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ROLE OF EXTRACELLULAR MICROENVIRONMENT ON REGENERATIVE BONE TISSUE CAPACITY

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Mesenchymal stem cells (MSCs) are multipotent stromal cells capable of self-renewal and multilineage mesenchymal differentiation (Jackson, Nesti, & Tuan, 2012) (Figure 1). MSCs represent approximately 0.01% of mononuclear cells in the bone marrow, and provide functional and structural support to hematopoietic stem cells (HSCs) (Johnson & Dorshkind, 1986; Pittenger et al., 1999). MSCs were originally identified ex vivo in small numbers by plastic adherence from rat whole bone marrow cultures (BMMSC) (Friedenstein, Petrakova, Kurolesova, & Frolova, 1968). However, plastic adherence does not seem to represent an essential MSCs feature. Indeed, recent studies have shown the existence of a non-adherent MSC subpopulation with multipotency characteristics, capable to migrate to the site of injury, and of tissue regeneration (Leonardi et al., 2009; Z. L. Zhang et al., 2009).

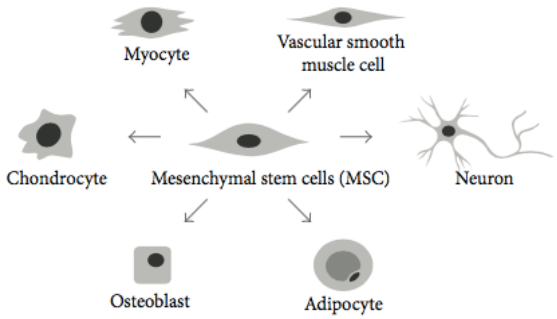

Figure 1. **MSCs multipotency**. MSCs can differentiate into osteoblasts, chondrocytes, adipocytes, astrocytes, myocytes, skeletal cells. (*James A. W. Review of Signaling Pathways Governing MSC Osteogenic and Adipogenic Differentiation. Scientifica (Cairo), v. 2013, p. 684736, 2013*)

MSCs can be virtually found in all postnatal organs and tissues, although they are most commonly isolated from bone marrow (BMMSC), adipose tissue (ADMSC), Wharton's jelly umbilical cord and dental pulp (DPSC) (Campagnoli et al., 2001; Caplan, 1991; In 't Anker et al., 2003; Nakahara et al., 1991; Zuk et

al., 2002). MSCs have been isolated and cultured from several species other than humans, including mice, rats, cats, dogs, rabbits, pigs, and baboons (Javazon, Beggs, & Flake, 2004). Phenotypically, MSCs express a number of markers, none of which, unfortunately, is specific to MSCs. It is generally agreed that adult human MSCs do not express the hematopoietic markers CD45, CD34, CD14, or CD11. They also do not express the costimulatory molecules CD80, CD86, or CD40 or the adhesion molecules CD31 (platelet/endothelial cell adhesion molecule-1), CD18 (leukocyte function-associated antigen-1), or CD56 (neuronal cell adhesion molecule-1), but they can express CD105 (SH2), CD73 (SH3/4), CD44, CD90 (Thy-1), CD71, and Stro-1 as well as the adhesion molecules CD106 (vascular cell adhesion molecule-1), CD166 (activated leukocyte cell adhesion molecule), intercellular adhesion molecule (ICAM)-1, and CD29 (Conget & Minguell, 1999; Galmiche, Koteliansky, Brière, Hervé, & Charbord, 1993; Haynesworth, Baber, & Caplan, 1992; Le Blanc, Tammik, Rosendahl, Zetterberg, & Ringdén, 2003; Pittenger et al., 1999; Sordi et al., 2005). It is generally accepted that all MSCs lack the pan-hematopoietic marker CD45 and the endothelial cell marker CD31, although MSCs from other species do not express all the same molecules as those on human cells (Peister et al., 2004). It is important to note that differences in cell surface expression of several markers may be influenced by factors secreted by accessory cells in the initial passages, and the in vitro expression of some markers by MSCs does not always correlate with their expression patterns in vivo (Gronthos, Simmons, Graves, & Robey, 2001). The mentioned variability in MSCs marker expression is also linked to variations in tissue source, method of isolation and culture, and species differences (Baddoo et al., 2003; Javazon et al., 2004).

Adult MSCs are widely studied in the prospective of clinical applications for their immunomodulatory properties and their ability to migrate to the injured site (homing), thus stimulating bone tissue regenerative process (Ciapetti, Granchi, & Baldini, 2012).

Bone tissue

Bone is a highly specialized form of connective tissue with a mineralized extracellular matrix made of calcium and phosphate in the form of hydroxyapatite, conferring marked rigidity and strength to the skeleton while maintaining some degree of elasticity. In addition to its supportive and protective functions, bone is a major source of inorganic ions, actively participating in calcium homeostasis in the body. Morphologically, bone tissue can be distinguished in cortical (compact) and cancellous (spongy). Cortical bone provides mechanical and protective functions, while cancellous bone is associated with metabolic functions (Horwitz et al., 2002). Bone is composed of different cell types, including osteoblasts, osteocytes, osteoclasts, and bone lining cells (Figure 2).

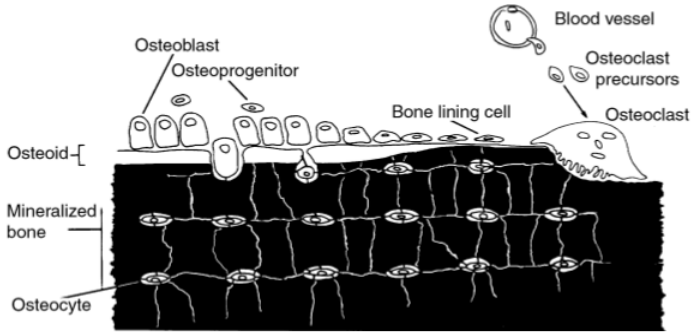

Figure 2. **Bone tissue with bone cells.** (*Marks S. C., Popoff S. N. Bone cell biology: the regulation of development, structure, and function in the skeleton. Am J Anat***,** *v. 183, n. 1, p. 1-44, Sep 1988*)

Osteoblasts, osteocytes, and bone lining cells originate from local osteoprogenitor cells, whereas osteoclasts arise from the fusion of mononuclear precursors, which originate in the various hemopoietic tissues. Osteoblasts, which mature into osteocytes, are responsible for the deposition of the mineralized

matrix and the secretion of the growth factors necessary for osteogenesis. Osteoclasts, derived from the monocyte-macrophage line, play a crucial role in the bone remodeling process by actively resorbing bone. Bone lining cells are flat, elongated, inactive cells covering bone surfaces that do not undergo either bone formation or resorption, but seem to act as precursors for osteoblasts (Chow, Wilson, Chambers, & Fox, 1998; Matic et al., 2016). The bone extracellular matrix is composed for approximately 95% by Type I collagen, and for the remaining 5% by proteoglycans and numerous noncollagenous proteins, such as osteocalcin, osteopontin, and bone sialoprotein (Kwan, Slater, Wan, & Longaker, 2008).

There are two processes resulting in the formation of normal bone tissue, or osteogenesis, both involving the transformation of a preexisting mesenchymal tissue into bone tissue. *Intramembranous ossification* is the direct conversion of mesenchymal tissue into bone. This process occurs mainly in the bones of the skull. Endochondral ossification is the process by which the mesenchymal cells differentiate into cartilage, and this cartilage is then replaced by bone.

The process of intramembranous ossification starts when a small group of MSCs begin to proliferate and form a small, dense cluster of cells, called nodule. Then, MSCs inside the nodule stop replicating and acquire morphological changes: the cell body becomes larger and rounder, the long thin cell processes disappear and the amount of Golgi apparatus and rough endoplasmic reticulum increase. Eventually, all of the cells within the nodule develop into, and show the morphologic characteristics of, an osteoprogenitor cell (Brighton & Hunt, 1991). Then, at this stage of development, osteoprogenitor cells may undergo further morphological changes that lead them to finally become osteoblast. In the process of bone formation, osteoblasts function in groups of connected cells. As mentioned above, osteoblasts secrete a collagen-proteoglycan matrix that is able to bind calcium salts. Through this binding, the prebone (osteoid) matrix becomes calcified, osteoblasts become osteocytes, and the osteoid mineralizes, giving rise to a nodule that contains osteocytes. The nodule has now become a rudimentary

bone tissue. Furthermore, the entire region of calcified spicules becomes surrounded by compact mesenchymal cells that form the periosteum, a membrane that surrounds the bone. The cells on the inner surface of the periosteum become osteoblasts and deposit osteoid matrix parallel to that of the existing spicules. In this manner, many layers of bone are formed.

Endochondral ossification is the process responsible for long bones development. In this process, blood vessels invade cartilage matrix at the center of the template, bringing this way osteoblasts for producing bone. After skeletal maturity, bone replaces most of the cartilage template, while, at the ends of the bone, it persists as the joint surfaces. The growth plate, that is a cartilaginous area localized near the ends of the bone between the cartilaginous epiphysis and the newly generated bone in the metaphysis, is responsible for longitudinal bone development during skeletal growth. The growth plate contains inside cells called chondrocytes and it is surrounded by the perichondrium, a layer of dense connective tissue. Through the resting, proliferating, prehypertrophic and hypertrophic stages, the chondrocytes experience a coordinated process of differentiation and eventually programmed cell death, arranging the scaffold on which new bone is formed. At the same time, blood vessels continue to bring in new osteoblasts to elongate the bone (Kronenberg, 2003).

MSCs immunomodulatory properties

MSCs have been reported to regulate the immune response in many diseases (Denburg, Inman, Sehmi, Uno, & O'Byrne, 1998; Puissant et al., 2005; Siegel, Schäfer, & Dazzi, 2009; Uccelli, Moretta, & Pistoia, 2006). Numerous reports have shown that adult MSCs can affect the immune T- and B-cell response: they suppress T-cell proliferation, cytokine secretion and cytotoxicity and regulate the balance of Th1/Th2 (Glennie, Soeiro, Dyson, Lam, & Dazzi, 2005; Puissant et al., 2005; Yañez et al., 2006). Adult MSCs regulate the functions of regulatory T cells (Tregs) (Selmani et al., 2008), increase B-cell viability but may also inhibit their proliferation and arrest the cell cycle; in addition, MSCs affect the secretion of antibodies and production of co-stimulatory molecules of B cells (Corcione et al., 2006). MSCs inhibit the maturation, activation and antigen presentation of dendritic cells (Burchell, Strickland, & Stumbles, 2010; Ramasamy et al., 2007), and interleukin-2 (IL-2)-induced natural killer (NK) cell activation (Spaggiari, Capobianco, Becchetti, Mingari, & Moretta, 2006).

Interestingly, current evidence suggests that MSCs exert variable immunomodulatory effects on the same types of immune cell depending on the local microenvironment or disease status. For example, MSCs decrease the Th1 response in patients with acute graft versus host disease (GvHD) (Le Blanc et al., 2004)and autoimmune diseases such as systemic lupus erythematosus (SLE) (Rafei et al., 2009). However, BMMSC lead to a shift from Th2 to Th1 responses in airway allergic inflammatory diseases, including allergic rhinitis (K. S. Cho & Roh, 2010) and asthma (Bonfield, Nolan Koloze, Lennon, & Caplan, 2010). Inflammatory conditions also have been proven to change immunomodulatory gene expression in MSCs or promote the cell–cell contact effect, resulting in an enhanced immunosuppressive response (Ren et al., 2010). These observations suggest that MSCs are capable of switching their effects to protect the body from disease in different situations.

Although the underlying mechanisms of MSC immunomodulation have yet to be elucidated, they are likely mediated by soluble factors and cell contact-dependent mechanisms in response to immune cells.

It has been shown that MSCs regulate the adaptive and innate immune systems by suppression of T cells and maturation of dendritic cells, reducing B-cell activation and proliferation and inhibiting proliferation and cytotoxicity of NK cells, and promote the generation of regulatory T cells via soluble factors or cell–cell contact mechanisms (Fu et al., 2012; Yagi et al., 2010).

The effect of soluble factors on the activity of MSCs may vary depending on the origin of the MSCs, target cells and the microenvironment.

MSCs homing

Homing is the process by which cells migrate to, and engraft in, the tissue in which they can exert local, functional effects. The high concentration of inflammatory chemokines released in the tissues after the injury can modulate the MSCs migratory activity, due to their expression of chemokine receptors, such as CCR2, CCR3, CCR4 and CCL5 (Tondreau et al., 2009). It has been demonstrated that bone marrow derived MSCs express many integrins on their surface (De Ugarte et al., 2003), including high levels of integrin β1 and α4 (Ip et al., 2007), which mediate cell-cell and cell-matrix interactions through the binding to the vascular adhesion molecule (VCAM-1) and the fibronectin V region. Ruster *et al. (2006)* have shown that MSCs interact in a coordinated fashion with endothelial cells, involving activation of both selectin-dependent and integrin-dependent binding. The C-X-C motif chemokine 12 (CXCL12) or stromal cell-derived factor 1 (SDF1) has been shown to play a central role in MSC homing phenomena, through its specific receptor CXCR4. However, cell surface receptor levels of CXCR4 on MSCs has been found to be low, with large amounts intracellularly instead, suggesting a storage function of this chemokine. The CXCR4 receptor is likely to be translocated to the surface upon chemokine stimulation, and thus contributing to MSCs migration (Wynn et al., 2004).

The exact mechanisms regulating MSCs migration are still unclear, and need further investigation, mainly because of their great interest for clinical applications.

Differentiation potential of MSCs

In addition to the identification of MSCs based on their morphologic or phenotypic characteristics, a further way to identify supposed MSC populations is by their capacity to be induced to differentiate into bone, fat, or cartilage in vitro.

Osteogenic differentiation

The classic method for differentiation of MSCs to osteoblasts in vitro involves incubating a confluent monolayer of MSCs with ascorbic acid, β-glycerophosphate, and dexamethasone for 2–3 weeks. The MSCs form aggregates or nodules and increase their expression of alkaline phosphatase; calcium accumulation can be detected over time (Pittenger et al., 1999). These bone nodules stain positively by alizarin red and von Kossa techniques. These conditions, however, are unlikely to reflect the physiological signals MSCs receive that induce osteogenesis in vivo. At a molecular level, the osteogenic differentiation process is managed by specific transcription factors. Runt-related transcription factor 2 (Runx2), also known as core-binding factor subunit alpha-1 (Cbfa1), has a crucial role in osteoblast differentiation and bone formation (Komori et al., 1997; Otto et al., 1997). Mice with a homozygous mutation in Runx2 die just after birth and show complete absence of bone formation (Komori et al., 1997; Otto et al., 1997). Runx2 has also been shown to participate in early chondrogenesis, and to inhibit adipogenesis by interfering with peroxisome proliferation-activated receptor 2 (PPAR-γ) action (Sanchez-Gurmaches & Guertin, 2014). On the other hand, the transcription factor Osterix suppresses chondrogenesis, and triggers osteogenesis, in the late steps of the process. Low levels of Osterix are enough to inhibit chondrogenesis, while high levels are necessary to promote osteogenic differentiation (Oreffo, Kusec, Romberg, & Triffitt, 1999). Osteoblasts in adult animals are thought to originate from multipotent MSCs resident in the bone marrow. Recently, a number of markers have been postulated to identify this cell population. The best-characterized marker in human

bone marrow is CD146, which labels a perisinusoidal cell population (Sacchetti et al., 2007), while in mice, markers used to identify MSCs include CD51, CD105, CD90, stem cell antigen-1 (Sca1), platelet-derived growth factor α (PDGFRα), α-smooth muscle actin (αSMA), Nestin, Leptin receptor (Lepr), and relative combinations (Chan et al., 2015; Z. Kalajzic et al., 2008; Méndez-Ferrer et al., 2010; Pinho et al., 2013; Qian et al., 2013).

Adipogenic differentiation

To promote adipogenic differentiation, MSCs are typically treated in culture with dexamethasone, insulin, isobutyl-methylxanthine, and indomethacin. More recently, some groups have replaced indomethacin by adding rosiglitazone, hydrocortisone and triiodothyronine to the basic adipogenic ingredients isobutyl-methylxanthine and dexamethasone (M. J. Lee, Wu, & Fried, 2012; Lequeux, Auxenfans, Mojallal, Sergent, & Damour, 2009; Skurk, Ecklebe, & Hauner, 2007). There is an accumulation of lipid-rich vacuoles within cells, and they express PPAR-γ, lipoprotein lipase, and the fatty acid-binding protein aP2 (Pittenger et al., 1999). Eventually, the lipid vacuoles combine and fill the cells. Accumulation of lipid in these vacuoles can be detected histologically by oil red O staining. PPAR-γ plays a crucial role in the adipogenic differentiation process, by regulating specific adipocytes gene expression (Tang & Lane, 2012). PPAR-γ interacts with CCAAT/enhancer binding protein (C/EBP) family members in the latest stage of the adipogenic process(Tang & Lane, 2012).

Chondrogenic differentiation

To promote chondrogenic differentiation, MSCs are centrifuged to form a pelleted micromass and cultured in the presence of transforming growth factor-β (Mackay et al., 1998). The cell pellets develop a multilayered, matrix-rich morphology, and histological analysis shows strong staining with

toluidine or Alcian blue, indicating an abundance of glycosaminoglycans within the extracellular matrix (Kopen, Prockop, & Phinney, 1999). The cells also produce type II collagen, which is typical of articular cartilage (Pittenger et al., 1999). During the chondrogenesis, the gene SRY-box9 (Sox 9) interacts with the proteins Sox5 and Sox6 in order to induce chondrocyte proliferation, and the consequent cartilage matrix deposition and maturation (Gruber, Norton, Ingram, & Hanley, 2005). Sox9 is expressed in all chondroprogenitors and differentiated chondrocytes, and is essential for differentiating chondrogenic mesenchymal condensations into chondrocytes, as well as for regulating every stage of chondrocyte differentiation (Akiyama, Chaboissier, Martin, Schedl, & de Crombrugghe, 2002; Bi, Deng, Zhang, Behringer, & de Crombrugghe, 1999). The transforming growth factor β (TGFβ), as well, plays a relevant role in chondrogenesis, and has been found to increase its expression when administered together with the bone morphogenic protein 2 (BMP2) (Shen, Wei, Tao, Diwan, & Ma, 2009).

Differentiation to other lineages

It has also been demonstrated that, when treated with 5-azacytidine and amphotericin B, MSCs differentiate into myoblasts that fuse into rhythmically beating myotubes (Wakitani, Saito, & Caplan, 1995), leading to a better cardiac function in animal models of infarcted myocardium (Choi et al., 2010). In addition, differentiation into neuron-like cells expressing markers typical for mature neurons has been reported (Kohyama et al., 2001; Kopen et al., 1999). However, Hofstetter and colleagues has reported that these neuron-like cells lack voltage-gated ion channels necessary for generation of action potentials; therefore, these cells cannot be classified as true neurons (Hofstetter et al., 2002).

MSCs *in vivo* identification

The definition of MSCs relies solely on the analysis of *in vitro* culture-expanded cell populations. Despite years of intense investigation, there is a lack of data about the *in vivo* properties of the native MSCs within their tissues and niches, mainly because of the absence of specific markers allowing their clear identification (Bianco, Robey, & Simmons, 2008; Morikawa et al., 2009). Moreover, MSCs phenotype and abilities vary between *in vivo* and *in vitro* settings due to the removal from their natural environment and the use of chemical and physical growth conditions that might alter their characteristics. MSCs are known to undergo phenotypic rearrangements during *ex vivo* manipulations, losing expression of some markers while also acquiring new ones (Jones et al., 2002). Inserting the *in vitro* results in the context of the organism is difficult also because the exact location and functions of MSCs *in vivo* remain elusive (da Silva Meirelles, Caplan, & Nardi, 2008).

A valid method to study MSCs *in vivo* properties resides in the use of murine models. However, in the murine system, some of the MSCs antigens are not universal for all the mouse strains, thus complicating the establishment of a panel of common surface antigens, and the consequent prospective isolation of these cells from mice of different strains (Anjos-Afonso & Bonnet, 2011). There is a general consensus that, like in human cells, murine MSCs do not express most common surface antigens that are found on haematopoietic and endothelial cells, such as Ter119, CD45, and CD31 (Anjos-Afonso & Bonnet, 2008; Peister et al., 2004; Phinney, Kopen, Isaacson, & Prockop, 1999). Therefore, the common view is that murine mesenchymal progenitor cells are confined in the $CD45^{-}Ter119^{-}CD31^{-}$ fraction. Most studies looking at identifying skeletal stem or progenitor cell populations focus on cells isolated from the bone marrow, or total bone (Chan et al., 2013; Chan et al., 2015; Morikawa et al., 2009). A number of cell surface marker combinations have been used, although there are differences between different groups. There is some solid evidence supporting the idea that Sca1 could be a potential positive marker for the

identification of murine mesenchymal progenitor cells. For instance, $Sca1^{-/-}$ mice present age-related osteoporosis characterized by weakening in bone tissue (Bonyadi et al., 2003). Decreased osteoprogenitors, osteoblasts and bone formation are observed in these mutant mice, as the result of reduced numbers of MSCs. Also, $Sca1^{-/-}$ mice display reduced adipogenesis in vitro (Bonyadi et al., 2003). In addition to these findings, some reports have shown that the tri-lineage potential of cells isolated based on $Sca1^{+}$ expression alone (Lundberg et al., 2007; Morikawa et al., 2009; Nakamura et al., 2010; Steenhuis, Pettway, & Ignelzi, 2008) or in combination with other antigens is confined to the $Sca1^{+}$ fraction whereas the $Sca1^{-}$ fraction displays mainly osteo- and chrondrogenic potential (Lundberg et al., 2007; Morikawa et al., 2009; Nakamura et al., 2010; Steenhuis et al., 2008). Sca1 has been used in combination with other markers such as CD166 (Alcam), CD51/Alpha V (integrin alpha V) and PDGFRα to further enrich murine mesenchymal progenitor cells in vivo (Arai, Ohneda, Miyamoto, Zhang, & Suda, 2002; Kitaori et al., 2009; Lundberg et al., 2007; Morikawa et al., 2009; Nakamura et al., 2010). Morikawa *et al.* (2009) have demonstrated that the $CD45^{-}Ter119^{-}Sca1^{+}PDGFR\alpha^{+}$ bone marrow subpopulation, derived from C57BL/6 mice, is enriched with mesenchymal progenitor cells with tri-lineage capacity, whereas other cellular fractions have a more restricted differentiation potential, mainly lacking adipogenic differentiation capacity (Morikawa et al., 2009). Specifically, flow cytometry analysis of clones from single cells revealed that subpopulation with multilineage differentiation potential were positive for the human MSCs markers CD105 and CD90, unlike those with limited differentiation potential. This observation suggests that also CD105 and CD90 may be useful indicators for multipotency of cultured murine MSCs. The authors used digested adult murine bone marrow/bone associated cells isolated from tibias and femurs, which were stained with antibodies to PDGFRα, Sca1, CD45, and TER119, and analyzed by flow cytometry. Sorted cells for the different subpopulation were then cultured for a traditional colony-forming unit-fibroblast (CFU-F) assay, which showed that the number of colonies

per 1000 cells was highest in the double positive population $CD45^{-}Ter119^{-}Sca1^{+}PDGFR\alpha^{+}$; however, the cells in the other subpopulations still produced a significant number of colonies (Morikawa et al., 2009). Although this study provides interesting developments in the identification of mesenchymal progenitor cells in mice, it is necessary to notice that most of the study was performed using cells isolated from bone fragments after collagenase digestion, so the isolated cells are mainly bone-derived and not bone marrow-derived. Chan *et al.* (2015) investigated the lineage origin of the mouse skeletal stem cells, by flow cytometry analysis. The markers analyzed were CD45, Ter119, Tie2, and AlphaV integrin (also known as CD51), corresponding to markers present on hematopoietic (CD45, Ter119), vascular and hematopoietic (Tie2), and osteoblastic (AlphaV integrin) cells. They further analyzed subpopulations of $AlphaV^{+}$ cells expressing different combination of markers such as CD105, Thy, 6C3, and CD200. The authors defined a lineage tree of skeletal stem/progenitor cells (Chan et al., 2015). In this scheme, the mouse skeletal stem cells are multipotent and capable of self-renewal and differentiation into more lineage-restricted progenitor cells, such as pre-BCSP (pre-*b*one, *c*artilage, and *s*tromal *p*rogenitors) and BCSP (*b*one, *c*artilage, and *s*tromal *p*rogenitors) (Chan et al., 2013). The mouse skeletal stem cells, pre-BCSPs, and BCSPs have been found capable of giving rise to bone ($CD45^{-}Ter119^{-}Tie2^{-}AlphaV^{+}Thy^{+}6C3^{-}CD105^{+}$), cartilage ($CD45^{-}Ter119^{-}Tie2^{-}AlphaV^{+}Thy^{+}6C3^{-}CD105^{+}CD200^{+}$), and hematopoietic supportive stroma ($CD45^{-}Ter119^{-}Tie2^{-}AlphaV^{+}Thy^{+}6C3^{+}CD105^{+}$). According to these data, bone, cartilage, and stromal tissue are clonally derived in vivo from lineage-restricted stem and progenitor cells that do not also give rise to muscle and fat, at least at the time points examined (Chan et al., 2015).

MSCs microenvironment

MSCs microenvironment is represented by their niche (Figure 3). The stem cell niche is the in vivo microenvironment where stem cells both reside and receive stimuli that determine their fate. Therefore, the niche should not be considered simply as a physical location for stem cells, but rather as the place where extrinsic signals interact and integrate to influence stem cell behavior. These stimuli include cell-to-cell and cell-matrix interactions and signals (molecules) that activate and/or repress genes and transcription pathways (Ferraro, Celso, & Scadden, 2010)**.** Schofield has first postulated the hypothesis of a specialized stem cell microenvironment in 1978 (Schofield, 1978). He proposed that niches have a defined anatomical location and also that removal of stem cells from their niche results in differentiation**.**

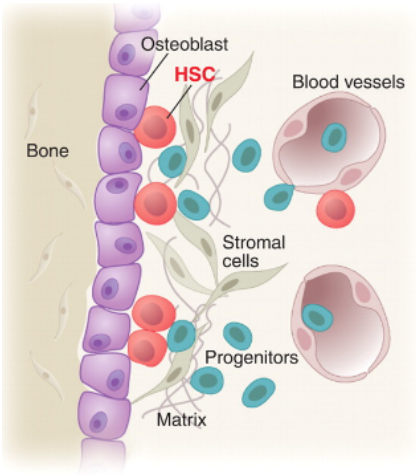

Figure 3. **HSCs niche in the bone marrow.** The stem cell niche is composed of both cells, such as stromal cells and progenitor cells, and matrix. (*Moore K. A., Lemischka I. R. Stem cells and their niches. Science, v. 311, n. 5769, p. 1880-5, 2006*)

Oxygen tension

Low oxygen tension is an important component of the stem and progenitor cell microenvironment since it provides signals that are conducive to the maintenance of stem-cell functions (Buravkova, Grinakovskaia, Andreeva, Zhambalova, & Kozionova, 2009). Hypoxia modulates the behavior of different stem cell types, such as embryonic stem cells (M. C. Simon & Keith, 2008), iPs (Yoshida, Takahashi, Okita, Ichisaka, & Yamanaka, 2009), and adult stem cells (Fotia, Massa, Boriani, Baldini, & Granchi, 2015). However, in many studies, cells are usually cultured under normoxic conditions equivalent to atmospheric oxygen, paying scarce attention to the niche metabolic environment in which stem cells grow or reside physiologically. Oxygen tension in adult tissues is considerably different from that in the inhaled atmosphere, which corresponds to 21% (160 mm Hg). The partial oxygen pressure (pO2) of the inhaled air decreases progressively after entering into the lungs and the bloodstream. When it reaches organs and tissues, pO2 level drops down to 2-9% (14-65 mm Hg) (Brahimi-Horn & Pouysségur, 2007; Silván et al., 2009). This pressure is dramatically different from the oxygen tension typically considered "normoxic" according to conventional cell culture standards. The key adaptive response to hypoxic conditions is the stabilization of hypoxia inducible factor (HIF)-1 (G. L. Wang & Semenza, 1993) (Figure 4). HIF-1 is the main regulator controlling the metabolic fate and multipotency of MSCs. HIF-1 is a heterodimer that consists of a constitutively expressed HIF- 1β subunit and a HIF-1α subunit; the expression of the latter is highly regulated and is determined by the relative rates of its synthesis and degradation. Synthesis of HIF-1α is regulated via oxygen-independent mechanisms, whereas its degradation is oxygen-dependent (Semenza, 2003). In the presence of high levels of oxygen (> 9%), HIF-1α is subjected to proteasome-dependent degradation. In hypoxic conditions, HIF-1α stabilizes, dimers with HIF-1β, and induces the expression of various genes involved in hypoxia-like processes such as glycolysis, erythropoiesis and angiogenesis (Semenza, 2003).

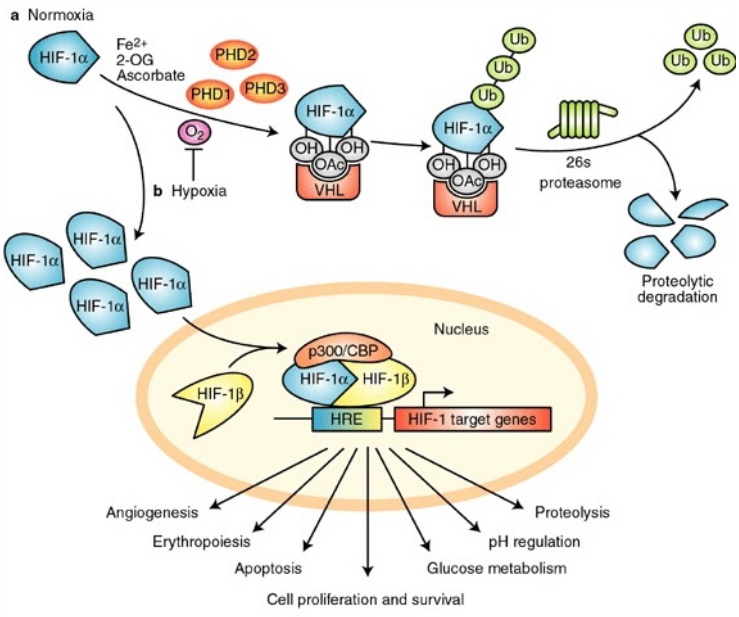

Figure 4. **HIF-1α regulation by proline hydroxylation.** In normoxic conditions, HIF-1α is hydroxylated by prolyl ydroxylases, and then degraded. In hypoxic conditions, HIF-1α translocates to the nucleus and activates its target genes implicated in several cellular processes. (*http://journals.cambridge.org/fulltext_content/ERM/ERM7_06/S14623994050 09117sup010.g*)

The effects of a low oxygen environment on MSCs behavior have been intensively investigated in the last 20 years, showing several conflicting results. Numerous studies have demonstrated that MSCs constantly propagated at low oxygen (1–5%) generally display enhanced proliferative potential (Basciano et al., 2011; D'Ippolito, Diabira, Howard, Roos, & Schiller, 2006; Dos Santos et al., 2010; Fehrer et al., 2007; Grinakovskaya, Andreeva, Buravkova, Rylova, & Kosovsky, 2009; Iida et al., 2010; López et al., 2013; Malladi, Xu, Chiou, Giaccia, & Longaker, 2006; Valorani et al., 2012; Zhambalova, Darevskaya, Kabaeva, Romanov, & Buravkova, 2009) and greater colony-forming activity as compared to cells cultured under normoxic conditions (21% O_2) (Diaz-Flores, Gutierrez, Lopez-Alonso, Gonzalez, & Varela,

1992; Dos Santos et al., 2010; Fehrer et al., 2007; Iida et al., 2010; López et al., 2013). However, these effects can be driven by specific circumstances, such as the culture passage (Basciano et al., 2011; Khan, Adesida, & Hardingham, 2007). The capacity for multilineage differentiation is one hallmark of MSCs. It is well established that under specific conditions, MSCs can differentiate into osteoblasts, adipocytes, and chondroblasts. However, some studies show that MSCs isolated from bone marrow in hypoxic conditions have a decreased ability to differentiate in adipocytes and osteoblasts, thus supporting the theory that low oxygen tension promotes an undifferentiated state of MSCs (D'Ippolito et al., 2006; Fehrer et al., 2007; Pasarica et al., 2010). Other studies report that MSCs cultured in hypoxic conditions have an improved differentiation capacity into the chondrogenic lineage (Adesida, Mulet-Sierra, & Jomha, 2012; Holzwarth et al., 2010). Summarizing, although the experimental results are highly variable for the different experimental conditions and the samples used (MSCs isolated from different sources), considerable data clearly demonstrate that hypoxia significantly modulates MSC properties, especially MSCs differentiation capacity.

Extracellular acidity

A less studied peculiar feature of the stem-cell niche microenvironment is its characteristic pH value, which is able to influence MSCs proliferation, differentiation and paracrine activity (Disthabanchong, Radinahamed, Stitchantrakul, Hongeng, & Rajatanavin, 2007). In physiological conditions, peripheral blood pH is approximately 7.4–7.35, while pH of the fluids flowing among cells in tissues is lower, and subject to alterations due to cell metabolism. Extracellular acidosis is caused by an increase in glycolytic and oxidative metabolism of the cells that leads to the production of high amount of carbonic and lactic acids, which are extruded to maintain an intracellular pH near the physiological value. As a consequence, an inverted membrane pH gradient is established: the extracellular pH (pHe) is

lower than the intracellular pH (pHi) (Gerweck & Seetharaman, 1996).

This altered acidic microenvironment is commonly associated with renal and respiratory diseases, but also with anaerobic exercise, gastroenteritis, excessive consumption of acidifying substances, diabetes, anemias, obesity, osteoporosis, and arthritis (Domínguez-Bendala, Lanzoni, Inverardi, & Ricordi, 2012; Garibotto et al., 1996; Lardner, 2001; X. Liu et al., 2014). Acidosis can also locally arise as a result of growth factor or cytokine stimulation of cell metabolism, vascular disease, ischemia, inflammation, infection, tumors, wounds, and fractures (T. R. Arnett, 2008).

Acidosis is reported to inhibit osteoblast function by decreasing expression of extracellular matrix genes, including collagen, as well as by reducing mineralization (Frick & Bushinsky, 1998; Sprague, Krieger, & Bushinsky, 1994). Brandao-Burch *et al.* (2005) has studied the effects of pH on osteoblasts function using primary rat osteoblast cultures. They found that acidification progressively reduces mineralization of bone nodules, reaching a complete abolition at pH 6.9 (Brandao-Burch et al., 2005). Alkaline phosphatase activity, which is required for bone mineralization, peaks strongly near pH 7.4 but is reduced by more than 90% at pH 6.9, whereas matrix Gla protein, an inhibitor of mineralization, is up-regulated. The pH reduction is associated with an increase in solubility for hydroxyapatite, thus indicating that acidosis exerts a selective, inhibitory action on matrix mineralization that is reciprocal with the osteoclast activation response (Brandao-Burch et al., 2005).

Cell culture experiments on rat osteoclasts have shown that protons exert a direct stimulatory effect on bone resorption (T. R. Arnett & Dempster, 1986). These experiments show that osteoclasts are almost inactive at a physiological pH value of 7.4, and that bone resorption increases as pH is reduced, reaching a plateau at about pH 6.8. Subsequent studies showed that avian (T. R. Arnett & Dempster, 1987) and human (T. R. Arnett, 2008) osteoclasts also exhibit acid-activation responses. Osteoclasts sensitivity to extracellular H^+ is such that pH reductions of <0.1 unit can cause a doubling of resorptive activity (T. R.

Arnett & Spowage, 1996). This effect is not subject to desensitization in longer-term cultures: acid-activated osteoclasts continue with their resorption activity over periods of 7 days or more, amplifying the effects of modest pH differences (T. Arnett, 2003). Acidosis is required for the initiation of resorption; once activated, osteoclasts can be further stimulated by factors such as receptor activator for nuclear factor kB ligand (RANKL) (Barzel, 1995), 1,25-dihydroxycholecalciferol (1,25D_3), parathyroid hormone (T. R. Arnett & Dempster, 1986), and ATP/ADP (Hoebertz, Meghji, Burnstock, & Arnett, 2001). Thus, osteoclasts stimulation is a two-step process, with acid-activation as the key initial requirement, and extracellular protons may be considered as osteoclasts activation factors. Acidification rapidly increases osteoclasts expression of carbonic anydrase II and the vacuolar-type H^+-ATPase, which is required for the generation and pumping of the protons that solubilize bone mineral (Biskobing & Fan, 2000; Nordström et al., 1997), and strongly up-regulates cathepsin K, involved in organic matrix degradation (Muzylak, Arnett, Price, & Horton, 2007). Other resorption-associated factors upregulated or stabilized by acidosis include tartrate-resistant acid phosphatase, TNF receptor-associated factor 6, and the transcription factor NFATc1 (Komarova, Pereverzev, Shum, Sims, & Dixon, 2005).

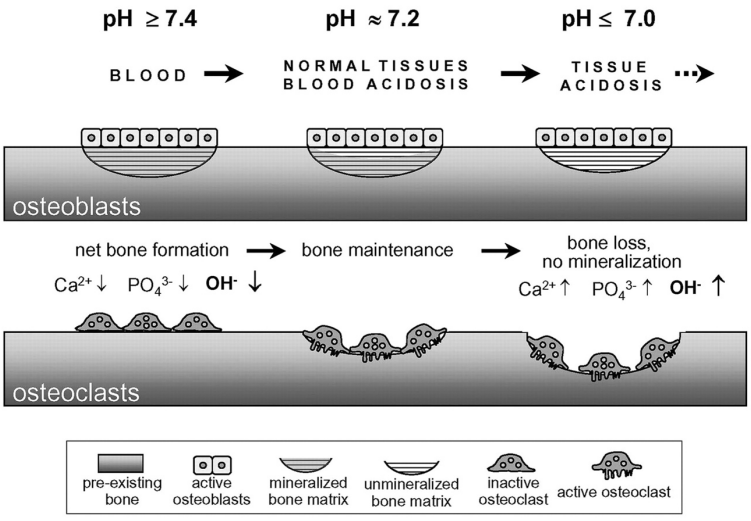

Figure 5. **Effects of extracellular pH on bone formation and resorption.** Matrix mineralization is strongly inhibited by acidosis, and osteoclasts resorption activity is activated. (*Arnett T. R. Extracellular pH regulates bone cell function. J Nutr, 138(2), 415S-418S, 2008*)

Acidic microenvironment and autophagy

According to recent works, an acidic microenvironment is able to modulate stemness and regenerative potential of MSCs (Mohyeldin, Garzón-Muvdi, & Quiñones-Hinojosa, 2010). Similarly, a cell process called autophagy has been associated with the regulation of MSC stem-like features, as well as with senescence and cell death/survival (Eom et al., 2014), and can be induced by acidic conditions.

Autophagy is a catabolic and degradative process activated by cells under stressful conditions, consisting of three different forms: microautophagy, which implies the direct uptake of soluble cytosolic substrates in the lysosomes via invagination of the lysosomal membrane (W. W. Li, Li, & Bao, 2012); chaperone-mediated autophagy, which degrades specific proteins carrying the peptide motif KFERQ and translocated to lysosomes via chaperone protein Hsc70 (heat shock cognate 70) (Kaushik & Cuervo, 2012); and macroautophagy (referred to as autophagy), involving the formation of double-membrane vesicles (autophagosomes) containing an autophagic cargo and their fusion with lysosomes (Mehrpour, Esclatine, Beau, & Codogno, 2010). Independently of the type of autophagy, the autophagic cargo is degraded by lysosomal acidic hydrolases and cathepsins and the molecules produced are released into the cytoplasm and re-used as building blocks in different anabolic pathways (Mizushima & Komatsu, 2011). Basal autophagy allows the removal of redundant or damaged and potentially toxic organelles and protein aggregates, thus representing an important system for quality control in cellular homeostasis. Autophagy can be upregulated by conditions of stress represented by hypoxia, nutrient deprivation, metabolic, oxidative and proteotoxic stress (Kroemer, Mariño, & Levine, 2010). Although starvation-induced autophagy is a non-selective process degrading bulk cytosolic material to provide

nutrients and support cellular metabolism and survival in stress conditions, there are several types of selective-autophagy, where specific organelles and substrates like mitochondria, lipid droplets, protein aggregates and ferritin are targeted to the autophagosome and delivered to lysosomes for degradation (Stolz, Ernst, & Dikic, 2014). Many types of selective-autophagy rely on the recognition of poly-ubiquitylated targets by specific autophagy receptors, including sequestosome 1 (SQSTM1 or p62), neighbor of BRCA1 (NBR1) and Optineurin (Lippai & Lőw, 2014). The autophagic process (Figure 6) starts with the elongation of membrane precursors and the formation of a double membrane vesicle (autophagosome), which engulfs bulk cytosolic material and targeted cellular organelles. Autophagy-related proteins (ATG) are responsible for the elongation and formation of the autophagosome (Lamb, Yoshimori, & Tooze, 2013). Moving along the microtubules network (Aplin, Jasionowski, Tuttle, Lenk, & Dunn, 1992), the autophagosome fuses with a lysosome whose acidic lumen activates hydrolytic enzymes that degrade the content of the autolysosome, giving rise to amino acids, fatty acids, nucleosides and other metabolites released into the cytosol and recycled in anabolic cellular metabolism (Rabinowitz & White, 2010).

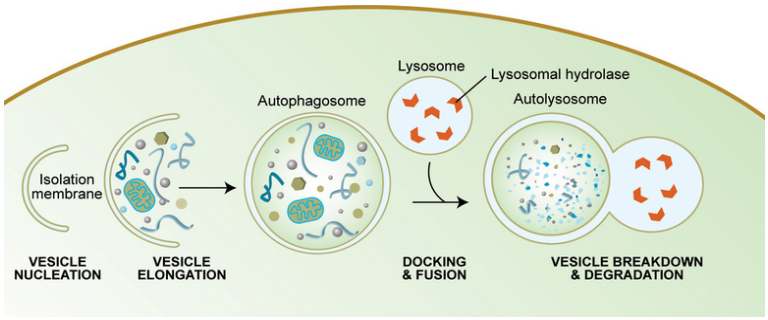

Figure 6. **Representative scheme of the steps of autophagy.** Autophagy begins with the formation of the phagophore (vesicle nucleation), which then expands becoming an autophagosome (vesicle elongation). The autophagosome engulfs bulk cytoplasm cargo. When the outer membrane of the autophagosome fuses with a lysosome (docking and fusion steps), it forms an autophagolysosome.

Eventually, the sequestered material is degraded inside the autophagolyosome (vesicle breakdown and degradation) and recycled. (*Meléndez A., Levine B. Autophagy in C. elegans WormBook, p. 1-26, 2009*)

At the molecular level, autophagy is negatively regulated by the mammalian target of rapamycin complex-1 (mTORC1), which in the presence of sufficient nutrients and growth factors phosphorylates and inactivates the serine/threonine kinase ULK1/2 (J. Kim, Kundu, Viollet, & Guan, 2011). The complex containing ULK1/2 regulates the initiation of the autophagic process and is activated by AMPK-mediated phosphorylation and in presence of inactive MTORC1 due to nutrients starvation and/or growth factors deprivation (J. Kim et al., 2011). The ULK1 complex activates the class III PI3K complex, containing Beclin-1 and the class III phophatidylinositol 3-kinase (PtdIns3K) protein VSP34 (Funderburk, Wang, & Yue, 2010). VPS34 is a lipid kinase producing phosphatidylinositol-3- phosphate (PI3P), essential for autophagosomal membrane elongation and for the recruitment of proteins with PI3P binding domains (e.g. WIPI1/2) involved in vesicle elongation, followed by the activity of two ubiquitin-like conjugation systems. The first is the complex ATG12– ATG5-ATG16L, an E3-like ligase that mediates the lipidation of the light chain 3 (LC3) family members LC3, GATE16 and GABARAP, which will be then associated to the autophagosomal membrane. The ATG12–ATG5-ATG16L complex will dissociate from the membrane before closure while lipidated LC3 members (e.g. LC3-II or LC3- PE) will regulate final maturation of the autophagosome, being retained on the inner membrane. In the case of selective autophagy, autophagy receptors like SQSTM1 contain an LC3-interacting region (LIR) and a ubiquitin-associated domain (UBA), which allow the selective recruitment of ubiquitinated organelles or structures to the autophagosome (Figure 7).

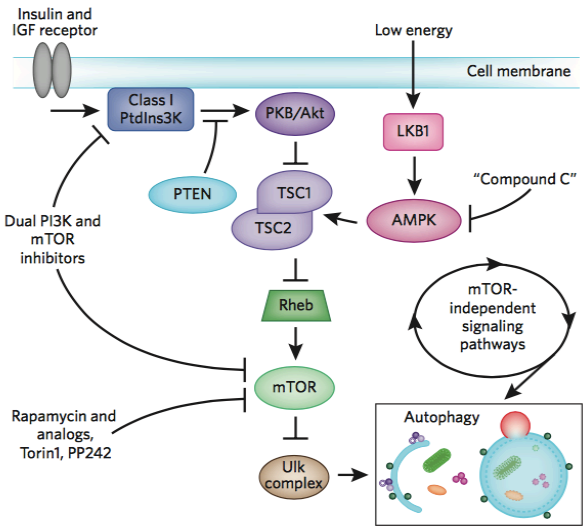

Figure 7. **Schematic representation of the mTOR signaling pathway.** Autophagy is negatively regulated by mTOR, a serine/threonine kinase, via the formation of the mTORC1 complex. This complex is inhibited by specific inhibitors, which therefore induce autophagy. (*Fleming, A., Noda, T., Yoshimori, T., & Rubinsztein, D. C. Chemical modulators of autophagy as biological probes and potential therapeutics. Nat Chem Biol, v. 7, n. 1, p. 9-17, 2011*)

Summarizing, autophagy is a crucial process that modulates the adaptive response to cellular stress and contributes to maintain cellular homeostasis in physiological conditions. Because of these important functions, alterations in autophagy are associated with several human conditions, including neurodegenerative, cardiovascular and infectious diseases, as well as cancer (Sridhar, Botbol, Macian, & Cuervo, 2012). Recently it has been shown that autophagy plays a basic role also in the commitment of MSCs to different lineages, especially in the osteoblastic lineage. Nuschke *et al.* (2014) have demonstrated that undifferentiated MSCs have an accumulation of undegraded autophagic vacuoles and a little autophagic turnover, while the stimulation of osteogenic differentiation leads to a consistent increase in turnover (Nuschke et al., 2014). According to these observations, autophagy seems to be of fundamental importance in the control of osteogenic differentiation. Also, SATB2, an AT-rich binding

protein, has the capacity of promoting osteogenic differentiation and bone defect regeneration of BMMSC, by upregulating pluripotency genes and autophagy-related genes which activate the mTOR signaling pathway (W. Dong et al., 2015). While mTOR signaling may function to affect osteoblastic differentiation, conflicting results have been reported on whether rapamycin decreases or increases osteogenesis, according to the cell type. In rats, rapamycin does not exhibit a spontaneous osteogenic effect on MSC, but inhibits the effect of osteogenic differentiation induced by dexamethasone (Isomoto et al., 2007). On the other hand, rapamycin promotes osteogenic differentiation in human embryonic stem cells (ESC) by blocking mTOR and stimulating BMP/Smad signaling pathway (K. W. Lee et al., 2010), once again suggesting that no general statement can be made on the role of autophagy.

Acidic microenvironment and inflammation

As previously mentioned, local acidosis can trigger the activation of inflammatory pathways (Lardner, 2001). Systemic inflammation characterizes several chronic conditions, such as rheumatoid arthritis (RA), diabetes mellitus, and systemic lupus erythematosus (SLE) (Hardy & Cooper, 2009), that are closely associated with bone loss and secondary osteoporosis, and, consequently, increased fracture risk (Hardy & Cooper, 2009). Although fracture treatment has improved considerably in the last decades, a large proportion still exhibit a delayed healing and several complications, including non-union. The outcome of fracture healing depends on a number of factors, such as trauma severity (Fong et al., 2013; Karladani, Granhed, Kärrholm, & Styf, 2001), quality of fracture reduction (realignment), fracture fixation technique and presence of comorbid diseases (Hayda, Brighton, & Esterhai, 1998), such as those mentioned above. In a retrospective study, fracture healing in patients with RA was associated with higher complication rates, including non-unions, but the intrinsic molecular mechanisms are still unknown (Strömqvist, 1984). Clinical studies have shown impaired fracture healing in patients with diabetes mellitus (Loder, 1988), and the results of experiments in animal models suggest that disrupted

repair is at least partly caused by inflammatory mediators. Specifically, TNF was shown to be associated with increased chondrocyte apoptosis, pre-mature loss of cartilage and enhanced osteoclasts formation during diabetic fracture healing (Alblowi et al., 2009; Kayal et al., 2010; Kayal et al., 2007). Dominak *et al.* (2005) found immunoglobulin and complement deposition at the site of a non-healing fracture in a patient with SLE; the authors of this study concluded that disease-related autoantibodies inhibited bone cell differentiation, resulting in non-union (Dominiak et al., 2005).

Inflammation is an important factor during bone healing, with molecular factors and immune cells appearing locally at the fracture site in a distinct spatial and temporal way. Perturbations of this finely tuned sequence of events leads to impaired fracture healing (Claes, Recknagel, & Ignatius, 2012; Gerstenfeld, Cho, et al., 2003; Wallace, Cooney, Englund, & Lubahn, 2011; Yang et al., 2007). Studies show that in a sheep bone-healing model, in which healing was mechanically impaired through flexible fixation, a higher number of cytotoxic T cells and other leukocytes was detected in the early fracture hematoma and the bone marrow close to the fracture gap, compared with more rigid fracture fixation (Schmidt-Bleek et al., 2012). A prolonged inflammatory phase was observed in the impaired healing group (Schmidt-Bleek et al., 2012). Likely, cytotoxic T cells release cytokines that can prolong the presence of proinflammatory M1 macrophages, possibly by delaying their differentiation into the more anti-inflammatory and proangiogenic M2 macrophages. Grundnes *et al.* (Grundnes & Reikeraas, 2000) supported this theory with their study, in which they observed impaired fracture healing after local stimulation of macrophage to secrete proinflammatory cytokines (Grundnes & Reikeraas, 2000).

In conclusion, a balance between the different inflammation stimuli at the fracture site seems to exist, and be dependent on the quantitative, qualitative and temporal composition of the fracture callus. However, further studies are needed to clarify the consequences of inflammation on fracture healing outcome, perhaps starting from a better characterization of this phase of fracture repair and its

relationship to the healing outcome.

Therapeutic potential of MSCs

MSCs specific features, such as multipotency, immunoregulatory properties, and ability to migrate to the site of injury, are critical for their clinical applications. As a matter of fact, MSCs can be easily isolated from bone marrow, adipose tissue, and dental pulp (R. H. Lee et al., 2004), and eventually administered to the patients with an autologous transplant, avoiding immune reactions.

Tissue repair

MSCs have shown a strong propensity to ameliorate tissue damage in response to injury and disease. MSCs actively respond to stress or injury in a manner that is very similar to how the adaptive and innate immune system cells respond to pathogen exposure or apoptosis. When supplied exogenously, MSCs home to sites of injury primarily inflamed or with impaired blood supply. MSCs secrete soluble factors that alter the tissue microenvironment, promote angiogenesis, regeneration, remodeling, immune cell activation or suppression, and cellular recruitment, thus triggering the regenerative process (Ciapetti et al., 2012; Prockop, 2007). The repair process consists in regulating extracellular matrix deposition, collagen synthesis, fibroblast proliferation, platelet activation, fibrinolysis, and angiogenesis. MSCs were originally evaluated for their capacity to repair skeletal defects, first in experimental animal models, and subsequently in human patients afflicted with osteogenesis imperfecta (OI), a genetic defect in bone and other tissues caused by mutations in the genes for Type I collagen (Horwitz et al., 2002).

Fracture healing process

Fracture healing (Figure 8) is a postnatal process that reflects many of the ontological events that take place during embryonic development of the skeleton (Einhorn & Gerstenfeld, 2015).

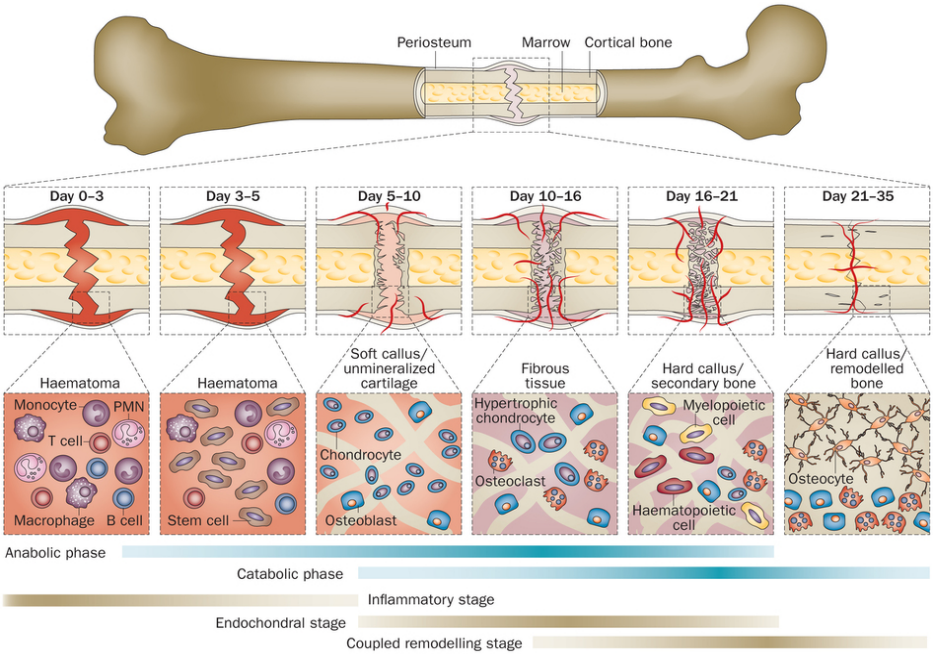

Figure 8. **Fracture healing.** Immediately after injury, a hematoma forms, followed by an inflammatory response at the fracture site. Within days of fracture, the periosteum, if intact, thickens because of extensive cell proliferation, and a callus made of fibrous and osteocartilaginous tissue forms. After mineralization, a hard callus forms and is eventually remodeled to regain the original bone anatomy. (*Einhorn T. A., Gerstenfeld L. C. Fracture healing: mechanisms and interventions. Nat Rev Rheumatol, v. 11, n. 1, p. 45-54, 2015*)

Even though there is still a lot to fully define the pathways of bone regeneration, several studies have been developed, providing a general understanding of how fracture healing occurs. The use of animal models has made it possible to investigate fracture healing from all the different aspects such as

histology, biochemistry and biomechanics, thus becoming significant tool in understanding fracture biology (Bonnarens & Einhorn, 1984).

Indirect (secondary) fracture healing is the most common form of fracture healing, which consists of both endochondral and intramembranous bone regeneration processes (Gerstenfeld et al., 2006), and typically occurs in non-operative fracture treatment (Green, Lubahn, & Evans, 2005).

Acute inflammatory response

Immediately after the trauma, a hematoma consisting of cells from both peripheral and intramedullary blood, as well as bone marrow cells, forms. An inflammatory response is initiated at the injury site, causing the hematoma to coagulate, thus generating a template for the callus formation (Gerstenfeld, Cullinane, Barnes, Graves, & Einhorn, 2003). The acute inflammatory response peaks within the first 24 hr and is complete after 7 days (T. J. Cho, Gerstenfeld, & Einhorn, 2002). The initial proinflammatory response involves secretion of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, IL-11 and IL-18 (Gerstenfeld, Cullinane, et al., 2003). These factors recruit inflammatory cells and promote angiogenesis (Tohma et al., 2017). TNF-α is expressed by macrophages and other inflammatory cells, and has been shown to act as a chemotactic agent responsible for recruiting the cells necessary to the injury acute response (Kon et al., 2001). IL-1 and IL-6 are believed to be most important interleukins involved in fracture healing. IL-1 is produced by macrophages in the acute phase of inflammation; it stimulates the generation of the soft cartilaginous callus, and promotes angiogenesis at the injured site. IL-1 is also responsible for the induction of IL-6 secretion from osteoblasts (Kon et al., 2001; S. K. Lee & Lorenzo, 2006). IL-6 is only produced during the acute phase of inflammation; it stimulates angiogenesis, vascular endothelial growth factor (VEGF) production, and the differentiation of osteoblasts and osteoclasts (Yang et al., 2007).

Recruitment of MSCs

In order for bone to regenerate, specific MSCs have to be recruited, proliferate and differentiate into osteogenic cells. Exactly where these cells come from has not been completely defined yet. Most studies indicate that MSCs involved in fracture healing derive from the surrounding soft tissues and bone marrow. Periosteum is also considered a major source of MSCs for bone repair, and it has been shown that the healing is delayed without it (Colnot, Zhang, & Knothe Tate, 2012; Grcevic et al., 2012). Furthermore, studies support the thesis of a systemic recruitment of circulating MSCs to the injured site for an optimal healing response (Granero-Moltó et al., 2009; Kitaori et al., 2009). Molecular events mediating this recruitment are still under debate.

Current data suggest that SDF-1 and its G-protein-coupled receptor CXCR-4 form an axis (SDF-1/CXCR-4) that is a key regulator of recruiting and homing specific MSCs to the site of trauma (Granero-Moltó et al., 2009; Kitaori et al., 2009; Ma et al., 2005). These reports show that SDF-1 expression is increased at the fracture site, especially in the periosteum. They also demonstrate that SDF-1 has a specific role in recruiting CXCR-4 expressing MSCs to the injured site during endochondral fracture healing (Kitaori et al., 2009). The importance of this axis has been further verified as treatment with an anti-SDF-1 antagonist or genetic manipulation of SDF-1 and CXCR-4 impairs fracture healing. It has also been shown that transplanted MSCs only home to the fracture site if they express CXCR-4, whereas CXCR-4 negative MSCs do not have this ability (Granero-Moltó et al., 2009; Kitaori et al., 2009).

Generation of a soft and a hard callus

Following the previously described formation of the primary hematoma, a soft callus made of fibrous tissue and cartilage forms (Rahn, Gallinaro, Baltensperger, & Perren, 1971). At this stage, the cartilage constituting the soft callus is fibrocartilage, which expresses Type I and Type II collagen, in contrast with hyaline cartilage of the joints. The fibrocartilage cells eventually become osteoblasts after

the hypertrophic stage (Stump, 1925). In animal models (rat, rabbit, mouse) the peak of soft callus formation occurs 7–9 days post trauma, characterized by an increased presence of both Type II procollagen and proteoglycan core protein extracellular markers (Einhorn, 1998). After cartilage mineralization, a hard callus forms and is ultimately remodeled to regain the original bone anatomy. It is the final bridging of this central hard callus that ultimately provides the fracture with a semi-rigid structure which allows weight bearing (Gerstenfeld et al., 2006).

The generation of these callus tissues is dependent on the recruitment of MSCs from the surrounding soft tissues, cortex, periosteum, and bone marrow as well the systemic mobilization of HSCs into the peripheral blood from remote hematopoetic sites. Once recruited, a molecular cascade involves collagen-I and collagen-II matrix production and the participation of several peptide signaling molecules. In this process the TGF-β superfamily members have been shown to be of great importance. TGF- β2, -β3 and GDF-5 are involved in chondrogenesis and endochondral ossification, whereas BMP-5 and -6 have been suggested to induce cell proliferation in intramembranous ossification at periosteal sites (T. J. Cho et al., 2002; Marsell & Einhorn, 2009).

Vascularization and neoangiogenesis at the fracture site

An important requirement for fracture healing is blood supply, indeed revascularization is essential for successful bone repair (Kanczler & Oreffo, 2008). In order to allow blood vessels growth at the injured site, chondrocytes undergo apoptosis, and cartilage is degraded, thus removing unnecessary cells and extracellular matrix (Ai-Aql, Alagl, Graves, Gerstenfeld, & Einhorn, 2008).

Once this structural pattern is achieved, the vascularization process is mainly regulated by two molecular pathways, an angiopoietin-dependent pathway, and a vascular endothelial growth factor (VEGF)-dependent pathway (Tsiridis, Upadhyay, & Giannoudis, 2007). The angiopoietins, specifically angiopoetin-1 and 2, are vascular morphogenetic proteins, whose expression is induced early in the

healing cascade. The angiopoietins promote an initial vascular growth from existing vessels in the periosteum (Lehmann et al., 2005), while the VEGF pathway is considered to be the key regulator of vascular regeneration (Keramaris, Calori, Nikolaou, Schemitsch, & Giannoudis, 2008). It has been shown that both osteoblasts and hypertrophic chondrocytes express high levels of VEGF, thereby promoting the invasion of blood vessels and transforming the avascular cartilaginous matrix into a vascularized osseous tissue (Keramaris et al., 2008). VEGF promotes both vasculogenesis, identified in the aggregation and proliferation of endothelial mesenchymal stem cells into a vascular plexus, and angiogenesis, representing the growth of new vessels from existing ones (Kanczler & Oreffo, 2008). VEGF importance in these processes is further supported by the observation that addition of excessive VEGF promotes fracture healing, whereas blocking of VEGF-receptors inhibit vascular growth and delays or disrupts the regenerative process (Ai-Aql et al., 2008; Kanczler & Oreffo, 2008).

Mineralization of the soft callus

In order to progress in bone repair, the initial soft cartilaginous callus needs to be replaced by a hard-bony callus. During this final step of fracture healing, a combination of cellular proliferation and differentiation occurs, with an increase in cellular volume and matrix deposition (Breur, VanEnkevort, Farnum, & Wilsman, 1991). Recent studies have focused their attention on the understanding of the role of the Wnt-family molecules, shown to give an important contribution in the bone healing process. The Wnt-family is thought to regulate the differentiation of pluripotent MSCs into the osteoblastic lineage and, at later stages of development, to positively regulate osteoblastic bone formation (Y. Chen & Alman, 2009). Chondrocytes in the callus proliferate, become hypertrophic, and the extracellular matrix calcifies. The mechanism of calcification involves mitochondria, which accumulate calcium-containing granules that, after being elaborated into the cytoplasm of fracture callus chondrocytes, are transported into the extracellular matrix where they precipitate with phosphate and form initial mineral deposits.

These deposits of calcium and phosphate will evolve into apatite crystals (Ketenjian & Arsenis, 1975). The peak of the hard callus formation is usually reached by day 14 in animal models, as confirmed by the detection of extracellular matrix markers, such as Type I procollagen, osteocalcin, alkaline phosphatase and osteonectin (Einhorn, 1998).

Bone remodeling

In order to achieve the completed restoration of the biomechanical properties of normal bone, the fracture healing process initiates a second resorptive phase, this time to remodel the hard callus into a lamellar bone structure with a central medullary cavity (Gerstenfeld, Cullinane, et al., 2003). This phase is biochemically played by IL-1 and TNF-α, both found to be highly expressed during this stage, as opposed to most members of the TGF-β family whose expression is strongly decreased by this time (Ai-Aql et al., 2008; Mountziaris & Mikos, 2008). However, some TGF- β family members such as BMP2, seem to be involved in this phase with reasonably high expression levels, as well (Marsell & Einhorn, 2009).

The remodeling process is carried out by a balance of hard callus resorption by osteoclasts, and lamellar bone deposition by osteoblasts. This process is initiated after 3–4 weeks from the day of fracture in animal and human models, and may last years before completely restoring a regenerated bone structure (WENDEBERG, 1961). As just mentioned, bone remodeling is the result of a combined activity of osteoclasts and osteoblasts, and has been shown to be a result of production of electrical polarity created when pressure is applied in a crystalline environment (Marsell & Einhorn, 2011). Axial loading of long bones occurs, creating one electropositive convex surface, and one electronegative concave surface, activating osteoclastic and osteoblastic activity respectively. By these actions the external callus is gradually replaced by a lamellar bone structure, whereas the internal callus remodeling re-establishes a medullar cavity characteristic of a diaphyseal bone (Marsell & Einhorn, 2011).

The major bone compartment responsible for bridging the callus formation and participating to endochondral and intramembranous ossifications during fracture healing is the periosteum. As a matter of fact, most cells involved in the regenerative process come from this bone compartment (Ferretti & Mattioli-Belmonte, 2014).

Periosteum

Periosteum is a structure comprised of two layers that surround the cortical bone (Figure 9).

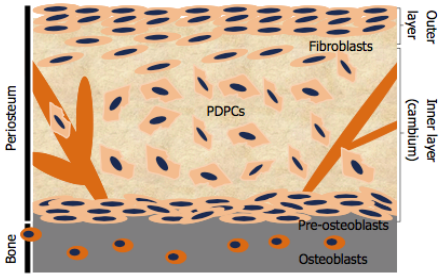

Figure 9. **Schematic representation of periosteum.** The periosteum is the membrane composed of both cells and matrix that surrounds the diaphyseal bone. PDPCs: Periosteum-derived precursor cells. (*Ferretti C., Mattioli-Belmonte M. Periosteum derived stem cells for regenerative medicine proposals: Boosting current knowledge. World J Stem Cells, v. 6, n. 3, p. 266-77, 2014*)

The outer layer provides mechanical stability to the periosteum, and is made of fibroblasts, collagen, and elastin along with a nerve and microvascular network (Allen, Hock, & Burr, 2004). The inner layer is enriched in cells that regulate bone formation and bone repair, represented by adult mesenchymal skeletal progenitor cells, osteoblasts, fibroblasts, and sympathetic nerves (Allen et al., 2004). For the properties listed above, the periosteum can be considered a structure with regenerative capacity. Due to its high vascularity, there are also numerous pericytes within the periosteum (Diaz-Flores et al., 1992). Pericytes are cells distributed around capillary endothelial cells, which can differentiate into several cell types, including osteoblasts (Reilly, Seldes, Luchetti, & Brighton, 1998). Thus, the pericytes could be considered a supplemental source of osteoprogenitor cells within the periosteum (Diaz-Flores et al., 1992). The periosteum is known to make major contributions to the bone-healing process. Within 24 to 48 hours following injury, an acute inflammatory reaction develops (Colnot, Thompson, Miclau, Werb, & Helms, 2003; Lu et al., 2005), periosteal cells start to proliferate, and a thickening of

periosteum is observed. This process is defined as periosteal activation. It has been shown that expansion of the mesenchymal progenitor cell population from the periosteum largely accounts for the formation of cartilage and bone within the callus (Colnot, 2009; Grcevic et al., 2012; X. Zhang et al., 2005). Conversely, cells belonging to the bone marrow or endosteum compartments remain in the marrow cavity and do not migrate out to form the callus (Colnot, 2009). These results indicate that the contributions of periosteal cells are critical for callus formation. In fact, activation of periosteum-derived progenitor cells (PDPCs) induces robust chondrogenesis and osteogenesis, together with marked induction of angiogenesis, which eventually leads to vascularization and remodeling of bone grafts (Colnot et al., 2012; X. Zhang et al., 2005). Different groups have demonstrated that the healing is impaired when the periosteum is absent (Colnot, 2009; Ozaki, Tsunoda, Kinoshita, & Saura, 2000). Ozaki *et al.* (2000) observed only little chondrocyte generation when the periosteum had been removed in induced-tibia fracture rats; Colnot (2009) caused periosteal injuries in mice, by exposing the anterior-proximal tibia surface and stripping the periosteum with a razor blade, and, in this way, she showed that, in the absence of periosteum, bone bridging was reduced. It has been demonstrated that periosteal replacement can heal critical-sized bone defects in animal models (Chang & Knothe Tate, 2012; Knothe Tate, Ritzman, Schneider, & Knothe, 2007).

Other sources of mesenchymal progenitor cells have also been proposed to participate in bone-fracture healing, including local bone marrow, adjacent muscle tissue, and circulating mesenchymal stem cells (Colnot et al., 2012; R. Liu et al., 2011; D. Park et al., 2012). However, the relative contribution to healing by each cellular phenotype is still unclear. Liu *et al.* (2011) showed that myogenic progenitors contribute to open fracture repair. Specifically, Liu *et al.* (2011) compared closed tibial fractures, presenting an intact periosteum, with an open, highly-traumatic fracture model, featuring periosteal stripping and local tissue trauma. A major contribution by cells expressing the myogenic specific marker MyoD was observed in the open fracture model, at all stages of the repair process. In contrast to the traumatic

open fracture scenario, MyoD-lineage cells made an insignificant contribution to closed fracture repair. This may suggest that the intact periosteum contribution to bone healing is sufficient for bone repair, and does not necessary require alternative osteoprogenitor sources.

Fracture healing is a regenerative process that involves coordinated responses of many cell types, but the characterization of these specific cell populations in this process has been limited. However, recent lineage-tracing studies during fracture healing in mice have shed a first light on the markers that could be used to identify skeletal progenitor cells in vivo. Transgenic Cre reporter mice have become a useful tool for studying specific cell populations *in vivo*. These reporter mice are genetically engineered to encode a fluorescent marker that is only expressed upon removal of a STOP codon cassette that is flanked by lox P sites that are recognized and excised by Cre recombinase. Therefore, the use of reporter mice that express Cre recombinase under the control of tissue-specific gene promoters has enabled the identification and lineage tracing of various stem cell types *in vivo*, such as progenitor cells (Grcevic et al., 2012; Z. Kalajzic et al., 2008; Kawanami, Matsushita, Chan, & Murakami, 2009; Matthews et al., 2014; Murao, Yamamoto, Matsuda, & Akiyama, 2013; D. Park et al., 2012; Roguljic et al., 2013; Zhou, Yue, Murphy, Peyer, & Morrison, 2014). Park et al. (2012) showed that *Mx-1*-Cre recombined widely among hematopoietic cells, PDGFRα^{+} stromal cells, and CFU-Fs in the bone marrow, and these cells gave rise to most of the osteoblasts formed in adult bone marrow (D. Park et al., 2012). Kawanami *et al.* (2009), using a with Prx1–CreER–GFP transgenic mice, has shown that PRX1-expressing cells in the periosteum are osteochondroprogenitor cells that give rise to chondrocytes and osteoblasts in the fracture callus (Kawanami et al., 2009). A recent study by Murao *et al.* (2013) further revealed that while no SOX9-positive cells are detected in resting periosteum, these cells quickly arise after bone fracture (Murao et al., 2013). Zhou *et al.* (2014) showed that LepR^{+} cells are highly enriched for CFU-F and uniformly express *Prx1*-Cre, PDGFRα, and CD51, markers expressed by bone marrow MSCs. LepR^{+} cells were the main source of new osteoblasts and adipocytes in adult bone marrow and could form bony

ossicles that support hematopoiesis in vivo (Zhou et al., 2014). In previous studies, Grcevic et al. have used alpha smooth muscle actin(αSMA)–CreERT2 transgenic mice to demonstrate that the majority of the callus cells, including chondrocytes and osteoblasts, are derived from αSMA-expressing cells (Grcevic et al., 2012). αSMA is a marker of pericytes and myofibroblastic cells that identifies mesenchymal progenitor cells with proliferative and multi-lineage differentiation potential in bone marrow stromal cell cultures in vitro (Z. Kalajzic et al., 2008), and osteoblast precursors in the bone marrow and periodontium in vivo (Grcevic et al., 2012; Roguljic et al., 2013). Although the origin of αSMA^{+} cells is still unknown, they are most likely cells derived from the local vascular system adjacent to the fracture site (Grcevic et al., 2012). When periosteal αSMA^{+}cells were isolated, they exhibited skeletal progenitor cell characteristics, including the expression of several mesenchymal progenitor cell markers and the ability to differentiate along the osteogenic, chondrogenic and adipogenic lineage (Grcevic et al., 2012; Matthews et al., 2014).

Molecular signaling during periosteal activation

Until recently, little was known about the molecular signaling that controls the periosteal responses during callus formation. With the development of genetically modified mouse models, studies have probed several signaling pathways and their effects on periosteal cells. BMP, Wnt, Notch, PDGF, FGF, Hedgehog, and inflammatory mediators have all been shown to contribute to the healing process, and therapies that target these pathways are currently under development for bone metabolic diseases.

Bone morphogenetic protein-2 (BMP2)

BMP2 is well-known for its powerful effect on bone development and regeneration (Lowery et al., 2011). The scientific community had agreed for years that BMP2 is an intrinsic initiator of bone healing. However, there was a limited number of studies to support this theory, since global BMP2-deletion results in embryonic lethality (Lowery et al., 2011). Tsuji *et al. (2006)* developed a mouse model in which the *Bmp2* gene is conditionally inactivated in the mesoderm by means of a Prx-1-Cre deleter mouse. A delayed periosteal activation response and a complete absence of bridging callus upon fracture were found in these *Bmp2* conditional knockout mice, underlining the crucial role of BMP2 in the initiation of fracture healing. Similarly, Wang *et al. (2011)* deleted *Bmp2* postnatally at the initiation stage of fracture healing using a Tamoxifen-inducible CreER mouse model, and found that *Bmp2* inactivation at the early stages of healing impaired the bone and cartilage callus formation derived from the periosteum. Further analysis of the periosteal callus showed that most of the *Bmp2* conditional knockout progenitors remained undifferentiated (Q. Wang et al., 2011). Recently, Mi *et al*. (2013) conditionally knocked out the *Bmp2* gene in chondrocytes and in osteoblasts by means of Col2-Cre and Col1-Cre deleter mice. They showed that the lack of *Bmp2* expression in chondrocytes led to prolonged cartilage callus formation and delayed osteogenesis initiation. However, when the *Bmp2* gene was inactivated in osteoblasts by the Col1-Cre deleter, the mice showed no significant difference in fracture

healing. These results indicate that, during fracture repair, expression of BMP2 is required in undifferentiated preosteoblasts but not in mature osteoblasts. Interestingly, the skeletal-specific inactivation of *Bmp4* and *Bmp7* by means of a Prx1-Cre deleter shows no effects on fracture repair (Bandyopadhyay et al., 2006), suggesting that, *in vivo*, under physiological conditions, BMP2, not BMP4 or BMP7, represents a primary modulator of periosteum-mediated fracture repair. These results indicate that the use of recombinant BMP2 in bone repair procedures may require the presence of the periosteum to reach a maximal therapeutic efficiency.

Wnt family members

Many members of the Wnt family are expressed in the injured periosteum (Y. Chen et al., 2007; J. B. Kim et al., 2007). It has been shown that the inactivation of the Wnt/β-catenin signaling pathway in chondrocytes leads to compromised bone repair due to reduced and delayed cartilage formation (Huang et al., 2012). Kim *et al.* (2007) observed that the inhibition of Wnt signaling in bone healing by using the Wnt inhibitor *Dkk1* reduces bone regeneration by 84%, and the addition of recombinant DKK1 or other Wnt inhibitor Sclerostin to primary periosteal cells reduces proliferation and increases apoptosis (H. K. Kim, Oxendine, & Kamiya, 2013). Therefore, it is assessed that Wnt signaling contributes significantly to fracture healing. In confirmation of these observations, gain-of-function of Wnt signaling seems to promote bone fracture healing. In fact, expression of an activated form of beta-catenin in osteoblasts dramatically enhanced bone healing in mice (Y. Chen et al., 2007). Additionally, gene knockout of *Axin2*, a negative regulator of Wnt signaling, resulted in accelerated bone healing after injury, which was related to more robust proliferation and earlier differentiation of skeletal stem and progenitor cells (Minear et al., 2010). Also, the administration of DKK1 neutralizing antibody in bone healing improved bone repair and regeneration in mice (Agholme, Isaksson, Kuhstoss, & Aspenberg, 2011; X. Li et al., 2011). *In vivo* deletion of the secreted Frizzled Related Protein 1 (sFRP1), one of several frizzled related

Wnt antagonists, improves fracture repair by promoting early bone union (Gaur et al., 2009). However, studies have shown that Wnt-responsive cells appear to be located mainly on the endosteal surface of injured bone, in contrast to the BMP target cells resident in the periosteum (Minear et al., 2010), leading to consider that, during fracture repair, BMP signaling and Wnt signaling act in different bone compartments. Thus, a Wnt-based therapy may not directly target the periosteum but may indirectly enhance periosteal-mediated bone regeneration. It is therefore possible that therapies under development that target the Wnt pathway, such as therapies based on DKK1 and SOST neutralizing antibodies, may be beneficial not only for bone endocrinology disorders but for periosteal-mediated bone-regenerative therapy as well.

Notch signaling

Notch signaling has been shown to regulate skeletal development (Tao, Chen, & Lee, 2010). In the skeleton, disruption of Notch signaling promotes osteoblast formation, and markedly increases osteoprogenitor differentiation (Tao et al., 2010), while ultimately depleting the mesenchymal progenitor pool (Y. Dong et al., 2010; Hilton et al., 2008). There has been limited characterization of the role of Notch signaling in fracture repair, although regulation of Notch signaling is important during healing in a number of other tissues (Chigurupati et al., 2007; Conboy, Conboy, Smythe, & Rando, 2003; Gude et al., 2008). Interestingly, Dishowitz and colleagues recently showed that Notch signaling pathway components expression is upregulated during fracture healing as *Jag1* and *Notch2* expression is localized in the undifferentiated mesenchymal cells of the healing periosteum (Dishowitz, Terkhorn, Bostic, & Hankenson, 2012). Another study showed that genetically inducible systemic inhibition of Notch signaling prolonged the inflammatory phase of fracture healing and altered cartilage formation, but it is unclear which cell types are responsible for this change (Dishowitz et al., 2013). A recent study showed that αSMA^{+} cell population presents a downregulation of many components of the Notch signaling

pathway in the early phases after fracture, including all four receptors and two of the canonical target genes. These changes have been observed specifically in the αSMA^+ population. It has been also demonstrated that forced expression of Notch in αSMA^+ cells from periosteum and bone marrow blocks differentiation into osteoblast, chondrocyte, and adipocyte lineages in vitro and reduced bone formation in an in vivo implant (Matthews et al., 2014). Downregulation of Notch signaling in these cells may be important for their contribution to fracture callus formation. However, further studies are required to clarify the role of Notch signaling in different lineage populations in vivo at different stages of fracture healing.

Platelet-derived growth factors (PDGFs)

PDGFs are involved in the wound-healing processes of various tissues, including bone. PDGF ligands and receptors have been shown to be expressed in osteoblasts, chondrocytes, and mesenchymal stem cells (Kaigler et al., 2011). Deletion of *PDGF receptors* α and β in mice showed that the primary effects of PDGF signaling in bone healing are proliferation and migration responses (Tokunaga et al., 2008). Recombinant PDGF-BB is currently utilized for periodontal regenerative therapy (Kaigler et al., 2011), but if it can be utilized for periosteal-mediated bone regeneration has not been proven yet.

Fibroblast growth factors (FGFs)

The role of fibroblast growth factors (FGFs) and their receptors in fracture healing has been extensively investigated over the years (Du, Xie, Xian, & Chen, 2012). *Fgf2, 5*, and *6* are strongly upregulated during early callus formation and maintain high levels of expression throughout the healing process. Other FGFs, such as *Fgf16* and *Fgf18*, reached their peak expression at the chondrogenesis stage of callus formation, whereas FGF receptors such as *Fgfr1* and *Fgfr2* were shown to be expressed in the proliferating periosteum stage (Du et al., 2012). Many studies have explored the use of FGFs,

especially FGF2, for promoting fracture healing. In the mouse, rat, rabbit, and non-human primates, FGF2 has been found to stimulate the proliferation of periosteal cells, osteoprogenitors, and chondrogenitors, enhancing callus formation (Du et al., 2012). However, some studies have shown that FGF2 treatment did not lead to increased bone mineral density and increased mechanical strength of the callus (Nakajima, Nakajima, Ogasawara, Moriya, & Yamazaki, 2007). To explain this discrepancy, additional studies suggested that the effect of FGF on bone formation is biphasic, with inhibitory effects at high doses (Du et al., 2012). All up to date data about FGF and bone healing indicate that a precise, time-controlled modulation of FGF signaling may be useful in bone-regenerative procedures (van Gastel et al., 2014).

Indian hedgehog (Ihh)

The Indian hedgehog (Ihh) pathway is known to be a master regulator in chondrocyte differentiation, proliferation, and maturation, especially during endochondral ossification (Lai & Mitchell, 2005). Loss of hedgehog signaling in postnatal chondrocytes, either genetically or pharmacologically, leads to loss of epiphyseal growth plates, with premature fusion and shortened stature. However, Ihh also plays critical roles in periosteum-mediated fracture healing. Studies have shown that *Ihh* is highly expressed during callus formation of both non-stabilized and stabilized fractures (Le, Miclau, Hu, & Helms, 2001), and appears to be expressed within the newly formed cartilaginous tissues, close to the bone surface of the callus (Q. Wang, Huang, Zeng, Xue, & Zhang, 2010). All Hh signals are transduced by the transmembrane protein known as Smoothened (Smo). By using a tamoxifen-inducible cre-mediated gene inactivation mouse model, Wang *et al. (Q. Wang et al., 2010)* showed that deletion of Smo at the onset of bone autograft repair resulted in a nearly 50% reduction in periosteal bone callus formation at the cortical bone junction. From the healing periosteum, they also isolated a population of early periosteum-callus-derived mesenchymal stem cells; gene knockout of *Smo*

markedly reduced osteogenic differentiation of these progenitor cells (Q. Wang et al., 2010). Thus, targeting the hedgehog pathway may represent an approach to bone anabolism as well as an approach to periosteal-mediated bone regeneration.

Inflammatory mediators

A wide variety of inflammatory signals are thought to regulate the periosteal response during fracture healing. Indeed, matrix metalloproteinase-9 (*Mmp9*), strongly expressed by neutrophils and macrophages in the early fracture callus, is involved in bone healing. Colnot and her group have shown that *Mmp9*$^{-/-}$ mice have non-unions and delayed unions of their fractures caused by persistent cartilage at the injury site (Colnot et al., 2003). It has been further demonstrated that MMP9 mediates its effects on skeletal cell differentiation by regulating the inflammatory response and the distribution of inflammatory cells, leading to the local regulation of periosteal cell differentiation (X. Wang et al., 2013). Cyclooxygenase 2 (COX-2) is the inducible isoform of cyclooxygenase, an enzyme involved in the prostaglandin biosynthesis pathway, which is a critical regulator in inflammation. *Cox-2* was found in chondroprogenitors and mesenchymal cells along the periosteal surface, and its expression was correlated with the early induction of chondrogenesis and early expansion of the cartilaginous callus (Xie et al., 2009). Administration of a COX-2 inhibitor in the early phase of the healing process compromised fracture repair (A. M. Simon, Manigrasso, & O'Connor, 2002). Furthermore, genetic ablation of *Cox-2* impaired periosteal progenitor cell proliferation and delayed subsequent endochondral and intramembranous repair, leading to reduction of bone formation, persistence of cartilaginous tissue, and high incidence of non-unions. Contrarily, overexpressing *Cox-2* locally accelerated fracture healing and bone unions (X. Zhang et al., 2002). Although further studies are needed, molecules involved in the inflammatory process may represent a valid target in periosteal-mediated bone-regenerative therapy.

Aim of the study

The extracellular microenvironment of the stem cell niche is known to be determinant for the regulation of MSCs behavior. Low oxygen tension is an important component of the stem and progenitor cells microenvironment since it provides signals that are conducive to the maintenance of stem cell functions (Buravkova et al., 2009), modulating the behavior of different stem cell types, including adult stem cells (Fotia et al., 2015). A less studied but peculiar feature of the stem cell niche microenvironment is the pH levels of the interstitial space, which is able to influence MSCs proliferation, differentiation and paracrine activity (Disthabanchong et al., 2007). Specifically, an acidic microenvironment is able to modulate stemness and regeneration of MSCs (Mohyeldin et al., 2010). Similarly, autophagy, that can be induced by acidic conditions, has been associated with the regulation of MSC stem-like features, as well as with senescence and cell death/survival (Eom et al., 2014). Furthermore, the activation of inflammatory pathways (Lardner, 2001) can be triggered by acidosis a condition typically found in several chronic inflammatory diseases associated with bone loss, secondary osteoporosis, and, as a consequence, an increased fracture risk with impaired bone healing (Hardy & Cooper, 2009). Inflammation is an important factor during bone healing, with molecular factors and immune cells acting locally at the fracture site in a finely tuned sequence of events. Changes in the pH value of the extracellular microenvironment affect bone generation and mineralization, since acidosis inhibits the deposition of mineral matrix by osteoblasts and promotes bone resorption by osteoclasts (T. R. Arnett, 2010).

Even though there is still a lot to fully define the pathways of bone regeneration, several studies have been developed, providing a general understanding of how fracture healing occurs (Bonnarens & Einhorn, 1984). Fracture healing is a regenerative process that involves coordinated responses of many cell types, but the characterization of these specific cell populations has been limited. Recent lineage-tracing studies during fracture healing in mice have shed a first light on the markers that could be used

to identify skeletal progenitor cells *in vivo,* even though, in the murine system, some of the MSCs antigens are not universal for all the mouse strains. The common view is that murine mesenchymal progenitor cells are confined in the $CD45^{-}Ter119^{-}CD31^{-}$ fraction (Anjos-Afonso & Bonnet, 2008; Peister et al., 2004; Phinney et al., 1999). Several studies support the idea that Sca1 could be a potential positive marker for the identification of murine mesenchymal progenitor cells, alone or in combination with other markers (Lundberg et al., 2007; Morikawa et al., 2009; Nakamura et al., 2010; Steenhuis et al., 2008). Other potential markers defined as possible candidates to identify progenitor cells in mice model *in vivo* are CD51/Alpha V (integrin alpha V), PDGFRα, CD105 and CD90 (Arai et al., 2002; Kitaori et al., 2009; Lundberg et al., 2007; Morikawa et al., 2009; Nakamura et al., 2010). In previous studies from Kalajzic's lab, Grcevic *et al*. (2012) have used alpha smooth muscle actin(αSMA)–CreERT2 transgenic mice to demonstrate that the majority of the callus cells, including chondrocytes and osteoblasts, are derived from αSMA-expressing cells (Grcevic et al., 2012). αSMA is a marker of pericytes and myofibroblastic cells that identifies mesenchymal progenitor cells with proliferative and multi-lineage differentiation potential in bone marrow stromal cell cultures in vitro (Z. Kalajzic et al., 2008), and osteoblast precursors in the bone marrow and periodontium in vivo (Grcevic et al., 2012; Roguljic et al., 2013). Specifically, αSMA^{+} cells isolated from periosteum exhibited skeletal progenitor cell characteristics, including the expression of several mesenchymal progenitor cell markers and the ability to differentiate along the osteogenic, chondrogenic and adipogenic lineage (Grcevic et al., 2012; Matthews et al., 2014).

In this study, we first investigated how variations in extracellular pH can affect MSCs behavior, in light of their great importance in clinical applications of regenerative medicine. We therefore evaluated the effect of acidity on MSCs proliferation, osteogenic potential, activation of survival mechanisms, such as autophagy, and release of inflammatory molecules. We also aimed to identify progenitor cells activated during the bone healing process in a mouse model *in vivo*. We looked for potential markers to

define osteo- and chondro-progenitor populations, focusing our attention on the periosteum, which is the bone compartment that gives the major contribution to bone healing (Ferretti & Mattioli-Belmonte, 2014).

Materials and methods

Cell cultures

Human bone marrow-derived mesenchymal stem cells (BMMSCs) and human adipose tissue-derived mesenchymal stem cells (ADMSCs) were purchased from Lonza (Euroclone) and from the American Type Cell Culture Collection (ATCC), respectively. Cells were cultured in Minimum Essential Medium Eagle Alpha Modified (αMEM) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), penicillin (100 U/ml), and streptomycin (100 mg/ml) (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C and 5 % CO2. BMMSCs and ADMSCs were always used before the 5–6 passage in culture. Specific pH of the culture medium (acidic pH of 6.5 or 6.8, and physiological pH of 7.4) was maintained by using different concentrations of sodium bicarbonate, according to the Henderson-Hasselbach equation. At the endpoint of each experiment, the final pH of the supernatant was always measured to ascertain the maintenance of the pH value along the incubation time.

Proliferation assay by Ki67 staining

BMMSCs were seeded on glass chamber slides ($8x10^3$ cells/well), and synchronized in the G0 phase of the cell cycle by starvation treatment for 48 hr (Rosner & Hengstschläger, 2011). Cells were then cultured with αMEM complete medium at different pHe (6.5 or 7.4) for 36 hr. Subsequently, cells were fixed with 3.7 % paraformaldehyde (Sigma-Aldrich) in PBS for 20 min at room temperature (RT) and permeabilized with HEPES-triton for 5 min. Ki67 was revealed with a monoclonal anti-Ki67 antibody (1:75, DAKO, Santa Clara, CA, USA). After washing, cells were incubated with the secondary antibody Alexa Fluor 488 nm (1:1000, Life Technologies) and 0.02 % Evans blue for 1 hr. Cells were analyzed using a fluorescence microscope. The Ki67 index was determined as the percentage of cells with Ki-67-positive nuclear staining in respect to the total cell population.

Osteogenic differentiation

To evaluate the effect of extracellular acidosis on osteogenic differentiation, BMMSCs were seeded at a density of 1×10^4 cells/cm^2 in αMEM supplemented with 10% FBS, 50 mg/mL L-ascorbic acid 2-phosphate, 10^{-8} M dexamethasone (Sigma-Aldrich), at different pHe (6.5 and 7.4).

The deposition of mineralized matrix was evaluated after 21 days of treatment with osteogenic medium at different pHe. Cells were fixed in 3.7 % paraformaldehyde for 20 min and stained with 2% alizarin red at pH 4.2 (Sigma-Aldrich) for 1 hr at RT. Then, the staining was eluted with a solution of 10% cetylpyridinium chloride (CPC) (p/v) (Sigma-Aldrich) and the absorbance at 570 nm was quantified by using a microplate-reader (Tecan Infinite F200pro, Männedorf, Switzerland). The results were expressed as mean optical density (OD).

Western blotting

BMMSCs were cultured at both physiological (7.4) and acidic (6.8) pH for different time points (1, 3 and 7 days), to analyze protein expression of typical autophagic markers by western blotting. The inhibitor of autophagy bafilomycin A1 (BafA1) (Yamamoto et al., 1998), was added during the last 2 hr of incubation to emphasize the autophagic process detection. Whole-cell extracts were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1% Nonidet P-40 [Sigma, I3021], 0.1% SDS and 0.5% sodium deoxycholate) supplemented with protease and phosphatase inhibitors. Equal amount of lysate was analyzed by SDS–polyacrylamide gel electrophoresis (PAGE), followed by immunoblotting overnight at 4°C with anti-human LC3B (Cell Signaling Technology, Danvers), anti-human p62 (BD Biosciences, San Jose, CA) antibodies, or anti-human ACTB (Dallas, TX) antibody, as reference. The next day membranes were washed and incubated for 1 hr with HRP-conjugated secondary antibody and the binding was

detected by the ECL system. Whole-cell extracts signals from each band were quantified by dedicated software (Quantity One, Biorad Laboratories Headquarters, Hercules, CA).

Electron microscopy

BMMSCs were cultured at both physiological (7.4) and acidic (6.8) pH for 3 days, in order to explore the cells content with a transmission electron microscope. After the 3 days incubation, the cells were sedimented and immediately fixed with 2.5% glutaraldehyde 0.1 M cacodylate buffer pH 7.6 for 1 hr at RT. After post-fixation with 1% osmium tetroxide in cacodylate buffer for 1 hr at 4°C, pellets were dehydrated in an ethanol series and embedded in Epon resin. Ultrathin sections stained with uranyl-acetate and lead citrate were observed with a Jeol Jem-1011 transmission electron microscope.

Cytokines secretion analysis

MSCs from different sources (BMMSCs and ADMSCs) were seeded at $3x10^4$ cells/well into 24-well plates for protein quantification by specific ELISA assay. After adhesion, cells were incubated with 0.1% FBS αMEM at different pH. After 24 hr, culture supernatants were collected, spun and tested for the release of inflammatory cytokines. IL-6 secretion was quantified with Human IL-6 DuoSet, IL-8 with Human CXCL-8/IL-8 Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA). Data were normalized to the total protein content quantified by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA, USA). Colorimetric reactions for all the ELISA assays were quantified by a microplate-reader (Tecan Infinite F200pro, Männedorf, Switzerland).

Real time PCR

BMMSCs and ADMSCs were cultured at both physiological (7.4) and acidic (6.8) pH for different

time points (3, 6, 24, 168 hs), to analyze inflammatory cytokines mRNA by Real time PCR. Total RNA was extracted from cells cultured at different pH by using RNeasy Mini Kit (Qiagen GmbH). Total mRNA was reverse transcribed by the Advantage RT-for-PCR Kit (Roche). The expression of the genes of interest was evaluated by using the Light Cycler instrumentation (Roche Diagnostics) by amplifying 1 μg of cDNA and the Universal Probe Library system (Roche Applied Science). Probes and primers were selected by using a web-based assay design software (ProbeFinder https://www. roche-applied-science.com): IL6-fwd 5'-GATGAGTACAAAAGTCCTGATCCA-3'; IL6-rev 5'-CTGCAGCCACTGGTTCTGT-3'; IL8/CXCL8-FWD 5'-GAGCACTCCATAAGGCACAAA-3'; IL8/CXCL8-5'-ATGGTTCCTTCCGGTGGT-3'). The protocol of amplification was: 95 °C for 10 min; 95 °C for 10s, 60 °C for 30s, and 72°C for 1s for 45 cycles; 40°C for 30s. The results were expressed as ratio between the gene of interest and rRNA18s (rRNA18s-fwd 5'-GCAATTATTCCCCATGAACG-3'; rRNA18s-rev 5'-GGGACTTAATCAACGCAAGC-3'), according to the 2-ΔΔCT method (Livak & Schmittgen, 2001).

Mouse strains

All animal procedures were approved by an institutional animal care and use committee, and performed in an AAALACi accredited facility. Mice were group housed in individually ventilated cages (Thoren Caging, Hazleton, PA) with a photoperiod of 12:12. The room temperature and humidity were maintained at 22°C and 30–70% respectively. Mice were fed irradiated Rodent Diet (Teklad 2918, Invigo, Indianapolis, IN), made available in pellets, and water (reverse-osmosis) ad libitum. Vendor surveillance and colony sentinel monitoring results show that the colony is free from all pathogens tested. The αSMACreERT2 (Grcevic et al, 2012) transgenic mouse has been previously described. The SMACre/Ai9 dual transgenic mice were generated by breeding αSMACreERT2 mice with Ai9 reporter mice (B6;129S6-Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/J, stock # 007905), obtained from Jackson Laboratory (Bar Harbor, ME, USA). SMACre/Ai9/Col2.3GFP transgenic mice were generating by breeding

SMACre/Col2.3GFP mice (I. Kalajzic et al., 2002), previously described, with Ai9 mice. The Ai9 mice harbor a targeted mutation of the Gt(ROSA)26Sor locus with a loxP-flanked STOP cassette preventing transcription of a CAG promoter-driven red fluorescent protein variant (tdTomato).

Immunodeficient NSG mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ, stock # 005557), obtained from Jackson Laboratory (Bar Harbor, ME), carry two mutations on the NOD/ShiLtJ genetic background, severe combined immune deficiency (*scid*) and a complete null allele of the IL2 receptor common gamma chain (*IL2rg*^{null}). The *scid* mutation is in the DNA repair complex protein *Prkdc*and renders the mice B and T cell deficient. The *IL2rg*^{null} mutation prevents cytokine signaling through multiple receptors, leading to a deficiency in functional NK cells.

Mice known as R26-M2rtTA/TetOP-H2B-GFP (B6;129S4-*Gt(ROSA)26Sor*$^{tm1(rtTA*M2)Jae}$ *Col1a1*$^{tm7(tetO-HIST1H2BJ/GFP)Jae}$/J, stock # 016836), obtained from Jackson Laboratory (Bar Harbor, ME), allow doxycycline-inducible, fluorescent labeling of cells. In the first targeted mutation, Human *HIST1H2BJ* (histone cluster 1, H2bj) produces a GFP fusion protein under the direction of the tetO minmal CMV promoter downstream of the *Col1a1* (collagen, type I, alpha 1) gene; in the second mutation, *Gt(ROSA)26Sor* drives expression of an optimized rtTA.

In order to activate the tetO system, thus enhancing GFP expression, we fed donor mice for transplantation with doxycycline containing food starting 3 days prior to sacrifice. Doxycycline food contained 625 mg/kg doxycycline (Harlan Laboratories, Indianapolis, IN), resulting in an estimated daily doxycycline dose of approximately 1.6-2.7 mg/day. Recipient mice for transplantation (NSG) were also fed with food containing the same dose of doxycycline mentioned above, starting a few days prior to surgery, which was switched with doxycycline water at a dose of 2mg/ml in 5% sucrose after the transplant.

In order to label αSMA-expressing cells, tamoxifen (Sigma Aldrich, St. Louis, MO) dissolved in corn oil was administered by intraperitoneal injection at a dose of 75 μg/g of bodyweight.

Primary cell isolation for mice studies

Cells from bone marrow, periosteum and endosteum bone compartments were isolated from mice tibias and femurs. Mice bones were dissected, muscle and tendon tissue were removed, and bone marrow flushed. After flushing, bone marrow was seeded at a density of approximately $1x10^7$ cells/100 mm dish in αMEM supplemented with 20% FCS, and placed in a low oxygen incubator (5% O2) for traditional BMMSC adherent culture; media was changed after 4 days of incubation, and the cells were confluent after 7 days.

BMMSC were also used for flow cytometry analysis, and, in this case, cells were incubated with red blood cells (RBC) lysis buffer (155 mM NH4Cl, 10 mM KHCI3, 0.1 mM EDTA in ddH2O). RBC was added to the cells for 5 min incubation, at room temperature; lysed cells were then washed with staining media (SM) made of PBS containing 2% FBS and 1 mM EDTA, and filtered. Periosteum was scraped from the diaphyseal cortex, then digested in PBS containing 0.05% collagenase P (Roche, Mannheim, Germany), and 0.2% hyaluronadase (Sigma, St. Louis, MO, USA) for 1 hr at 37°C with agitation; cells were then resuspended in culture media containing 10% FBS, and passed through a strainer; after spinning, cells were washed again in a large volume of PBS (~40 mL), and then resuspended in SM. Mice bones were then washed repeatedly in PBS, and cut in smaller pieces, and endosteum cells were digested in PBS containing 0.05% collagenase A (Roche, Mannheim, Germany) for 1 hr at 37°C with agitation; the bones were crushed after the first 30 min incubation; crushed bones were then washed twice with PBS, and filtered, and the cells were eventually resuspended in SM.

Bone injury procedures

Closed transverse diaphyseal fractures of the right tibias were created in 2-months-old mice. Briefly, before fracture, a 0.38-mm-diameter stainless steel pin was inserted into the medullary canal.

Fractures were created 1 to 2 mm proximal to the distal tibia-fibula junction using a drop-weight blunt guillotine device. X-ray (Faxitron LX-60) was used to confirm pin and fracture placement.

An alternative to fracture in which the exclusive contribution of periosteum is more clear is the scratch injury. For this procedure, mice were anesthetized with 2-3% isoflurane in oxygen, and then scratched on all four limbs using a needle through the skin and the muscle. In this way, there is a substantial thickening of the periosteum, and the activation of all the cells involved in injury repair that are coming from this specific bone compartment.

Histology

Mice bones were fixed in 4% paraformaldehyde for 2-4 days at 4°C, the intramedullary pins were removed from fractured samples, and bones were incubated overnight in 30% sucrose/PBS, and embedded in Cryomatrix (Thermo Fisher Scientific, Waltham, MA, USA). Sections of 7 µm were obtained on a cryostat (Leica, Wetzler, Germany) using a tape transfer system (Section-lab, Hiroshima, Japan). Sections were coverslipped in 50% glycerol containing DAPI. Imaging was performed using appropriate filter cubes on an Axioscan (Carl Zeiss, Thornwood, NY, USA). Consistent exposure times were maintained to allow for image analysis.

Immunostaining

After fluorescent imaging, coverslips were removed and immunostaining was performed for the chondrocyte marker Sox9 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), for the tibias samples. Sections for immunostaining were rehydrated in PBS, permeabilized in 0.1% Triton X-100 for 15 min, and blocked with 2% BSA/PBS for 1 hr, in a humidified chamber at room temperature. Primary antibody diluted in PBS 2% BSA was applied overnight at 4°C. After washing, secondary antibody diluted 1:300

was applied for 1 hr at room temperature (goat anti-rabbit Alexa Fluor 647, Life Technologies, Carlsbad, CA, USA). Sections were then coverslipped again, and the images analyzed.

Image analysis

αSMA^+ cells contribution to fracture healing was quantified in histological sections using ImageJ (NIH). Callus area was split in three parts, upper, lower and central, referring to a 2-mm line drew starting from the fracture point. Within each callus region of interest (ROI), individual cells were defined using the DAPI channel, and separated using the watershed algorithm. Appropriate standardized thresholds for the red and green channels were determined, then signal levels in both channels were determined for each nuclear region, and used to count the number of red, green, and dual-labeled cells. The proportion of labeled osteoblasts was determined by calculating the proportion of green cells that were also red. Chondrocytes were quantified by drawing ROIs around areas of chondrocytes, as determined by SOX9 staining and confirmed by cells morphology, then calculating the proportion of red nuclei in these ROIs. To ensure values were representative of all the samples, 3-4 sections were analyzed per sample.

Flow cytometry

Periosteum was used to sort cells for low density cultures and to perform cell surface marker analysis, together with bone marrow and endosteum. After processed as described above, cells resuspended in SM were stained with the following antibodies: CD45 (clone 30-F11), CD31 (clone 390), Ter119 (clone TER-119), Sca1 (clone D7), CD105 (clone MJ7/18), PDGFRα (clone APA5), all purchased from eBioscience (San Diego, CA, USA), and CD51 (clone RMV-7) and CD90 (clone 30-H12) purchased from BioLegend (San Diego, CA, USA). FACSAria II (BD Biosciences, San Jose, CA, USA) was used for cell sorting, while LSR II (BD Biosciences, San Jose, CA, USA) was used for cell analysis. Data were analyzed

with FlowJo (TreeStar, Ashland, OR, USA) and FACS Diva (BD Biosciences, San Jose, CA, USA) software. Voltages and gates were set based on unstained samples from Cre-negative and Cre-positive animals.

Colony Forming Unit (CFU) assay

Approximately 0.5-1×10^3 sorted cells per well were seeded on a 6-well plate in αMEM supplemented with 20% FCS, and placed in a low oxygen incubator (5% O2). Based on previous experience sorting on similar cell populations, we found a cell survival rate of about 50%, so we considered this to calculate the proper density to seed. Groups of cells of consistent amount were also seeded at higher densities, generally the negative population. Total media was changed after 4 days, and, after other 3-4 days, different tests were performed. PrestoBlue cell viability reagent (ThermoFisher Scientific) was used at a concentration of 5% in αMEM 10% FCS to test cell viability. Our purpose was to evaluate cell growth keeping the cells alive, in order to decide when the culture was ready to be stopped. PrestoBlue reagent is a resazurin-based solution that functions as a cell viability indicator by using the reducing power of living cells to quantitatively measure the proliferation of cells. Cells were incubated at 37°C for 3 hs, then 200 µL of each well supernatant was transferred to a 96-well plate, and the fluorescence was measured by using the excitation (570 nm) and emission (583 nm) peaks for resorufin, with a microplate reader (Tecan Infinite F200pro, Männedorf, Switzerland).

Alkaline phosphatase (ALP) assay was performed to detect ALP activity of the cells (CFU-ALP). Cells were fixed with 10% formalin for ~5 min, washed with water, then ALP reaction mix (Sigma-Aldrich) was added to the wells. After 20 min in the darkness, wells were gently washed with water and left to dry.

Crystal violet staining (250 mg of Crystal violet powder (Sigma-Aldrich) dissolved in 500 µL of ethanol, and 500 mL of water) was performed to visualize the colony formation (CFU-F). Cells were stained for ~3 min, then gently washed with water and left to dry.

Plates were scanned, and colonies were counted using ImageJ (NIH). Adherent cell clusters containing >50 cells were counted as a colony.

Calvarial transplantation

NSG mice were anesthetized with ketamine (87 mg/kg) and xylazine (13 mg/kg), and two 3.5-mm diameter calvarial bone discs were extracted from both sides of the suture, carefully avoiding damaging the dura mater. Scaffold discs of 3.5 mm of diameter and 0.5 mm of thickness were cut from a hydroxyapatite/collagen matrix (HEALOS, DePuy Spine, Inc., Raynham, MA, USA), to be placed into the defect area. Different numbers of sorted cells were loaded on each scaffold in 5-7 μL of media; tdTomato-negative loaded cells were 20000, tdTomato-positive loaded cells were 4-5000, $CD51^+$ and $CD51^+Sca1^+$ loaded cells were 13-20000, $Sca1^+$ loaded cells were 2-4000. All the positive cells were loaded together with 250000 BMMSC as carrier. All the mice woke up and recovered well after transplantation, and were maintained for 5 weeks after surgery.

Statistical analysis

Statistical analysis of human MSCs was performed by the StatViewTM 5.0.1 software for Windows (SAS Institute, Cary, NC, USA). To evaluate Ki67 index for cell proliferation, Wilcoxon Rank for paired analysis was used. To evaluate cytokines secretion, Mann–Whitney U test was used as unpaired comparison for two independent variables. Results were reported as mean ± standard error. Only $p<0.05$ were considered as statistically significant.

Statistical analysis of cells isolated from mice was performed by the GraphPad Prism statistical software (Graph Pad, Inc, San Diego, CA). Data are reported as the mean value ± standard deviation. Values were analyzed by ANOVA with post-hoc analysis by the Tukey's test method for multiple comparisons

between pairs or by Student's t-test. Values were considered significant if $p<0.05$ in comparison to negative controls.

Results

Acidic microenvironment impairs BMMSCs proliferation and osteogenic differentiation.

MSCs behavior is influenced by the niche microenvironment *in vivo* (Disthabanchong et al., 2007; Sacchetti et al., 2007). On these basis, we mimicked a peculiar microenvironment *in vitro*, by culturing MSCs in acidic medium, and then analyzed their proliferation and differentiation activity. Even though systemic pH of 7.0 or below is generally only seen clinically in multi-organ failure with profound hypoperfusion, local tissue pH can reach even lower levels. Hence, in bone trauma, it is conceivable that intracellular and interstitial fluid pHs in the immediate area of blood supply disruption could have pHs down to 6.4 – 6.5 (Bischoff, Zhu, Makhijani, & Yamaguchi, 2008).

First, we verified if the culture medium pH values, that we chemically adjusted to simulate different extracellular pHe, were maintained over the incubation period. After 24 h, the culture medium that was originally buffered at the physiological value of pH 7.4 showed a slight decrease to pHe 7.2 (Figure 1**A**), which was maintained over time. For the medium originally buffered at acidic pHe 6.5, the pH value was more stable and constant at all the examined time points (Figure 1**A**). We then analyzed BMMSCs proliferation in low pH conditions, and observed a significant reduction, correlated with a lower number of cycling cells, as revealed by the Ki67 index that significantly decreased in a pH-dependent manner (Figure 1**B**).

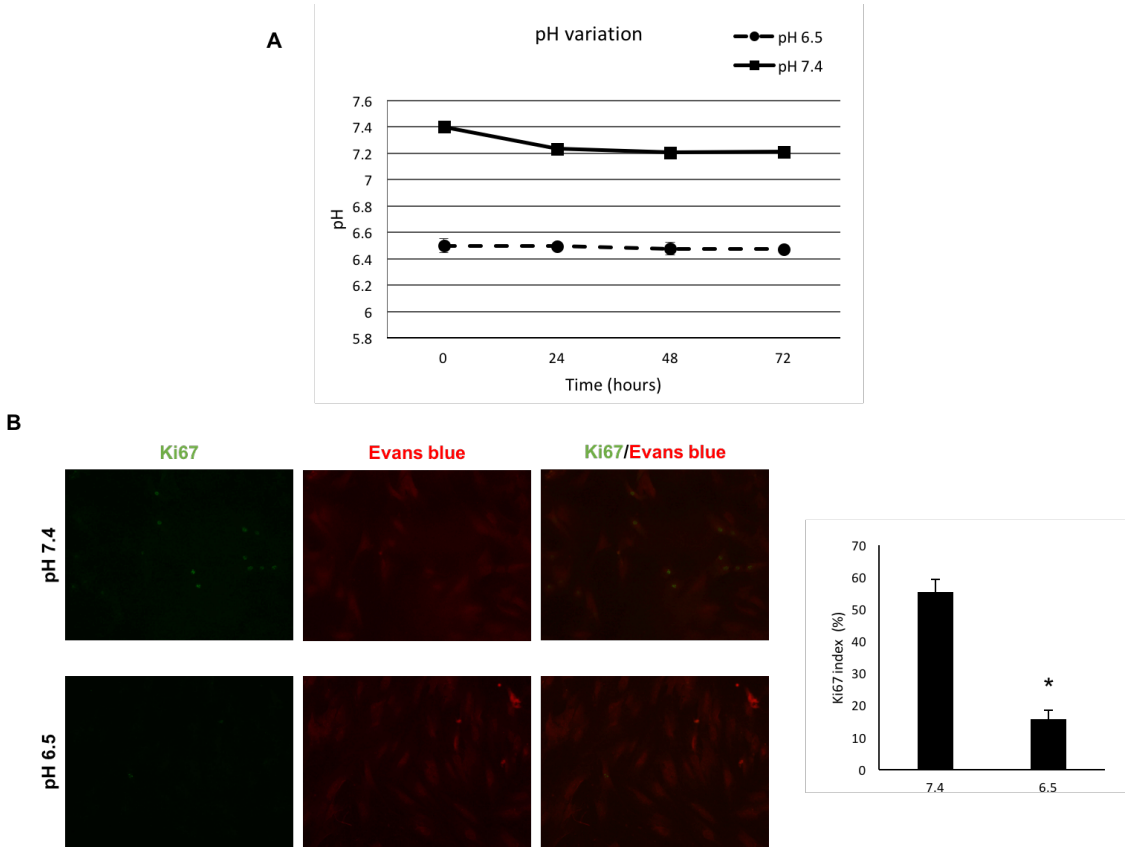

Figure 1. Extracellular acidosis inhibited BMMSCs cell proliferation. Cells were seeded and incubated at the specified pH. pH maintenance of media with different sodium bicarbonate concentrations over the culture period (**A**). Representative images of Ki67 staining of BMMSCs at different pHe (**B**). Nuclear Ki67 (green) and cytoskeletal staining by Evans blue (red). Quantification of the Ki-67 index. Results were obtained with two different donors and three technical replicates; * $p < 0.05$.

We also observed that extracellular acidosis impairs BMMSCs osteogenic differentiation (Figure 2). Indeed, the formation of mineral nodules, an indirect index of osteogenic differentiation, was strongly inhibited at pHe 6.5 compared to pHe 7.4 (Figure 2).

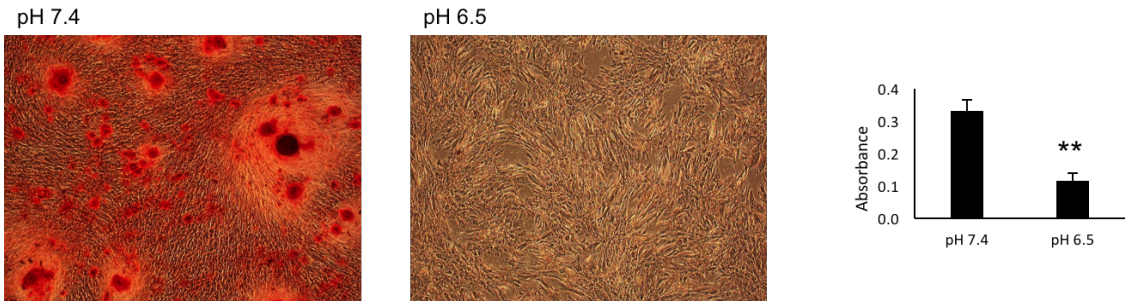

Figure 2. **Extracellular acidosis impaired osteogenic differentiation of BMMSCs.** Representative pictures of alizarin red assay after 21 days of incubation with osteogenic medium at different pH, and the respective quantification (absorbance at 570 nm). Results were obtained with three different donors and two technical replicates, ** $p < 0.01$.

Autophagy is activated by BMMSCs at both acidic and physiological pH.

It has been shown that cells activate autophagy as a survival mechanism in microenvironmental stressful conditions (Kroemer et al., 2010). Autophagy has been associated with the regulation of MSC stem-like features (Sordella, Jiang, Chen, Curto, & Settleman, 2003), and can be induced by acidic conditions. The autophagic flux of BMMSCs was evaluated by protein expression of typical autophagic markers (LC3-II, p62 and Beclin1) at lower (pH 6.8) or neutral (pH 7.4) pH (Figure 3**A**, 3**B**). Despite we could observe a detectable autophagic activity of BMMSCs in both culture conditions, as shown by LC3-I to LC3-II conversion, no significant variations between pH conditions could be observed, leading to the speculation that autophagy is not the main mechanism by which MSC face an acidic microenvironment. Ultrastructural analysis was used to confirm our findings: we observed the typical features of autophagy, such as formation of autophagic vacuoles containing cytoplasmic components and organelles (Figure 3**C**), but no qualitative differences among different pHe.

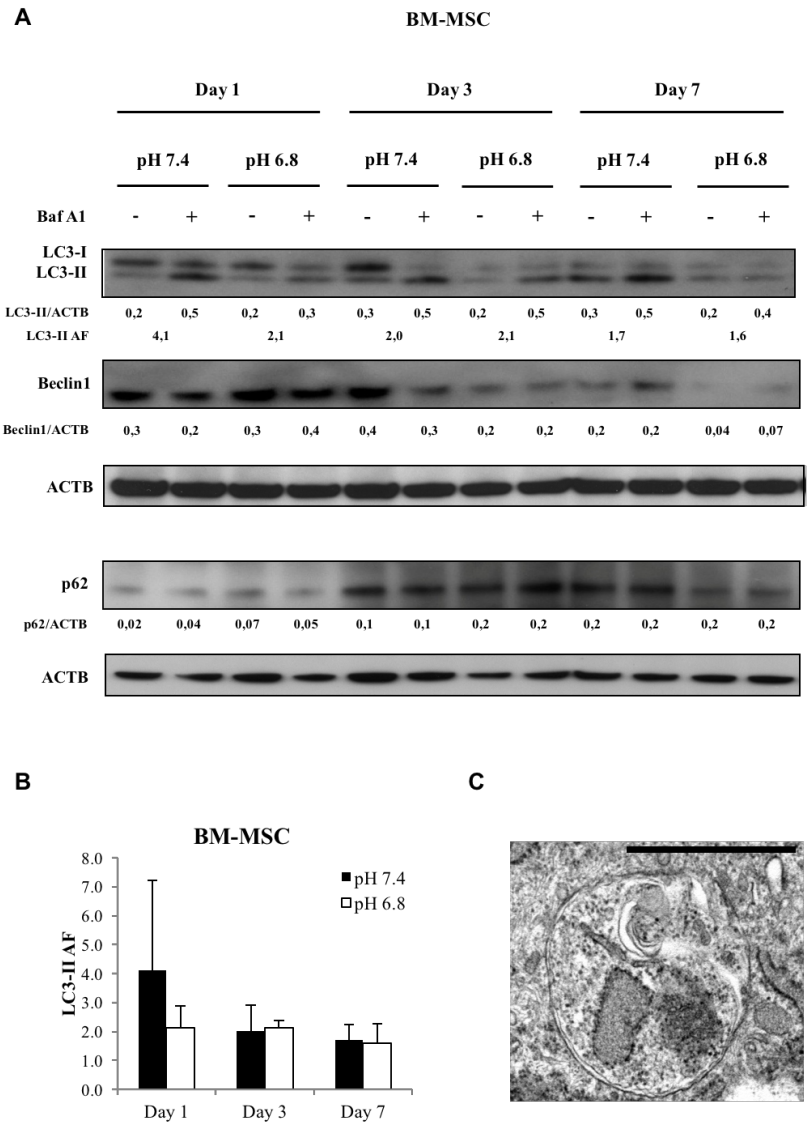

Figure 3. Autophagic flux in BMMSCs was detected at both pH 7.4 and pH 6.8. Protein expression of typical autophagic markers (LC3-II, p62 and Beclin1) evaluated by Western blot (**A**), and autophagic flux (AF) quantification (**B**). Representative transmission electron microscopy image of a double-membrane autophagosome in BMMSCs cultured at acidic pH (**C**). Scale bar=1 µm.

Acidic microenvironment triggers BMMSCs secretion of inflammatory cytokines.

Acidic pH values of 6.8 are commonly associated to several pathological conditions (Bischoff et al., 2008), and are known to induce the activation of inflammatory pathways with consequent release of

cytokines (Lardner, 2001). On these bases, we evaluated the paracrine activity of the MSCs most used sources in clinical application (BMMSCs and ADMSCs) when cultured in acidic conditions, at both protein and mRNA levels. An ELISA assay showed a significant increase in IL-6 and IL-8 secretion at pH 6.8, compared to pH 7.4 (Figure 4**A**). At the mRNA level, inflammatory cytokines presented a time-dependent regulation, with a very high variability due to the intrinsic differences between the different MSCs sources used (Figure 4**B**). We thus observed an increased release of inflammatory cytokines at both protein and mRNA levels (Figure 4**A** and 4**B**).

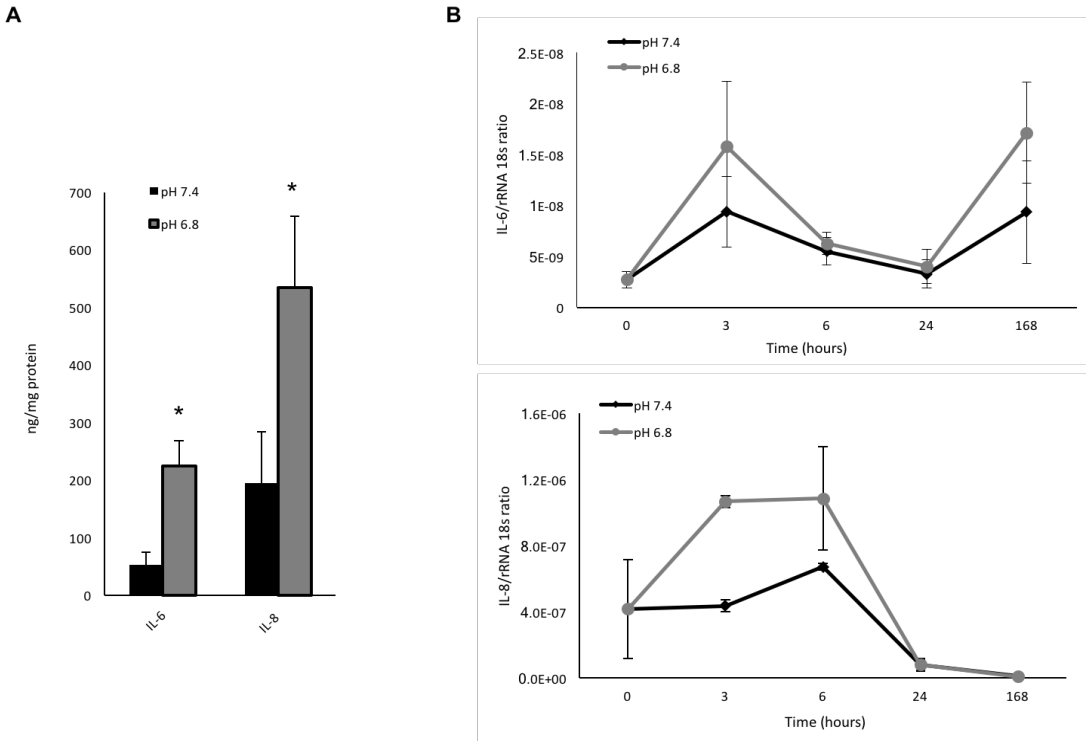

Figure 4. **Extracellular acidosis promotes the secretion of inflammatory cytokines IL-6 and IL-8**. IL-6 and IL-8 protein expression after 24 hr of incubation with acidic medium by using specific ELISA (**A**). IL-6 and IL-8 mRNA analysis of BMMSCs and ADMSCs at different time points of incubation with acidic medium. Results were obtained with three different donors and two technical replicates, * $p < 0.05$.

Periosteum is significantly enriched in progenitor cell markers, including αSMA, compared to endosteum and bone marrow.

MSCs *in vivo* properties are mostly studied by using murine models. Some of the MSCs antigens are not universal for all the mouse strains, however it is assessed that murine mesenchymal progenitor cells are confined in the $CD45^{-}Ter119^{-}CD31^{-}$ fraction (Anjos-Afonso & Bonnet, 2011). Most studies focus on cells isolated from the bone marrow, or total bone (Chan et al., 2013; Chan et al., 2015; Morikawa et al., 2009), and use a wide number of cell surface marker combinations, although there are differences between different groups. Sca1, PDGFRα, CD105, CD90, and CD51 seem to be the most promising candidates as markers to identify progenitor cell populations in bone (Chan et al., 2013; Chan et al., 2015; Lundberg et al., 2007; Morikawa et al., 2009; Nakamura et al., 2010; Steenhuis et al., 2008). Grcevic *et al.* (2012) previously demonstrated that the majority of the callus cells, including chondrocytes and osteoblasts, are derived from αSMA-expressing cells, thus qualifying αSMA as a marker of osteo- and chondro-progenitors. αSMA is known to be a marker of pericytes and myofibroblastic cells that identifies mesenchymal cells with proliferative and multi-lineage differentiation potential (Grcevic et al., 2012; Z. Kalajzic et al., 2008; Roguljic et al., 2013). Specifically, it has been observed that αSMA^{+} cells isolated from periosteum exhibited skeletal progenitor cell characteristics, including the expression of several mesenchymal progenitor cell markers and the ability to differentiate along the osteogenic, chondrogenic and adipogenic lineage (Grcevic et al., 2012; Matthews et al., 2014). Periosteum is the bone compartment from which most of the cells come during the bone repair process, so it is of great interest in the study of identification of progenitor cell populations.

The mice used in this study (SMACre/Ai9) combine a tamoxifen-inducible Cre transgene driven by the αSMA promoter, and a Cre-activated fluorescent reporter tdTomato. This allows the tracking of developmental progression of αSMA-expressing cells by activating the expression of the tdTomato visual

reporter through Cre-mediated recombination. In order to characterize the periosteum cell population, we performed flow cytometry analysis for stem cell surface markers expression. Mice were treated twice with tamoxifen before the sacrifice, and cells from the different bone compartments were collected. We excluded from the analysis the cells belonging to the hematopoietic and the endothelial lineages, gating for CD45/Ter119/CD31 markers. We observed that all the stem cell surface markers analyzed are expressed in periosteum cells. Specifically, the flow analysis showed a substantial higher frequency of markers, such as Sca1, PDGFRα, and CD90, in the periosteum compartment respect to endosteum or bone marrow, where they are expressed in a very low proportion of cells. (Figure 5).

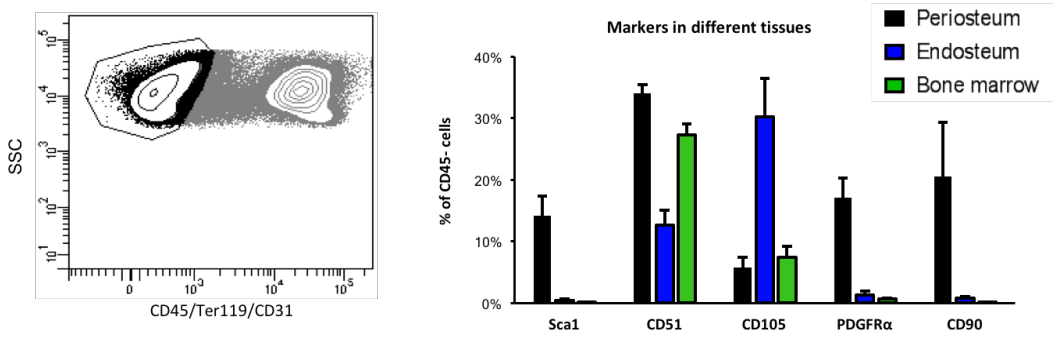

Figure 5. **Stem cell surface markers are expressed in a high number of cells in periosteum, compared to endosteum and bone marrow.** Cells from the different bone compartments were collected and analyzed for stem cell surface markers expression by flow cytometry. Cells of the hematopoietic and the endothelial lineages were excluded from the analysis. The graph shows an experiment in which four groups of two female-9-weeks-old mice each have been used (n=4). We repeated the same experiment two other times, and we obtained the same trend of markers frequency.

αSMA has been found to be a promising marker for the identification of progenitor cells (Grcevic et al., 2012; Matthews et al., 2014), thus we analyzed the frequency of αSMA-labeled cells in the different

bone compartments, within the $CD45^{-}Ter119^{-}CD31^{-}$ population. We observed that αSMA-labeled cells are more abundant in periosteum compared to endosteum, and that they are almost absent in the bone marrow (Figure 6).

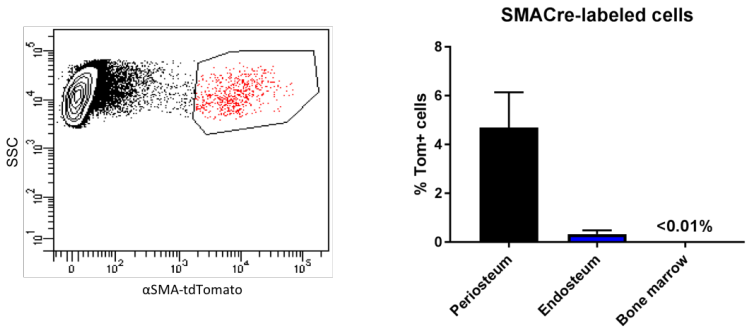

Figure 6. αSMA-labeled cells are more abundant in periosteum compared to the other bone compartments. αSMA-labeled cells frequency in the different bone compartments was analyzed by flow cytometry. The results were obtained from five different male mice of 7-8 weeks of age (n=5).

αSMA-labeled cells contribute to callus osteoblasts and chondrocytes formation in a time-dependent way, and part of them constitutes a local population.

Previous studies in Kalajzic's lab have shown that αSMA-expressing cells contribute to chondrocytes and osteoblasts formation during the regenerative process (Grcevic et al., 2012; Matthews et al., 2014). Our interest was then related to this cells activation timing and their origin. On these bases, we hypothesized the existence of αSMA-expressing long-term osteo- and chondro- progenitors in the periosteum, and we investigated on whether these αSMA-expressing cells were tissue-resident. For this study, we used a tibial fracture mice model. To track the population of αSMA-expressing cells that contribute to generate mature osteoblasts, we bred SMACre/Col2.3GFP mice (Matthews et al., 2014) with Ai9 mice, obtaining SMACre/Ai9/Col2.3GFP mice. Different groups of mice were treated twice with

tamoxifen at different time points, which led us to permanently label αSMA-expressing cells, and to trace their progeny during the fracture-healing process (Figure 7).

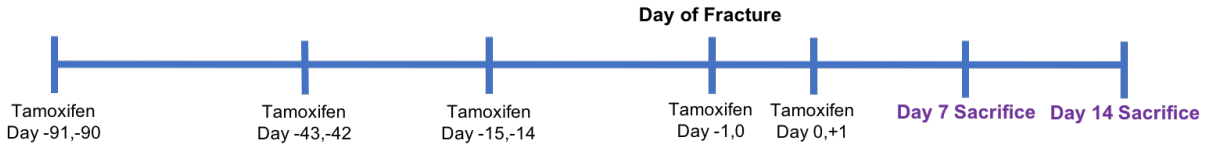

Figure 7. **Experimental timeline.** Mice were injected twice with tamoxifen 3 months, 6 and 2 weeks prior to fracture, at the fracture time, and after the fracture. All the groups of mice were sacrificed 7 and 14 days after fracture.

After the formation of the fracture callus, we collected and processed the tibias to quantify the fluorescent signals: red-tdTomato signal for αSMA-expressing cells, and green-Col2.3GFP signal for mature osteoblasts. The colocalization of the two fluorescent signals is represented by the yellow color (Figure 8**A**). We observed that, in the groups of mice sacrificed 7 days after fracture (Figure 8**B**), about 80% of osteoblasts in the callus are also αSMA-expressing cells when the label occurs soon (day 0, day +1) after fracture, and about 60% when it occurs on the day of fracture (day -1, day 0). In the groups of mice sacrificed 14 days after fracture (Figure 8**C**), we found about 60% of αSMA+ osteoblasts in the callus when the label occurred on the day of fracture (day -1, day 0). These findings led us to hypothesize that αSMA identifies the majority of osteoprogenitors that differentiate into osteoblasts in the fracture callus. This percentage decreases as the labeling is induced at farther time points from the day of fracture. Based on these observations, we hypothesized that αSMA+ periosteal cells may not have significant self-renewing capabilities. After labeling the αSMA-expressing cells, 3 months or 2-6 weeks later, some of those cells had either died or differentiated into a type of cell that does not proliferate and aid in the fracture healing. There are most likely other αSMA-negative populations in the periosteum that also contribute to osteoblasts and chondrocytes in the callus.

Interestingly, we still observe a subset of αSMA-expressing cells that contribute to osteoblast formation in the callus at least three months after labeling, representing a long term osteoprogenitor population (Figure 8). However, this result is not statistically significant due to the low number of replicates, thus it will be interesting to repeat this experiment to consolidate our hypothesis.

The group of mice that did not get tamoxifen injections showed only a very small percentage of labeled cells, due to some spontaneous activation of the Cre recombinase. This negative control group is important to validate the reliability of the mouse model system we used for the study.

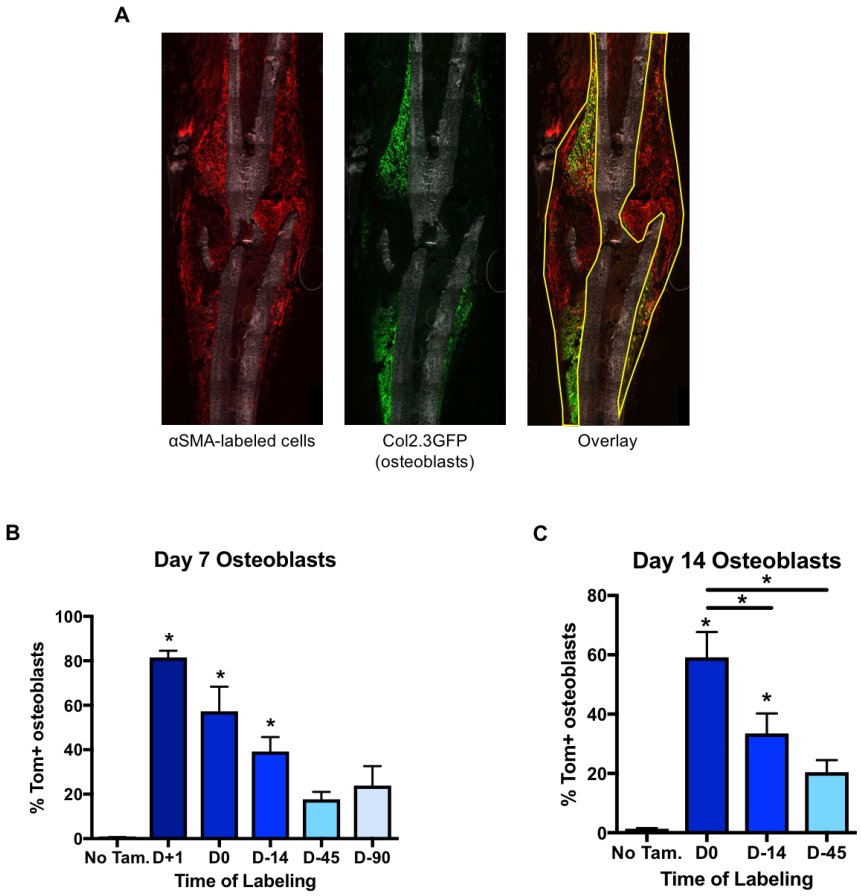

Figure 8. αSMA identify the majority of osteoprogenitors that contribute to the early stages of the healing process. Representative images of a tibia fracture callus, with ROIs drawn (right image) to show how the image analysis was performed (**A**). αSMA-labeled cells are detected in the red fluorescence

channel, Col2.3GFP-labeled osteoblasts in the green one. The overlay between the red and the green channels is represented by the yellow color. Different percentages of dual labeled cells have been found in the fracture callus at different time points of tamoxifen injection, in groups of mice sacrificed 7 (**B**) and 14 (**C**) days after fracture. The results were obtained from groups of 5-7 female mice of 8 weeks of age at time of tamoxifen injection. Tukey's multiple comparison test (ANOVA) was applied. Each group of mice that got injected with tamoxifen was compared to the negative control group. * $p < 0.05$

We also investigated αSMA-labeled cells that contribute to cartilage formation. According to the well-known fracture-healing process, a soft callus is formed within 5-10 days after injury, and it is made of fibrous tissue and cartilage. On these bases, we performed Sox9 staining on day 7 fracture sections to detect the presence of chondrocytes. We detected non-specific staining in some sections, so we analyzed the images drawing ROIs that had both Sox9 fluorescent signal and chondrocyte morphology (Figure 9). We observed that about 40% of chondrocytes in the callus are also αSMA-expressing cells when the label is induced one day after fracture, and about 30% when it is induced on the day of fracture, and that this value decreases depending on the time of labeling. As for osteoblast formation, we can conclude that αSMA-expressing cells contribute to chondrocytes generation after fracture, although a lower proportion of cells is labeled, thus suggesting that there may be some differences in the cells that contribute to osteoblasts respect to those that contribute to chondrocytes.

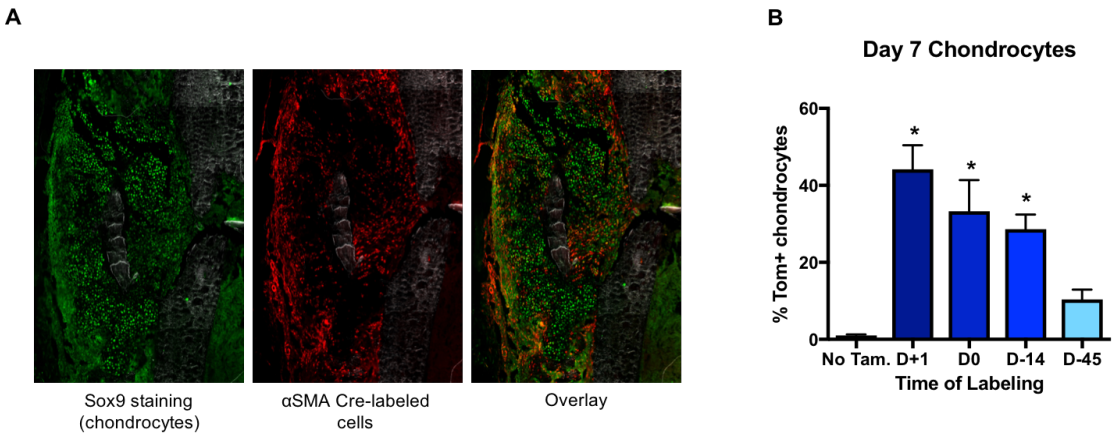

Figure 9. **αSMA-expressing cells contribute to chondrocytes generation after fracture.** Representative images of a tibia fracture callus (**A**). Sox9-positive chondrocytes are detected in the green fluorescence channel, αSMA-labeled cells, in the red one. The overlay between the green and the red channels is represented by the yellow color. Chondrocytes can be distinguished by their morphology, too. Different percentages of dual labeled cells have been found in the fracture callus (**B**) at different time points of tamoxifen injection. The results were obtained from groups of 5-7 female mice of 8 weeks of age at time of tamoxifen injections. Tukey's multiple comparison test (ANOVA) was applied. Each group of mice that got injected with tamoxifen was compared to the negative control group. * $p < 0.05$

We were also interested in investigating if αSMA-expressing cells contributed equally to the types of cells throughout the callus. Based on this question, we compared the different (middle and distal) callus areas in terms of percentage of αSMA-labeled cells, 7 (Figure 10**A**) and 14 (Figure 10**B**) days after fracture. We observed that the proportion of αSMA-labeled cells is related to the timing of tamoxifen administration, and that these cells show similar prevalence in both central and distal areas of the fracture callus (Figure 10).

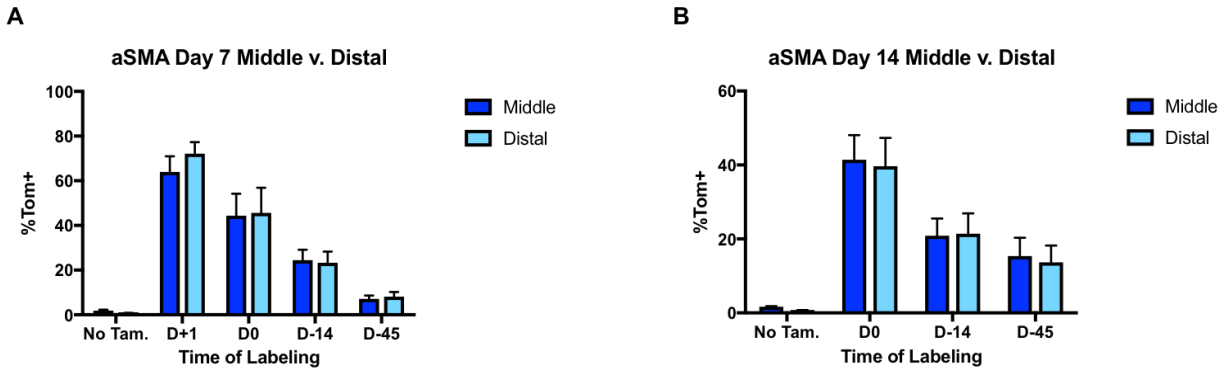

Figure 10. **Similar percentages of αSMA-labeled cells have been found in the different callus area, at all the different time points of tamoxifen injection.** A similar trend is shown 7 (**A**) and 14 (**B**) days after fracture. The results were obtained from groups of 5-7 female mice of 8 weeks of age at time of tamoxifen injection.

In order to confirm that the Col2.3GFP-labeled cells reflected what we already knew about the osteoblasts behavior in the fracture healing process, we compared the percentages of Col2.3GFP-labeled cells (osteoblasts) in the different callus area (Figure 11). We observed, as expected, that 7 days after fracture (Figure 11**A**) osteoblasts are more prevalent in the distal area with respect to the middle one, while 14 days after fracture (Figure 11**B**), the difference of osteoblasts prevalence through the callus is decreased, due to callus progression.

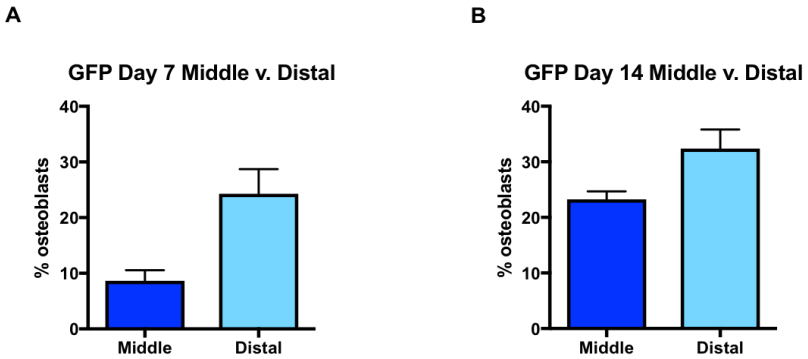

Figure 11. **The difference in the percentage of osteoblasts distribution in the callus decreases with its progression.** A higher prevalence of osteoblasts is observed in the distal callus area 7 days after fracture (**A**). This difference decreases 14 days after fracture due to callus mineralization (**B**). The results were obtained from groups of 5-7 female mice of 8 weeks of age at time of tamoxifen injection.

αSMA-labeled cells are capable of density-independent growth *in vitro*, and expand but do not show evidence of osteogenic differentiation, *in vivo*.

In order to test periosteum αSMA-expressing cells ability to form bone when isolated, we used SMACre/Ai9/Col2.3GFP mice, injected with tamoxifen, with or without bone injury. In this study, we injured the mice with a scratch injury, an alternative to the fracture in which the exclusive contribution of periosteum is more clear (Figure 12). For this procedure, anesthetized mice were scratched on the limbs using a needle through the skin and the muscle.

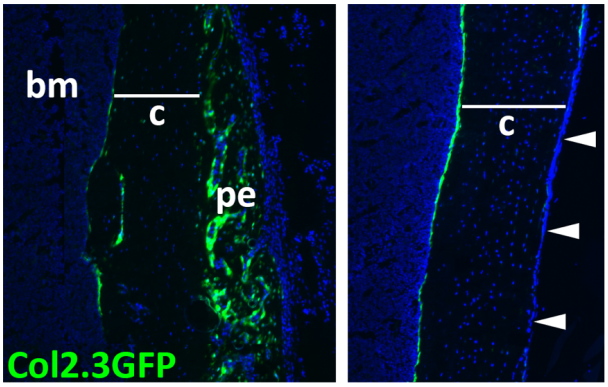

Figure 12. Scratch injury representative image. Periosteal activation after scratch injury is shown on the left image, as highlighted by the Col2.3+ cells (osteoblasts, green). Intact periosteum is shown in the right image, in which it is detectable as a thin layer. bm=bone marrow; c=cortical bone; pe=periosteum.

We sorted by flow cytometry tdTomato-positive cells and tdTomato-negative cells within the CD45$^-$ Ter119$^-$CD31$^-$ population. Part of these cell populations were used to establish low densities cultures, and part to perform *in vivo* transplantation into NSG mice with calvarial defect (Figure 13).

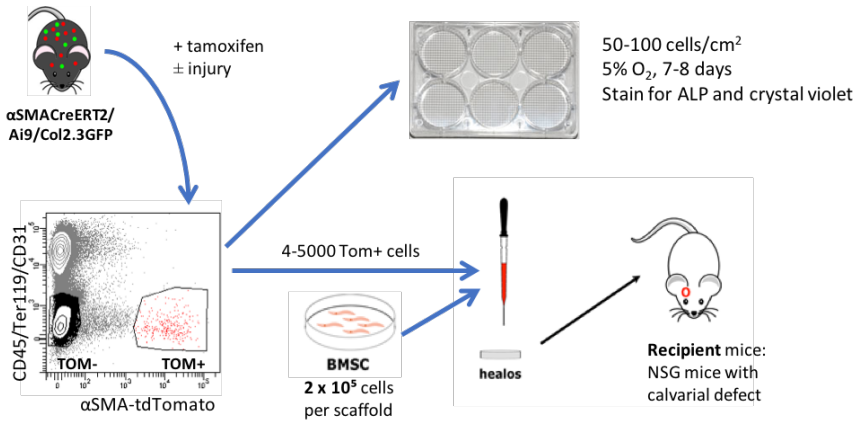

Figure 13. **Schematic representation of experimental procedure.** αSMA-expressing cells were tested for their progenitor properties, by *in vitro* low density culture and *in vivo* transplantation.

Sorted cells were plated at low density to investigate their ability to grow in colonies, in a density-independent way *in vitro*, which is a typical feature of progenitor cells (Figure 14). We visualized the colonies formation by performing Crystal Violet staining (Figure 14**A**). We also tested the Alkaline Phosphatase (ALP) enzyme activity of the cells (Figure 14**B**), as it represents a bone-related feature. tdTomato-positive cells enriched for colonies, especially in injured mice, while tdTomato-negative cells almost didn't enrich for colonies at all. ALP activity was detected only in tdTomato-positive cells isolated from injured mice.

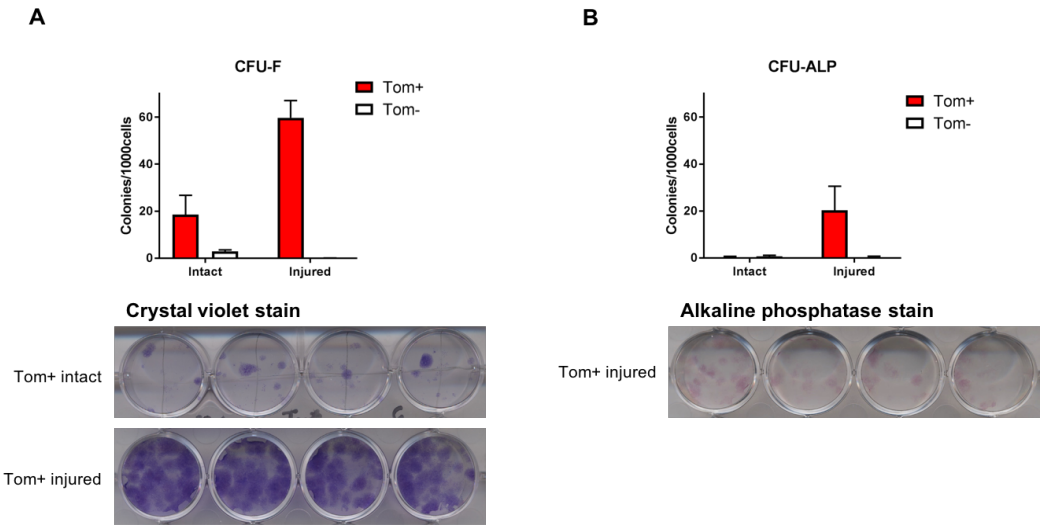

Figure 14. **Quantification of tdTomato positive and negative colonies, in intact *versus* injured mice.** Crystal violet stain (**A**) was detectable almost only in tdTomato-positive cells, with a higher prevalence in injured mice respect to intact mice. ALP activity (**B**) was detectable only in tdTomato-positive cells from injured mice. The number of colonies per 1000 seeded cells is shown in the graphs. The results were obtained from 2-3 groups of 2-4 male mice each, of 3-9 weeks of age, and two technical replicates.

Sorted tdTomato-positive cells from SMACre/Ai9/Col2.3GFP mice were also used to evaluate their ability to form bone *in vivo*. For this purpose, we chose a mouse model with calvarial defect, which reproduces a condition of critical size defect that can take place *in vivo*. Together with bone marrow stromal cells as carrier, 4-5000 tdTomato-positive cells were loaded on healos and placed into immunodepressed NSG mice with calvarial defect. After 5 weeks, mice calvaria were collected and processed for fluorescent image analysis. tdTomato-positive transplanted cells showed a good engraftment into the defect, however there was no bone formation, as highlighted by the absence of Col2.3GFP-positive cells (Figure 15). Interestingly, we observed some Col2.3GFP-positive cells in the transplanted tdTomato-negative

mice (data not shown), most likely due to osteogenic differentiation of other progenitor cells. This experiment was performed one time only, so it needs to be repeated.

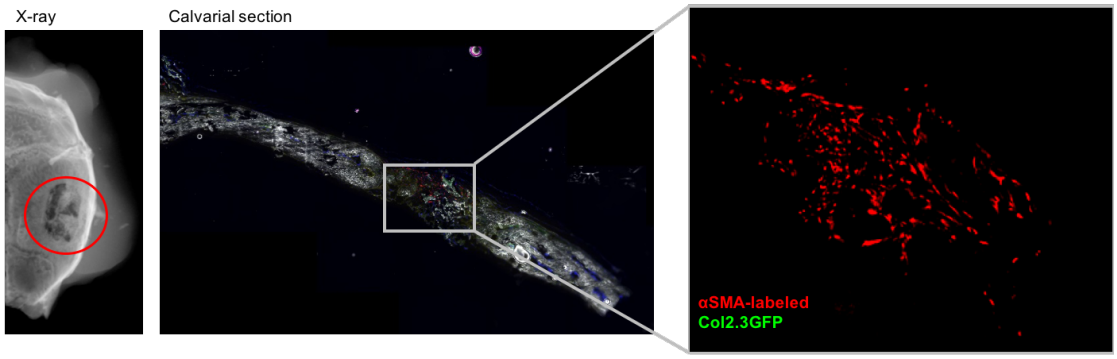

Figure 15. **tdTomato-positive transplanted cells showed a good engraftment into the defect, but no bone formation.** Mouse x-ray shows the calvarial defect of the skull filled with the scaffold as it appears 5 weeks after the surgery. Calvarial section image is here magnified to enable the visualization of the engraftment of αSMA-expressing cells (red color). Osteoblasts Col2.3GFP green signal was not detectable. This is a representative image from one mouse. The experiment has been performed one time.

Periosteum $CD105^+$ and $CD90^+$ cells are capable of density-independent growth *in vitro*

Surface markers such as CD105 and CD90 have been defined as useful indicators for multipotency of cultured murine progenitor cells (I. Kalajzic et al., 2002). We, therefore, tested periosteum cells for the presence of these surface markers. Cells isolated from periosteum of intact mice were sorted by flow cytometry by using antibodies for CD105 and CD90 markers (Figure 16). Cells belonging to the hematopoietic and the endothelial lineages were excluded from the sorted populations, gating for CD45/Ter119/CD31 markers. Sorted $CD105^+$, $CD105^-$, $CD90^+$, and $CD90^-$ cell

populations were plated at low density to investigate their ability to grow in colonies *in vitro* (Figure 17). Within 7-8 days from seeding, PrestoBlue assay was performed (Figure 17**A**), and we observed that both $CD105^+$ and $CD90^+$ cell populations were able to grow, as confirmed by the significant enrichment of colonies respect to the $CD45^-$ control population detected with Crystal violet stain. ALP assay showed a significant enzyme activity in the $CD90^+$ cell population, while it appeared almost absent in the $CD105^+$ cell population (Figure 17**B**). Both Crystal violet stain and ALP stain resulted almost undetectable in the negative populations.

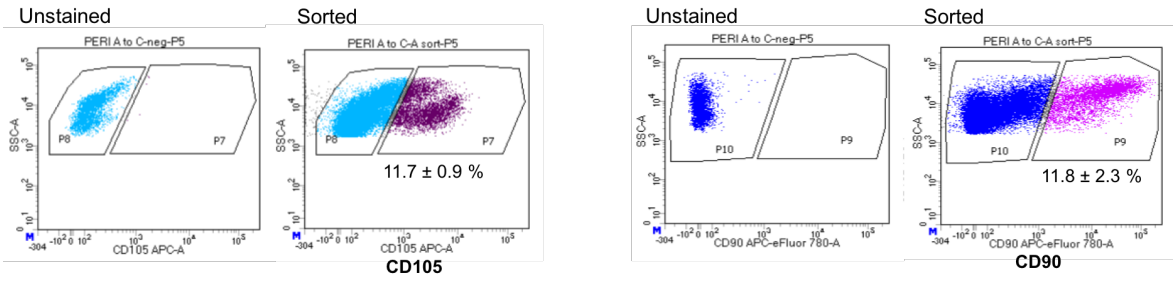

Figure 16. **Flow cytometry sorting for CD105 and CD90 cell surface markers.** Periosteum cells from intact mice were sorted by flow cytometry to select the CD105 and CD90 surface markers expressing populations. The hematopoietic and the endothelial cells were excluded from the sort.

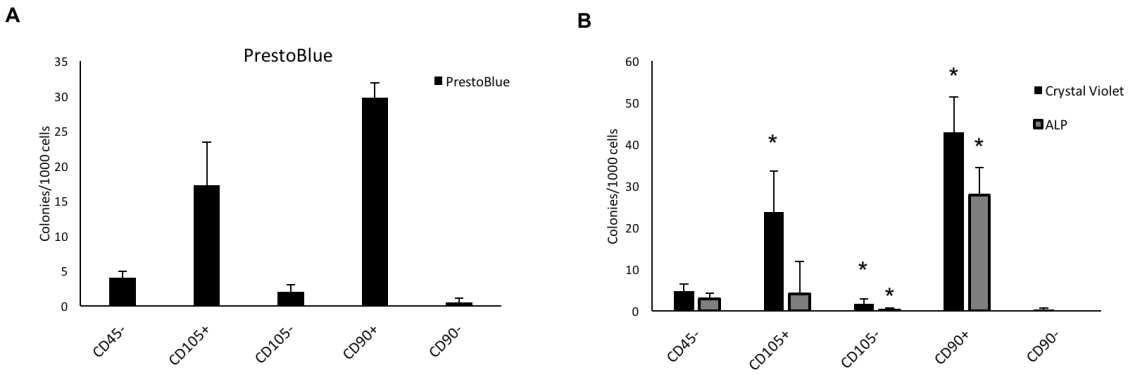

Figure 17. Periosteum CD105$^+$ and CD90$^+$ cells are capable of density-independent growth *in vitro.* PrestoBlue assay on sorted cells (**A**). Quantification of Crystal violet stain and ALP activity on sorted cells (**B**). CD105$^+$ and CD90$^+$ populations significantly enrich for CFU-Fs compared to the CD45$^-$ control population, while CD105$^-$ is significantly lower compared to the CD45$^-$ control. CD90$^+$ cell population significantly enriches for CFU-ALPs compared to the CD45$^-$ control population, while CD105$^-$ ALP activity is significantly lower compared to the CD45$^-$ control. The number of colonies per 1000 seeded cells is shown in the graphs. The results were obtained from 2-3 groups of 2-4 male mice each, of 5-8 weeks of age, and two technical replicates. * $p < 0.05$ (paired t test applied).

Periosteum Sca1$^+$, CD51$^+$, and double-positive Sca1$^+$CD51$^+$ cell populations are capable of density-independent growth *in vitro*, and show promising results of osteogenic differentiation *in vivo*.

Sca1 and CD51, as well as their combination, are potential marker for the identification of murine mesenchymal progenitor cells (Morikawa et al., 2009). On these bases, we tested Sca1$^+$, CD51$^+$, and double-positive Sca1$^+$CD51$^+$ periosteum cell populations for their ability to form colonies in a density-independent way *in vitro*, and to differentiate into osteoblasts *in vivo*. Cells isolated from periosteum of intact and injured SMACre/Ai9/Col2.3GFP mice were sorted by flow cytometry by using antibodies for Sca1 and CD51 markers (Figure 18). Cells belonging to the hematopoietic and the endothelial lineages were excluded from the sorted populations, gating for CD45/Ter119/CD31 markers. Sorted Sca1$^+$, CD51$^+$, double-positive Sca1$^+$CD51$^+$, and double-negative Sca1$^-$CD51$^-$ cell populations were plated at low density. The double negative population was plated at high density, too.

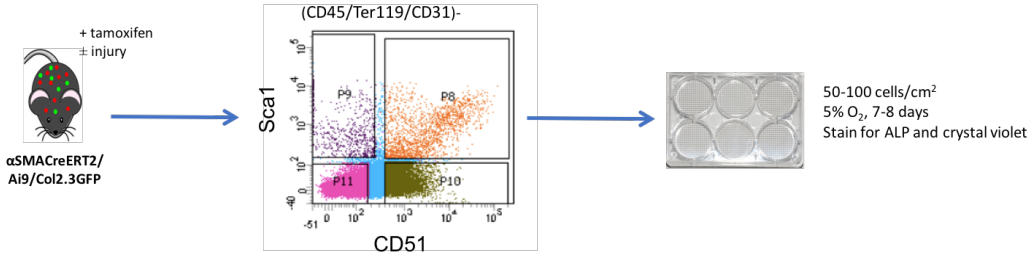

Figure 18. **Schematic representation of experimental procedure.** $Sca1^+$, $CD51^+$, double-positive $Sca1^+CD51^+$, and double-negative $Sca1^-CD51^-$ cell populations were tested for their progenitor properties by *in vitro* low density culture.

Within 7-8 days from seeding, PrestoBlue assay was performed for both intact and injured mice (Figure 19, **A** and **B**). All the positive populations grew, while the double negative one was not able to form colonies. Crystal violet stain showed that $Sca1^+$ and double-positive $Sca1^+CD51^+$ populations grew in a significantly higher number of colonies respect to their $CD45^-$ control in the intact mice group (Figure 20**A**), while only the $Sca1^+$ population significantly enriched for colonies in the injured mice group (Figure 20**B**). ALP assay showed a slight enzyme activity in the $Sca1^+$ and double-positive $Sca1^+CD51^+$ populations, only in the intact mice group (Figure 20**A**). Both Crystal violet stain and ALP stain resulted undetectable in the negative populations.

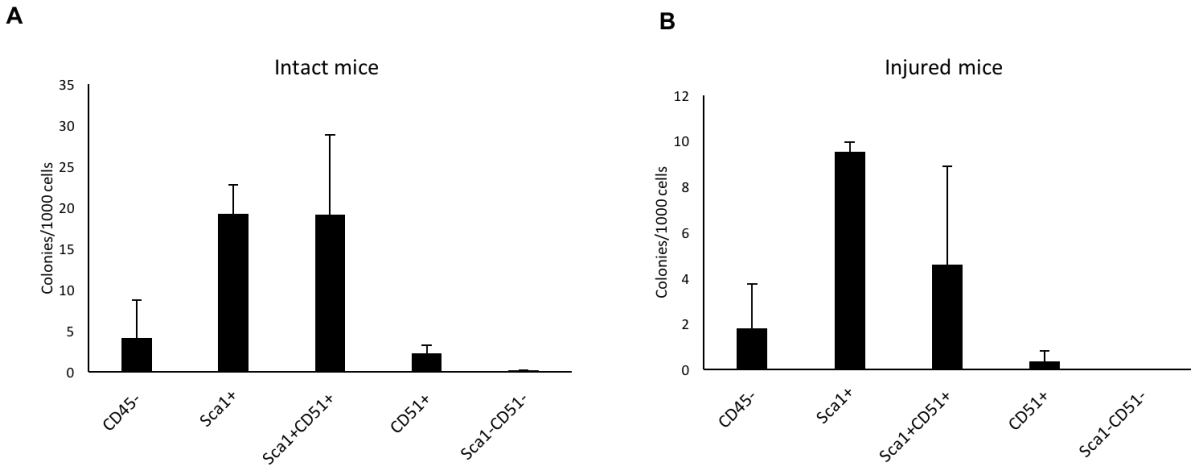

Figure 19. **Periosteum Sca1^{+}, CD51^{+}, and double-positive Sca1^{+}CD51^{+} cell populations are capable of density-independent growth *in vitro*.** PrestoBlue assay on sorted Sca1^{+}, CD51^{+}, double-positive Sca1^{+}CD51^{+}, and double-negative Sca1^{-}CD51^{-} cell populations, in the intact mice group (**A**) and in the injured ones (**B**). The double negative population was not able to grow. The number of colonies per 1000 seeded cells is shown in the graphs. The results were obtained from 1-2 groups of 2-3 male mice each, of 3-8 weeks of age, and two (injured)-three (intact) technical replicates.

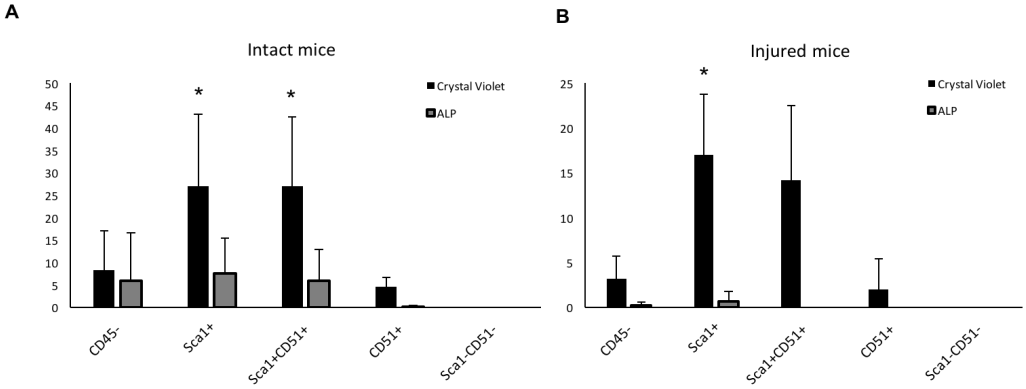

Figure 20. **Periosteum Sca1^{+}, CD51^{+}, and double-positive Sca1^{+}CD51^{+} show stem cell properties *in vitro*.** Quantification of Crystal violet stain and ALP activity on sorted cells from intact mice (**A**), and injured mice (**B**). Sca1^{+} and double-positive Sca1^{+}CD51^{+} populations significantly enrich for CFU-Fs

compared to the $CD45^-$ control population, in the intact mice only; $Sca1^+$ alone enriches for CFU-Fs in the injured mice only. The number of colonies per 1000 seeded cells is shown in the graphs. The results were obtained from 1-2 groups of 2-3 male mice each, of 3-8 weeks of age, and two (injured)-three (intact) technical replicates. * $p < 0.05$ (paired t test applied).

Interestingly, we also sorted endosteum-derived cells from some of groups of animals used in the experiments above by flow cytometry using antibodies for the above-mentioned progenitor cell markers. We observed that sorted cells were able to grow. Specifically, the $CD45^-$ cell population showed a mean CFU-F value of 0.75 colonies per 1000 seeded cells for the intact mice group versus a mean of 5.77 observed in periosteum, and 0.54 colonies per 1000 seeded cells for the injured mice group versus a mean of 2.48 observed in periosteum. These results confirmed that periosteum is the most progenitor-enriched bone compartment.

In order to test $Sca1^+$, $CD51^+$, and double-positive $Sca1^+CD51^+$ cells ability to differentiate into osteoblasts *in vivo,* sorted cells from the periosteum of R26-M2rtTA/TetOP-H2B-GFP mice were transplanted into immunodepressed NSG mice with calvarial defect (Figure 21). In order to activate the tetO system, thus enhancing nuclear GFP expression in cells undergoing mitosis, we fed donor and recipient mice with doxycycline containing food starting a few days prior to surgery. The recipient mice kept receiving the doxycycline diet after the transplantation, switching from food to water the last week before the sacrifice. $Sca1^+$, $CD51^+$, and double-positive $Sca1^+CD51^+$ loaded cells, which were respectively 2-4000 and 13-20000, were loaded on scaffolds together with BMMSC as carriers.

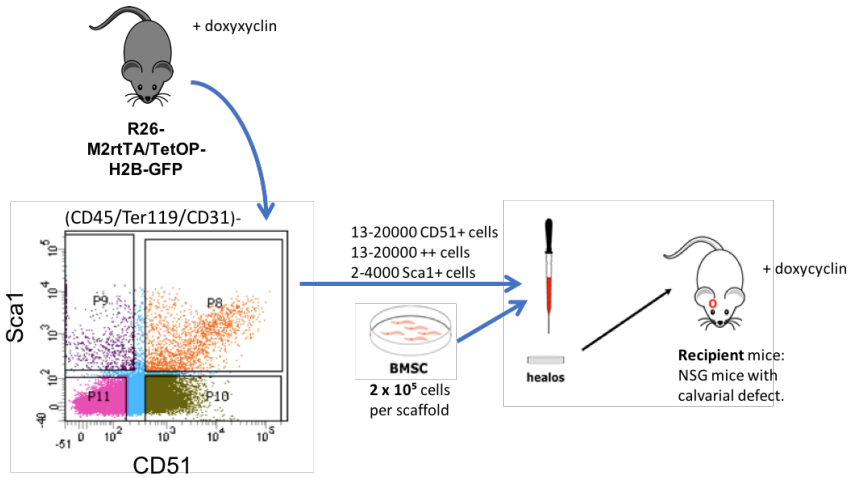

Figure 21. **Schematic representation of experimental procedure.** $Sca1^+$, $CD51^+$, and double-positive $Sca1^+CD51^+$ periosteum cell populations were tested for their progenitor properties by *in vivo* transplantation.

Five weeks after the transplant, calvaria were collected and processed for fluorescent image analysis. $CD51^+$ transplanted cells didn't engraft in the defect, while interesting results were shown by $Sca1^+$ (Figure 22**A**) and double-positive $Sca1^+CD51^+$ (Figure 22**B**) transplanted cells, which engrafted well in the defect, and seemed to give rise to osteoblasts, as shown by the colocalization of some of the green donor cells with the ALP stain (red color). Cell morphology and ALP stain can help in identifying potential osteoblasts, however we are looking for a better way to detect osteoblasts. Furthermore, this experiment was performed one time (n=2 sorts), so it would be interesting to repeat it.

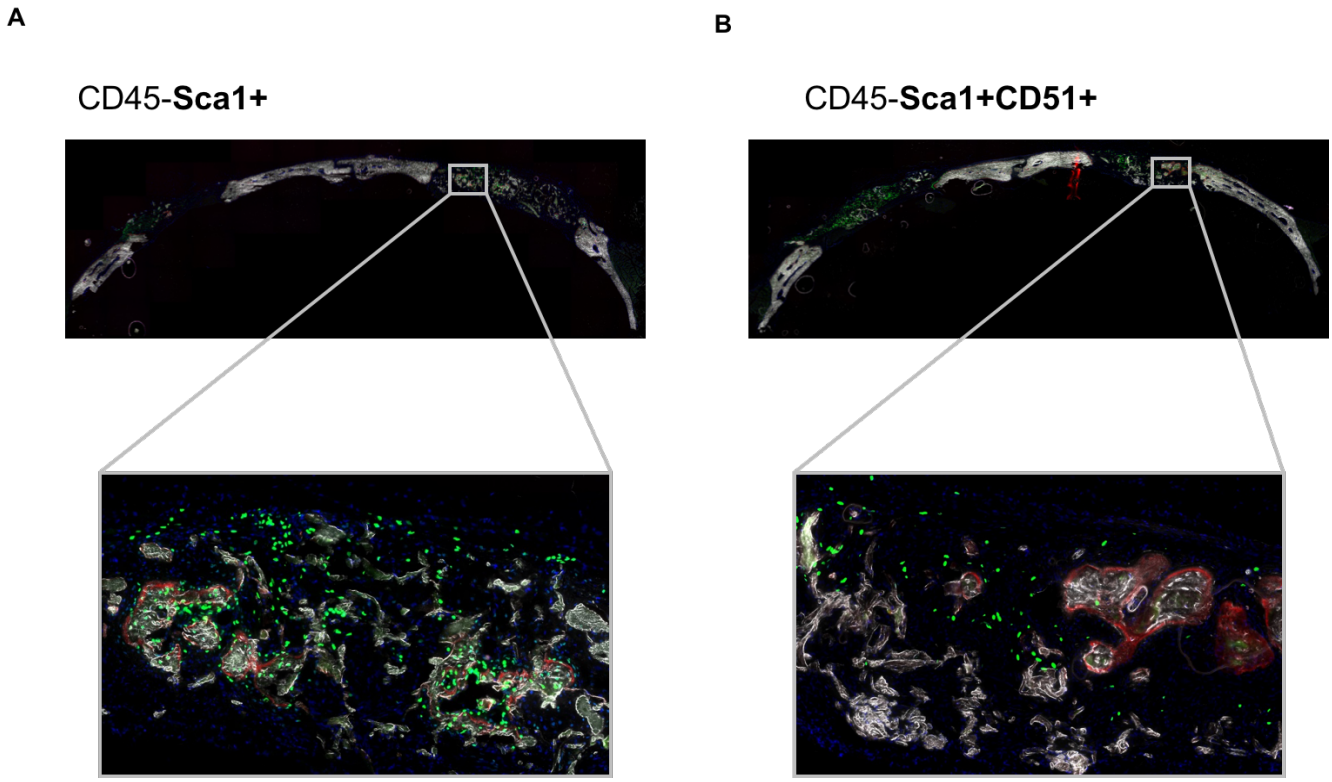

Figure 22. Periosteum Sca1^{+}, CD51^{+}, and double-positive Sca1^{+}CD51^{+} cell populations show promising results of osteogenic differentiation *in vivo*. Calvarial section images of transplanted Sca1^{+} (**A**) and double-positive Sca1^{+}CD51^{+} (**B**) cells in NSG mice. The images are here magnified to appreciate the engraftment of the donor cells (green color) and the colocalization of some of them (possibly osteoblasts) with the ALP stain (red color). This is a representative image from one mouse for each transplanted marker. The experiment has been performed one time.

Discussion

Interstitial acidic pH characterizes various pathological conditions (L. Dong et al., 2013; Hardy & Cooper, 2009; Kraut & Madias, 2010; Martin & Jain, 1994) and tissues with impaired vascularization, as well as fractures (T. R. Arnett, 2010; Newman, Stone, & Mukherjee, 1987).

These conditions are characterized by pH values of interstitial fluids ranging from pH 6.0 to pH 7.0 (L. Dong et al., 2013). It has been shown that extracellular acidosis is able to modulate MSCs proliferation, differentiation, paracrine activity (Disthabanchong et al., 2007), stemness and regenerative potential (Mohyeldin et al., 2010). Similarly, the cell process autophagy has been associated with the regulation of MSC stem-like features, as well as with senescence and cell death/survival (Eom et al., 2014), and can be induced by acidic conditions.

The activation of inflammatory pathways by local acidosis (Lardner, 2001) is a condition typically found in several chronic inflammatory diseases, associated with bone loss, secondary osteoporosis, thus increased fracture risk with impaired bone healing (Claes et al., 2012; Hardy & Cooper, 2009). Fracture healing is a regenerative process that involves coordinated responses of many cell types, but the characterization of these specific cell populations has been limited. In the bone repair process, MSCs are essential elements that can contribute to bone healing directly, since they can differentiate into osteoblasts, and indirectly, with the secretion of cytokines and growth factors involved in the modulation of vascularization and inflammatory response (Granero-Moltó et al., 2009). Therefore, the infusion of MSCs represents a therapeutic approach to fracture healing (Rosset, Deschaseaux, & Layrolle, 2014). However, the success of regenerative strategies may be impaired by a hostile extracellular microenvironment (Dimmeler, Ding, Rando, & Trounson, 2014; Sui et al., 2017).

In this study, we focused our attention on how acidic extracellular pH can affect MSCs behavior in terms of proliferation, osteogenic potential, activation of autophagy, and release of inflammatory molecules,

and looked for potential osteo- and chondro-progenitor cell populations activated during the fracture healing process in the periosteum of a mouse model *in vivo.*

We cultured human BMMSCs, representing the most commonly used source of MSCs in clinical applications, in acidic medium of pH 6.5 or 6.8, to reproduce the typical microenvironment of the above-mentioned pathological conditions, and in physiological medium of pH 7.4, as a standard-culture control. We observed a significant decrease in BMMSCs proliferation at low pH conditions, as revealed by the index quantification of the nuclear protein associated to cell proliferation Ki67 (Scholzen & Gerdes, 2000). Not only BMMSCs proliferation, but also their mineralization was found to be impaired by extracellular acidosis, as shown by the inhibition of mineral nodules formation observed at pHe 6.5, compared to pHe 7.4. The significantly reduced ability of BMMSCs to deposit mineralized matrix in an acidic microenvironment had been previously described (T. R. Arnett, 2008; Frick & Bushinsky, 1998; Kohn, Sarmadi, Helman, & Krebsbach, 2002). Arnett (2008) has connected it to a decreased alkaline phosphatase activity and an increased solubility of hydroxyapatite at low pH, which is in line with our observations. Interestingly, recent studies have demonstrated that a short-term exposure to pH 6.5 inhibits glycolysis in MSCs (Chano et al., 2016), and cell metabolism variations have been shown to exert an indirect control of cell differentiation (Agathocleous & Harris, 2013), leading us to speculate that MSCs metabolism is modulated by extracellular acidosis. Overall, our observations and considerations show a close correlation between inhibition of MSCs osteogenic mineralization and acidic pH of the extracellular microenvironment.

The survival mechanism of autophagy is known to be activated by cells under acidic conditions, and previous literature associated it with the regulation of MSCs stem-like features (Kroemer et al., 2010; Sordella et al., 2003). Therefore, we evaluated BMMSCs autophagic flux by protein expression of typical autophagic markers (LC3-II, p62 and Beclin1) at low and physiological pHs. We confirmed previous studies showing that the autophagic flux is constitutively activated in human MSCs (Oliver, Hue, Priault,

& Vallette, 2012; Salemi, Yousefi, Constantinescu, Fey, & Simon, 2012), as evidenced by LC3-I to LC3- II conversion detected in BMMSCs cultured at physiological pH. However, we did not observe significant differences between the two culture conditions, leading us to conclude that autophagy is not the main mechanism by which BMMSCs adapt to an acidic microenvironment.

Tissue acidification is commonly associated with inflammation (Punnia-Moorthy, 1987), and consequent release of cytokines (Lardner, 2001). Indeed, we detected, at the protein level, a significant increase in IL-6 and IL-8 secretion at the acidic pH, compared to the physiological one. This effect caused by extracellular pH was observed 24 hs after induction, by analyzing the cultures supernatant with an ELISA assay. At the mRNA level, we observed an increased release of IL-6 and IL-8 in acidic conditions, as well. The high variability detected in this analysis may be due to the use of both bone marrow-derived and adipose tissue-derived MSCs. It is well-known that MSCs isolated from different sources share similar biological features but display differences in immunophenotype, transcriptome, proteome, differentiation potential, and immunomodulatory activity (Jin et al., 2013; Strioga, Viswanathan, Darinskas, Slaby, & Michalek, 2012). According to these considerations and our results, it would be interesting to further characterize MSCs secretion of inflammatory molecules, specifically investigating the differences between different sources. These observations have allowed to deepen the knowledge of MSCs biology in a non-physiological context, which can be observed in patients with an altered acid-base balance. Restoring physiological pH is necessary to obtain new bone deposition, indeed variations in extracellular pH have been taken into consideration for the development of novel treatments of bone diseases and tissue engineering strategies.

Inflammation is an important factor during bone healing, with molecular factors and immune cells acting locally at the fracture site in a finely tuned sequence of events. Since the periosteum is an important source of cells during fracture healing, we focused on cells belonging to this bone compartment. The periosteum is a tissue that responds rapidly to injury, makes a major cellular contribution to both

cartilage and bone, and promotes angiogenesis during the healing process, such that healing is impaired when the periosteum is absent (Colnot, 2009; Ozaki et al., 2000; X. Zhang et al., 2005). Periosteal replacement can heal critical-sized bone defects in animal models (Chang & Knothe Tate, 2012; Knothe Tate et al., 2007). Cells isolated from the periosteum may also be suitable for tissue engineering applications (Agata et al., 2007; Ribeiro et al., 2010). However, the identity of mesenchymal progenitor cells within the periosteum is not well defined. Previous studies in Kalajzic's lab have identified αSMA as a marker of a population of mesenchymal progenitor cells in the periosteum that contributes to osteochondral elements during fracture healing (Grcevic et al., 2012; Matthews et al., 2014). Furthermore, a number of studies have shown that periosteal cells express markers such as CD105 and CD90 in culture, but there have been very few studies that have characterized freshly isolated cells (Sakaguchi, Sekiya, Yagishita, & Muneta, 2005; van Gastel et al., 2012).

We analyzed cell surface markers frequency in freshly isolated cells of the periosteum of a mouse model *in vivo*, and observed that periosteum is highly enriched for progenitor cell markers compared to the other bone compartments. This is a notable result, since it represents the evidence that periosteum is of great interest in the identification of progenitor cell populations. The current literature investigating the presence of cells expressing stem cell markers focuses mostly on cells isolated from bone marrow or total bone (Chan et al., 2013; Chan et al., 2015; Morikawa et al., 2009), while our result pave the way to further studies towards the analysis of the periosteum bone compartment as the main source of progenitors.

Periosteum has also been found to be enriched in αSMA^+ cells compared to the other bone compartments. There are some studies in literature showing that αSMA-expressing cells give a substantial contribution to osteoblasts and chondrocytes generation after bone injury. Kinner *et al.* (2002) detected αSMA^+ cells in a mouse tibia fracture model 3-7-14-21-28 days after fracture; this group observed the presence of αSMA^+ cells throughout all the phases of the heling process, specifically in the

periosteum prominent to the fracture site, and their co-expression with newly formed osteoblasts and chondrocytes (Kinner et al., 2002). Mori *et al.* (2016) has recently demonstrated the co-expression of αSMA^+ cells with $Dkk3^+$ cells after fracture; Dkk3 is a secreted protein of the Dickkopf (Dkk) family (Krupnik et al., 1999), whose physiological role and biological function of Dkk3 remain largely elusive; in the mentioned study, $Dkk3^+$ cells have been found to co-localize with αSMA^+ cells, and increase a few days after fracture, then originating fibrocartilage elements (Mori et al., 2016). Previous studies in Kalajzic's lab used a SMACre mouse tibia fracture model to analyze αSMA-expressing cells 2 and 17 days after fracture; the presence of αSMA^+ cells was detected in newly formed chondrogenic areas of the fracture callus and areas of the new bone formation (Grcevic et al., 2012). Matthews *et al.* (2014) confirmed αSMA^+ cells osteochondral contribution specifically to the earliest stages of fracture healing (2 and 6 days post fracture), and demonstrated αSMA^+ cells expression of progenitor cell markers after fracture (Matthews et al., 2014).

Our study aimed at investigating if this αSMA-expressing population involved in fracture healing identifies a long-term resident progenitor cell population. Although we confirmed the previously shown contribution of αSMA^+ periosteal cells in osteoblasts and chondrocytes generation in the fracture callus (Grcevic et al., 2012; Kinner et al., 2002; Matthews et al., 2014; Mori et al., 2016), we observed that some of these cells may not have significant self-renewing capabilities. Three months or six/two weeks before the injury event, some of the αSMA^+ periosteal cells that we labeled had either died or differentiated into types of cell that do not participate in the fracture healing, while perhaps other αSMA-negative populations in the periosteum contribute to osteoblasts and chondrocytes generation in the callus. However, a subset of αSMA-expressing cells has been still found active as a long term osteoprogenitor population in the fracture callus. Furthermore, we showed that αSMA^+ periosteal cells labeled the day before and the same day of the injury have the ability to grow *in vitro* in a density-

independent way, thus confirming their feature of progenitor cell population. Specifically, αSMA$^+$ enriched for about 20 colonies per 1000 seeded cells in the intact mice group, and about 60 colonies in the injured one. CFU-ALPs were observed only in the αSMA$^+$ cells from the injured mice group, and the transplantation of αSMA$^+$ cells in a mouse model with calvarial defect showed good engraftment without bone formation. According to our observations showing that injury stimulates αSMA$^+$ periosteal cells participation in osteoblasts generation during the bone healing process, it would be interesting to transplant periosteal αSMA$^+$ cells freshly isolated from injured mice, and investigate if they are able to form bone.

Several studies mainly conducted on bone marrow or total bone (Chan et al., 2013; Chan et al., 2015; Morikawa et al., 2009) identify markers such as Sca1, CD51/Alpha V, PDGFRα, CD105 and CD90 as potential positive markers for the identification of murine mesenchymal progenitor cells (Arai et al., 2002; Kitaori et al., 2009; Lundberg et al., 2007; Morikawa et al., 2009; Nakamura et al., 2010; Steenhuis et al., 2008). In the present study, we observed that all the periosteal cells expressing the above-mentioned surface markers display the typical stem cells ability to grow in a density-independent way *in vitro*. Specifically, CD90$^+$ sorted cells enriched the most for both CFU-Fs, by forming more than 40 colonies per 1000 seeded cells, and CFU-ALPs, with almost 30 colonies grew per 1000 seeded cells. Unexpectedly, injury did not seem to stimulate the progenitor phenotype of Sca1$^+$, CD51$^+$, and double-positive Sca1$^+$CD51$^+$ cell populations *in vitro*, as shown by the reduced ability to grow as colonies in a density-independent way observed in the injured mice group, compared to the intact one.

Interestingly, promising results are shown by Sca1$^+$, and double-positive Sca1$^+$CD51$^+$ cells after *in vivo* transplantation in mice with calvarial defect. While CD51$^+$ transplanted cells alone did not engraft in the defect, Sca1$^+$ and double-positive Sca1$^+$CD51$^+$ transplanted cells appeared to generate osteoblasts in

this model. However, the use of transplantation in mouse with calvarial defect as a system to analyze progenitor cells contribution to bone formation *in vivo* might not be the most appropriate one. The mouse calvarial defect model has been shown to permit evaluation of drugs, growth factor, or cell transplantation efficacy, together with offering the benefit of utilizing genetic models to study intramembranous bone formation within defect sites (Samsonraj et al., 2017; Wu, Wang, Deng, & Watts, 2014). However, recent studies underline the importance of ectopic bone formation models *in vivo*, such as the kidney capsule transplantation. The kidney capsule model is a method of ectopic bone formation, in which material is placed between the thin, fibrous capsule of the kidney and the underlying renal parenchyma. Significant features of the renal capsule model include increased blood flow to the implant, which helps engrafted cells to survive and proliferate once in place, theoretical lack of endogenous bone-forming stem cells, thus it is almost certain that the engrafted cells are responsible for any observed bone formation, and size limitations of the implant, a very determinant feature when the model used is a mouse (Chun et al., 2011; S. H. Park et al., 2006; Slater, Lenton, James, & Longaker, 2009; Yu et al., 2007; W. Zhang, Liu, Wang, & Li, 2011). In general, ectopic bone formation models reduce the number of variables involved in bone formation, thus it would be interesting to further investigate this kind of model for our progenitor cells studies *in vivo*.

Summarizing, in this study, we showed the close correlation between MSCs behavior and the surrounding extracellular microenvironment.

Specifically, MSCs proliferation and mineralization potential decrease at low pH conditions, while the release of cytokines such as IL-6 and IL-8 increases. Notably, we observed a high variability within cytokines mRNA analysis, which we attributed to the use of MSCs derived from different sources, BMMSCs and ADMSCs. This result led us to consider further studies aimed to clarify the discrepancies existing among the different MSCs sources of isolation, in the light of their importance in clinical

applications of regenerative medicine. Recent studies show that extracellular acidosis can be overcome by using specific drugs, and this may improve the process of bone regeneration, usually impaired in patients with chronic sub-acidosis (Amer et al., 2011; W. Chen & Abramowitz, 2014).

Autophagy was found to be activated by MSCs at the same level in both acidic and physiological conditions. We, thus, concluded that autophagy is not the main mechanism that BMMSCs activate in an acidic microenvironment, most likely there are other processes involved, so further studies are needed.

An altered extracellular microenvironment characterizes the early stages of the fracture healing process, and the identification of the cells participating in bone repair is necessary to eventually improve therapeutic approaches of regenerative medicine. The periosteum is the major contributor of progenitor cells during bone healing. Although we observed that αSMA^{+} periosteal cells may not have significant self-renewing capabilities, we demonstrated that markers previously characterized in bone marrow are suitable for identification of stem/progenitor cell populations in periosteum. Further studies are needed, especially aimed to better investigate different surface markers combination, and the ability of these progenitor cells to form bone *in vivo*, by increasing the number of replicates, and overcoming the technical issues we experienced.

In conclusion, we highlighted the importance of the extracellular microenvironment in the modulation of MSCs behavior, and contributed to the identification of osteo- and chondro-progenitors involved in bone repair, aiming to improve approaches of regenerative medicine, especially in patients in which the healing process is impaired by pathological conditions.

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