaging (MRI) could be an excellent tool for high resolution visualization of the fate of MSC and recently several publications have focused on the combined use of mammalian cells and super-paramagnetic nano particles (SPION) (Ahrens, *et al*, 2003; Guzman *et al*., 2007). However, further investigation about the effect of SPION incorporation by stem cells is essential for their approval in clinical applications (Jasmin *et al*., 2011)

In this project, the effects of a new generation of magnetic nano-particles on MSC biology were investigated. These nano-particles, developed by the group of Matteo Santin (Brighton University), have a Fe^{+} core upholstered with a monolayer of hyperbranched poly(epsilon-lysine) dendrons (G3CB). This modification allows the interaction with the glycocalix of the cell membrane avoiding their internalization and all possible drawback effects resulting by the internalization.

The potential application of MSC labeled with G3CB MNP will be to develop a new method to seed cells on a magnetic scaffold applying a magnetic field.

The protocol used in this study is different from those previously described. Several publications focused on the labeling of mammalian cells with SPION. These methods apply long time of incubation (24 hours or more) and cells in adhesion (Guo *et al.,* 2012; Jasmin *et al.,* 2011). The main innovation with G3CB-functionalized nano-particles is that the time of incubation was significantly reduced, since the labeling of 60% of the cells was already obtained after 5 minutes of incubation. On the contrary,

only 20% of the cells were labeled with control beads (MNP). Moreover, cells were labeled in suspension on an orbital shaker, indicating that this procedure could be used in an intra-operative approach. The percentage of labeled cells could be increased up to 80% after 30 minutes of exposure and G3CB-MNP were always localized on the cell membrane. The number of cells labeled with control MNP, instead, remained stable at 20% even after a longer incubation, indicating that the time points tested were not appropriate to increase the percentage of labeling. The results obtained from the labeling of MSC with three different concentrations of G3CB MNP shows that, after 30 minutes of exposure, the percentage of labeled cells is similar in all concentrations, indicating saturation even at the lower concentration. Furthermore, assays to determine cytotoxicity, and maintenance of proliferation and differentiation capacity showed that labeling of MSCs did not affect their progenitor properties.

Our results show the feasibility of a potential application of G3CB nano-particles in clinical trials.

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