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***IN VITRO* STUDY OF THE OSTEOCYTES RESPONSE TO HYPOXIA AND THEIR
REGULATION OF BONE HOMEOSTASIS**

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Chapter 1. ABSTRACT

Bone remodelling is a fundamental mechanism for removing and replacing bone during adaptation of the skeleton to mechanical loads. During periods of skeletal unloading, as seen with bed rest condition, the mechanism of bone remodelling is not in equilibrium and a loss in bone mass is observed. It was suggested that the loss of bone observed in disuse models was the result of osteocyte hypoxia, caused by deprivation of loading induced oxygen transport. Hypoxia, *in vivo*, is a physiological condition for osteocytes, in fact a concentration of approximately 5% O₂ is more likely physiological for osteocytes than a concentration of 20% O₂, as osteocytes are embedded deep inside the mineralized bone matrix and their nutrient availability is greatly dependent upon diffusion.

It has been hypothesized that severe oxygen deprivation, that hypothetically could be 1% of oxygen as in other pathological conditions, due to decreased mechanical loading could have an effect on osteocyte apoptosis and that this leads to osteoclast recruitment and bone resorption.

Since is well known that osteocytes orchestrate bone homeostasis by regulating both bone-forming osteoblasts and bone-resorbing osteoclasts, this study proposes to understand the molecular and cellular mechanism of hypoxia in osteocytes, using the MOL-Y4 osteocyte-like cell line.

Hypoxia and oxidative stress increase 150-kDa oxygen-regulated protein (ORP 150) expression in different cell types. ORP 150 is a novel endoplasmic-reticulum-associated chaperone induced by hypoxia/ischemia. It well known that ORP 150 plays an important role in the cellular adaptation to hypoxia, in fact it has been proposed to be involved in the prevention of apoptosis. It has been reported that the expression of ORP 150 is higher in osteocyte-like MLO-Y4 cells than in osteoblast-like MC3T3-E1 cells and is possible that

ORP 150 is required for osteocytes to survive in their physiological and not-physiological condition of oxygen deficiency.

The aims of the present study are 1) to determine the cellular and molecular response of the osteocytes at two different conditions of oxygen deprivation, 1% and 5% of O₂ compared to the atmospheric oxygen concentration at several time setting points, 8, 16, 24, 48 and 72 hours. 2) To clarify the role of hypoxic osteocytes in bone homeostasis, through the detection of releasing of soluble factors principally involved in a crosstalk with osteoclast and osteoblast (RANKL, OPG, PGE₂ and Sclerostin). 3) To detect the activation of osteoclast and osteoblast induced by condition media collected from hypoxic and normoxic osteocytes.

The data obtained in this study shows that hypoxia compromises the viability of osteocytes and induces apoptosis. Unlike in other cells types, ORP 150 in MLO-Y4 does not seem to be regulated early during hypoxia. The release of soluble factors and the evaluation of osteoclast and osteoblast activation shows that osteocytes, grown under severe oxygen deprivation, play a role in the regulation of both bone resorption and bone formation.

Chapter 2. *INTRODUCTION*

2.1 Bone tissue

The bone is a specialized connective tissue derived by mesenchyme with principal functions of locomotion, support for muscles and protection of vital organs and soft tissues; it is also involved in the maintaining of electrolyte balance, especially maintaining the calcium and phosphate ions homeostasis, and in the haematopoiesis that occurs in the marrow or medullary cavities of bone.

The bone tissue consists of organic and an inorganic matrices; the organic matrix, called **osteoid**, represents about 30% of the weight of the tissue and it is composed primarily of collagen fibres; type. Type I collagen fibres represent approximately 90% of the whole organic materials, but also type III and V collagen are present in bone. Due to the organisation in fibres, the hydroxylation and mature crosslinks found with type I collagen, it is essential for the bone strength and mechanical properties (Beraudi et al., 2010; George and De Crombrughe, 2008; Nair et al., 2013). Although collagen is the most abundant protein of the bone, a huge group of proteins, called non-collagenous proteins, also plays an important role in the bone structure and biology. Proteoglycans, glycosaminoglycans, glycoproteins, leucine-rech repeat proteins as well as fibronectin, osteopontin, osteocalcin, bone sialoprotein, dentin matrix protein 1 are involved in a broad kind of activity, regulating bone matrix organization, growth factor activity, cell attachment and proliferation, other molecular pathways, cellular signalling and mediation of hydroxyapatite deposition (Ritter et al., 1992; Robey, 2008). The inorganic matrix of the bone consists of 70% of the total weight. It is rich in calcium and phosphorus that form a complex structure with formula $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$, called hydroxyapatite. The bone structure is formed by different levels of hierarchy and reflects the material and mechanical

properties of each component. Mechanical and biological properties of bone depend on the interaction taking place across all levels of organization (Noor, 2013).

On the basis of the hierarchical organization of the bone tissue components, it is possible to distinct two kinds of tissue: cortical and trabecular bone. **Cortical bone**, also known as compact bone, constitutes the diaphysis of long bones and the thin shell that surrounds the metaphysis. It is extremely dense, hard and contributing 80% of the weight of a human skeleton.

Cortical bone consists of a functional structure, called **osteons** or haversian system, a quasi-cylindrically shaped element. The individual osteon is composed of concentric lamellae, about 3 to 7 μm thick, constructed from wrapped collagen fibres impregnated at regularly spaced sites with hydroxyapatite and other mineral crystals. The lamellae are arranged in a concentric way around a central channel called Haversian; a smaller size channel, Volkmann, runs transversely to the axis of the bone channels; the interconnection of this channel structure accommodate small arteries, arterioles, capillaries, and venues of the microcirculation system (Buckwalter and Cooper, 1987). The relationship between mechanical stress and the orientation of osteonal collagen in the various quadrants of each bone has been shown by Beraudi and collaborators that classified the different types of osteons in the cortical bone of a human fibula and identified a correlation between the orientation of the collagen within the osteons and the anatomical location (figure 1) (Beraudi et al., 2010).

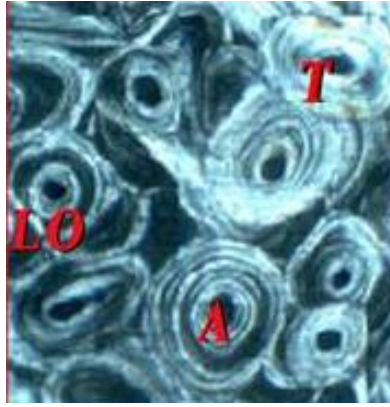


Figure 1. Cortical bone analysed by Circular Polarized Light Microscopy (CPL).

Beraudi et.al., 2010 classified three different osteons on the basis of collagen fibres orientation.

LO: LONGITUDINAL-HOOPED; T: TRANSVERSAL; A: ALTERNATE

Trabecular bone (also known as cancellous, or spongy) is a type of osseous tissue with a low density and strength but very high surface area, that fills the inner cavity of long bones, as a three-dimensional, interconnected network of trabecular rods and plates.

The cancellous bone is the typical structure of the bones short, typically of the vertebral bodies but also of the epiphysis and metaphysis of long bones. Here the lamellae are organized in structures flattened and irregularly shaped trabeculae, osteons are often incomplete and lacking of Volkman channels (figure 2) (Kragstrup, 1985).

The skeleton is a dynamic tissue that is constantly being remodelled in response to alterations in physical activity, dietary calcium levels, hormonal changes, and local paracrine signals within the bone microenvironment. Bone remodelling, in fact, is a fundamental mechanism of removing and replacing bone during adaptation of the skeleton that occurs through all the time life. Bone homeostasis is maintained via an equilibrium of bone resorption and bone formation (Sims and Walsh, 2012)(Sims and Walsh, 2012).

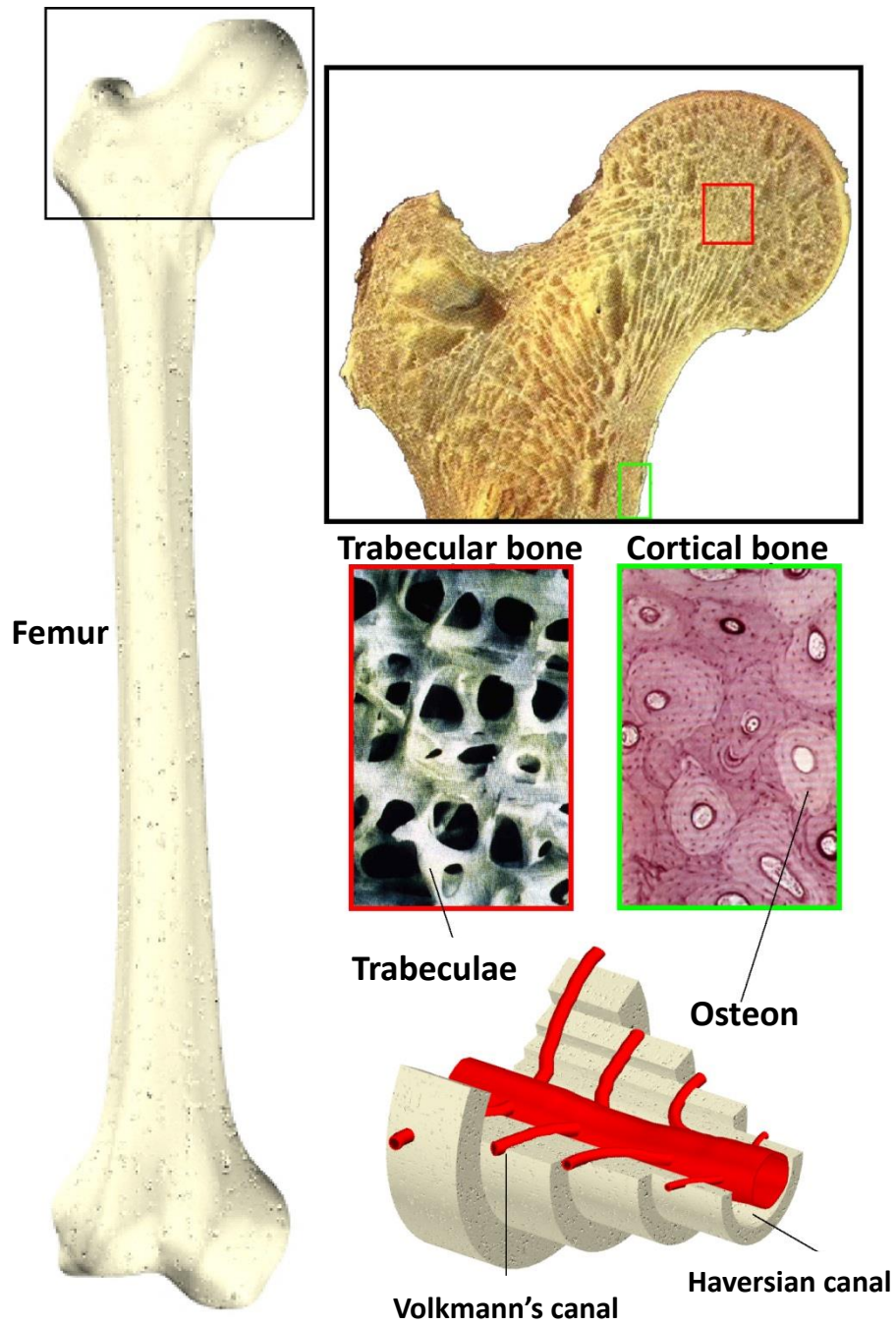


Figure 2. Representation of bone tissue structure.

2.2 Bone cells

The bone cell population consists of osteocytes, osteoclasts, osteoblasts and lining cells. The intercellular cross-talk that occurs among bone cells is a critical process for the maintenance of normal bone structure. **Osteocytes** are considered the mechanosensors of bone, and they are actively involved in the orchestration of both bone-forming **osteoblasts** and bone-resorbing **osteoclasts** (Bonewald, 2011; Nakashima et al., 2011). Osteoblasts derive from mesenchymal stem cells and probably based on a predetermined fate, can become osteocytes or lining cells. The transcription factors that control osteogenesis require the activation of runt-related transcription factor 2 (Runx2), essential for the cascade of transcriptional factors/cofactors involved in the osteoblast differentiation. Osterix (Osx), a zinc finger-containing protein, acts downstream of Runx2 to induce mature osteoblasts that express osteoblast markers, including osteocalcin, collagen type I, alkaline phosphatase (Harada and Rodan, 2003). Due to their effect on Runx2 and Osx expression, the bone morphogenetic proteins (BMPs) are considered very potent inducers of mesenchymal progenitor cell differentiation into osteoblasts; for these reasons, recombinant human BMP-2 and BMP-7 have been approved for clinical use in orthopaedic surgery (Fakhry et al., 2013).

Osteoclasts are multinucleated bone resorbing cells formed by cytoplasmic fusion of their mononuclear precursors derived from hematopoietic stem cells, through monocyte lineage progenitor cells (Miyamoto and Suda, 2003). The transformation to osteoclast requires expression in osteoclast precursors of different molecules, as c-Fos, receptor activator of nuclear factor- κ B ligand (RANKL) and many others; it has been also showed that the RANKL/RANK/osteoprotegerin (OPG) signalling system is the fundamental molecular pathway involved in proliferation and activation of osteoclasts (Boyce and Xing, 2007).

2.2.1 Osteocytes

Osteocytes are the most abundant cells in bone and they are actively involved in the maintenance of bone homeostasis (Bianconi et al., 2013; Bonewald, 2007; Busse et al., 2010). Osteocytes lie embedded deep inside the mineralized bone matrix, in holes called osteocyte lacunae, and their dendritic processes occupy tiny canals called canaliculi (figure 3). The so formed cellular network allows osteocytes to communicate with each other and with other cells on the bone surface, moreover their dendritic processes are in contact with the bone marrow giving them the potential to recruit osteoclast precursors to stimulate bone resorption and to regulate mesenchymal stem cell differentiation (Fakhry et al., 2013). Osteocytes are considered the principal cell type responsible for integrating the mechanical and chemical signals that govern bone modelling and remodelling. Recently, it has been demonstrated that osteocytes exert their regulatory role by participating in endocrine pathways that regulate phosphate metabolism (Harada and Rodan, 2003).



Figure 3. *Transversal section of cortical bone; the image shows the osteocytes inside the lacunae and the interconnected network of osteocytes dendritic process.*

Osteocytes originate from mesenchymal stem cells, as they are derived from a subpopulation of osteoblasts that undergo terminal differentiation (Miyamoto and Suda, 2003). However, so far, the osteocytogenesis is a process that is not well understood and several different theories exist about this process. On one side, osteocytogenesis has been proposed to be a passive process whereby osteoblasts become passively encased in a mineralized matrix (Boyce and Xing, 2007). With the increasing of knowledge about the osteocyte, it is becoming clear that osteocytogenesis is an active and controlled invasive process requiring cleavage of collagen and other matrix proteins, a strong change in morphology from a polygonal to a dendritic cells, and a fine regulation of molecular pathway (Gross et al., 2005; Rubin and Lanyon, 1984).

Bonewald and collaborators (Bonewald, 2011) defined the transitional stages of the osteocytogenesis process; i) pre-osteoblasts derive from mesenchymal stem cells that express markers such as Stro1, CD29, CD105, CD166, ii) once committed to a mature stage, osteoblasts able to produce mineralized bone matrix. It remains unclear if each osteoblast has a predefined cellular fate, yet, at the end of the bone formation phase osteoblasts can become embedded in bone as iii) osteocytes, or become iv) bone lining cells, or undergo to programmed cell death (apoptosis) (Dallas and Bonewald, 2010). Matrix-producing osteoblasts express Runx2 and Osterix, necessary for osteoblast differentiation, followed by alkaline phosphatase and collagen, necessary for the production of osteoid. Osteocalcin is produced by the late osteoblast and continues to be expressed by the osteocyte. Some designated cells begin to embed in osteoid (osteoid osteocytes) and begin to extend dendritic projections, keeping connections with already embedded cells and cells on the bone surface. Molecules such as E11/gp38 and MT1-MMP appear to play a role in dendrite formation, whereas molecules such as destrin and capping protein (actin filament) gelsolin-like (CapG) regulate the cytoskeleton, and phosphate regulating

endopeptidase homolog X-linked (PHEX), matrix extracellular phosphoglycoprotein (MEPE), and dentin matrix protein 1 (DMP-1) regulate biomineralization and mineral metabolism, and fibroblast growth factor 23 (FGF-23) that regulating renal phosphate excretion. All of these, are the typical markers of the later stage of differentiation, the mineralizing osteocytes. Sclerostin is a marker of the mature osteocyte and is a negative regulator of bone formation. Mature osteocytes seem to be also enriched in proteins associated with resistance to hypoxia, for example ORP 150, as one would expect from their location embedded within bone and the potential for a restricted oxygen supply (Hirao et al., 2007). It has been shown that oxygen tension may regulate the differentiation of osteoblasts into osteocytes, and osteocyte hypoxia may also play a role in disuse-mediated bone resorption (Gross et al., 2001).

In contrast to osteoclasts and osteoblasts, osteocytes are defined as mechanosensor of bone (Knothe Tate et al., 1998) because of their location deep within the bone matrix and their dendritic network that permits detection of variations in the levels of strain placed on bone (Bonewald, 2011; Gortazar et al., 2013). For this reason they represent the main model adopted in our study.

2.2.2 Intercellular Cross-Talk and bone homeostasis

Intercellular communication within the bone microenvironment is a critical process for the maintenance of normal bone structure, and osteocytes are considered the “master orchestrators of bone” (Schaffler et al., 2014).

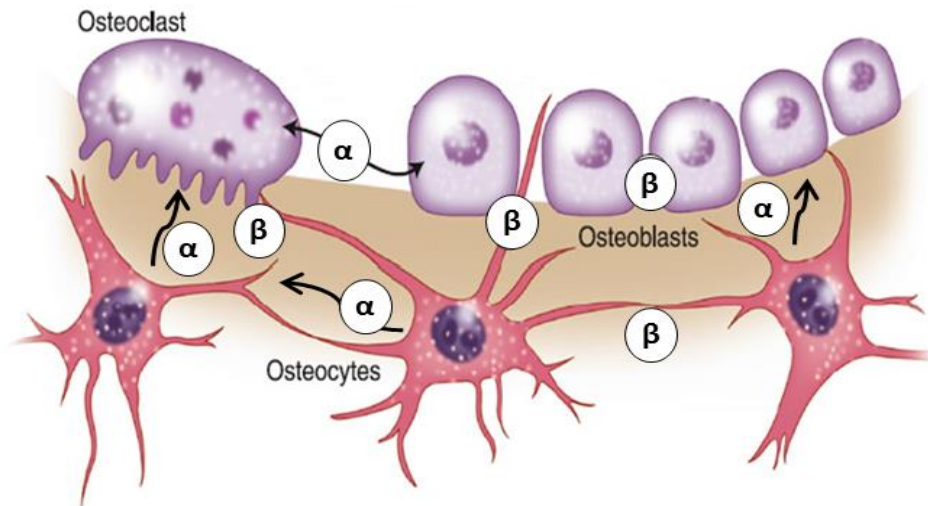


Figure 4. Mechanism of cross-talk among the bone cells that occurs through the release of factors (α) and cell-cell contact and interaction (β)

As above-mentioned, osteocytes carry out their role of biomechanical sensor thanks to their intricate network, that allow them to communicate to each other and to the other cells on bone surfaces; these cells use several mechanisms, both direct and indirect communication, to accomplish these signalling tasks (figure 4). Direct cell–cell communication through gap junction (the most common in osteocytes are connexin 43) allow the movement of molecules of about 1 kDa among the cells and the transmission of electrical potential within the osteocytic network. Another mechanism of osteocyte communication is the paracrine signalling by small metabolites, like prostaglandins, nitric oxide (NO), and ATP, and by macromolecules such Wnt, Sclerostin, FGF23 and RANKL involved in the cellular cross-talk (Schaffler et al., 2014).

Wnt/ β -catenin signalling, also known as canonical Wnt signalling, is a key pathway required for normal bone formation and for bone homeostasis and its activation is crucial for osteocyte viability and adaptation of different stimuli, including mechanical loading (Kramer et al., 2010).

Wnt/ β -catenin initiated by secreted Wnt ligands binding to a dual-receptor complex formed by low-density lipoprotein receptor-related protein 5 or 6 (Lrp5/6) and the seven-transmembrane domain receptor frizzled. The downstream signalling cascade leads to the β -catenin accumulation in the cytoplasm and translocation into the nucleus where β -catenin acts as a transcription factor to regulate expression of genes involved in the control of osteoblastogenesis. Thereby, inducing mesenchymal progenitor cells to differentiate into osteoblasts and in mature osteoblasts promoting proliferation and mineralization, while blocks apoptosis (Kubota et al., 2009).

Osteocyte to osteoblast communication involve specifically the Wnt/ β -catenin pathway (Javaheri et al., 2013) and the production of Sclerostin, an inhibitor of osteoblast activity in response to unloading (Robling et al., 2008). In addition to sclerostin, osteocytes express the Wnt inhibitors i.e. Dkk1 and secreted frizzled-related protein 1 (sFRP1), that inhibit osteoblast differentiation and bone formation by binding to Lrp5/6 (Burgers and Williams, 2013). Santos and collaborators in 2009 showed that MLO-Y4 osteocytes are also able to respond to fluid shear stress *in vitro* by modulating the expression of Wnts ligand, especially WNT3a, and the activation of Wnt responsive genes (Santos et al., 2009).

Osteoclast differentiation is also driven by Wnt/ β -catenin signalling, via the reduction of OPG expression, the osteoclasts differentiation inhibitor, produced by both osteoblasts and osteoclasts (Baron and Kneissel, 2013).

Recently, it has been shown that osteocytes express RANKL, a pre-osteoclastogenic cytokine involved in bone resorption activation (Nakashima et al., 2011; Paszty et al., 2010; Xiong and O'Brien, 2012). It is well known that osteoclast precursors require supporting cells for osteoclast formation and that cell-to-cell interactions play a pivotal role in regulation of osteoclast formation and bone resorption (Zhao et al., 2002). Lau E. and collaborators report that osteocytes release RANKL in response to mechanical stimulation

(Lau et al., 2010). The balance between RANKL and OPG defines the number of osteoclasts formed and their activity and, consequently, determines the rate of bone resorption. Moreover it is well known that the requisite for osteoclast formation and activation is the binding of RANKL to the RANK receptor on osteoclast precursor cells and that OPG, also secreted by osteoblastic cells, is a decoy receptor of RANKL. By blocking the RANKL–RANK interaction, OPG acts to antagonize the formation and survival of osteoclasts (Busse et al., 2010).

Another important signalling molecule that osteocytes release in response to mechanical stimuli is Prostaglandin E₂ (PGE₂) that has been reported to act on both osteoblasts and osteoclasts, and have both stimulatory and inhibitory effects. In particular, PGE₂ promotes the differentiation of osteoclasts in bone marrow cultures and the stage of osteoclast maturation. PGE₂ released by bone cells has been found to increase upon fluid flow stimulation and mediate downstream responses such as increased expression of gap junction protein connexin (Cx) 43 and decreased expression of OPG (Tanabe et al., 2005; Zhang et al., 2007).

In recent years it has become clear that the regulation of bone homeostasis is not a process regulated by unidirectional pathway, but that it implicates many coupling factors involved in a bone cellular cross-talk. In support of this, it has been shown that osteoclasts express cardiotrophin-1, a cytokine that stimulates bone formation *in vivo*, and EphrinB2 expressed in the osteoclast lineage and provided an intracellular signal that inhibits osteoclast formation by cell to cell contact. It has been also shown that osteoclasts are able to expressed different inhibitory factors such as Semaphorin 4D, which is consider the first negative regulator of osteoblasts function.

Also the osteoblast seems to be involved in the regulation of osteoclast activation; in fact, a cytokine signal, that involve interleukin (IL)-33, from the osteoblast lineage has been

shown to inhibit osteoclast formation through an indirect mechanism that leads with the induction of other osteoclast inhibitors, like granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-14, IL-13, and IL-10 (Sims and Walsh, 2012).

2.3 Bone unloading and hypoxia

Bone remodelling is a fundamental mechanism for removing and replacing bone during adaptation of the skeleton to mechanical loads. Mechanical loading placed on bone can result in mechanosensation by osteocytes via several potential mechanisms, which include changes in whole tissue strain, hydrostatic pressure, and streaming potentials generated by bone fluid flow through a charged bone matrix (Klein-Nulend et al., 2013).

During periods of non-load bearing, as seen with bed-rest condition, the mechanism of bone remodelling is not in equilibrium and a loss in bone mass is observed (Bikle and Halloran, 1999). It was suggested that the loss of bone observed in disuse models was the result of hypoxic osteocytes, caused by deprivation of loading, inducing a decreased oxygen transport (Stevens et al., 2006). Oxygen and other gases are transported to the nearest vascular canal by diffusion (due to Brownian motion) and by convection (due to the interstitial fluid flow). The later is produced in part by the pressure oscillations due to the cardiac output, and in part by the acceleration that the interstitial fluid experience due to the deformation of the mineralized extracellular matrix. Therefore, it is possible assume that:

$${}^{\text{ocy}}\text{pO}_2 = {}^{\text{S}}\text{pO}_2 + {}^{\text{d}}\text{pO}_2$$

where ${}^{\text{ocy}}\text{pO}_2$ is the partial pressure of oxygen in the osteocyte lacunae, ${}^{\text{S}}\text{pO}_2$ is the fraction of pressure due to diffusion and to cardiac output, and ${}^{\text{d}}\text{pO}_2$ is the fraction due to bone matrix deformation. The actual concentration of oxygen occurring inside of the osteocyte lacunae during skeletal unloading is remaining still unknown, because of the fluid pressure

and the oxygen concentration inside an osteocyte lacuna cannot be measured experimentally.

Most studies on bone microcirculation in unloading condition are done on mice and show that hindlimb suspension reduces mice femoral intramedullary pressure of 20-25% (Stevens et al., 2006; Zhang et al., 2007).

By using another classic disuse model (turkey ulna immobilization) originally developed by Lanyon and Rubin (Rubin and Lanyon, 1984), it has been shown that osteocytes become hypoxic when the bone is totally shielded from biomechanical deformation (Dodd et al., 1999). Also this observation suggests that a possible model for disuse osteopenia could be mediated by the osteocytes hypoxia (Gross et al., 2001).

In conclusion, skeletal loading is fundamental to the maintenance of the interstitial fluid flow necessary for nutrient and gaseous exchange and, consequently, for maintaining of osteocyte viability (Knothe Tate, 2003; Ontiveros et al., 2004).

2.4 Osteocyte hypoxia

Several studies showed that hypoxia is a physiological condition for osteocytes compared to other cell types. There is evidences that a pO_2 value of about 6% was measured in the normal human bone marrow aspirates (Harrison et al., 2002) and a concentration of approximately 5% O_2 is more likely to be physiological for osteocytes than a concentration of 20% O_2 , as osteocytes are embedded deep inside the mineralized bone matrix and their nutrient availability is greatly dependent on diffusion (Al Hadi et al., 2013; Arnett, 2010).

Cellular oxygen concentrations are normally maintained within a physiological range; as on one site the lack of oxygen can result in a production of ATP insufficient to maintain essential cellular functions, while on the other side the excess of oxygen may result in the generation of damaging reactive oxygen intermediates. Cells respond to changes in oxygen

tension (pO_2) *via* oxygen-dependent degradation of hypoxia inducible transcription factors (HIFs), a heterodimer containing α and β subunits (Riddle et al., 2011; Zahm et al., 2008). In the absence of sufficient oxygen, HIF- α becomes stabilized and is able to heterodimerize with its transcription partner HIF- β . This heterodimer binds hypoxia response elements (HREs) in target gene promoter sequences and initiates transcription of hypoxia-regulated genes involved in a variety of cellular processes including angiogenesis, by increasing of vascular endothelial growth factor (VEGF), energy metabolism, cell proliferation and survival (Semenza, 2009); the inhibitory actions of HIF-1 α on Wnt signalling by directly sequester of β -catenin in hypoxia, seems to be one of the mechanism involved in decreasing cell proliferation (Choi et al., 2010).

“Physiological hypoxia” might be involved in the transformation of osteoblasts to osteocytes although it is unclear what is the cellular mechanism regulating this process in response to altered oxygen tension. It has been reported that hypoxia promotes the synthesis of mineralized matrix by pre-osteoblast cells line *in vitro*, and increase the expression of late osteoblast and osteocyte markers, as connexin 43, dentin matrix protein 1 (Dmp-1), matrix extracellular phosphoglycoprotein (Mepe), and fibroblast growth factor 23 (Fgf23) expression, and increase the expression of protein that mediate adaptive responses to hypoxia and oxidative stress as the ORP 150 protein (150-kDa oxygen-regulated protein), a novel endoplasmic-reticulum-associated chaperone induced by hypoxia/ischemia (Hirao et al., 2007; Kuwabara et al., 1996).

However, acute disuse has been reported to induce **severe osteocyte hypoxia**, hypothetically pO_2 of about **1%** can be reached inside of osteocyte lacuna during unloading condition, leading to a severe environmental stress for the cells. It has been reported that severe hypoxia condition induces expression of molecules acting as chemotaxants for osteoclasts, as demonstrated with the direct upregulation of osteopontin

(OPN) induced by acute disuse in osteocytes both *in vivo* and *in vitro* (Gross et al., 2005). Moreover, some evidence shows that hypoxic osteocytes are also involved in human mesenchymal stem cells (MSCs) migration, through an OPN/CD44-mediated pathway (Raheja et al., 2008).

Furthermore, it has been postulated that severe oxygen deprivation could have an effect on osteocytes apoptosis (Dufour et al., 2008; Plotkin et al., 2005), leading to osteoclastic recruitment and bone resorption, probably by RANKL signal pathway (Aguirre et al., 2006; Noble et al., 2003; Plotkin, 2014).

Advancements show that osteocyte apoptosis plays a key role in orchestrating bone homeostasis, in fact, osteocyte apoptosis is elevated in bone with high rates of remodelling (Noble et al., 1997). Like many other cell types that undergoing to apoptosis or necrosis, osteocyte-like MLO-Y4 cell line release high mobility group box 1 (HMGB1) protein that stimulates the synthesis of RANKL, TNF α , and IL6, but inhibits the production of OPG (Bidwell et al., 2008). Apoptotic bodies produced during the last stages of apoptosis represent another potential means of communication with neighbouring viable cells; the diameters of apoptotic body generated from osteocyte-like cell line MLO-Y4, or apoptotic osteocyte-like cells derived from neonatal calvaria, allow them to circulate within the fluid space that surrounds the dendritic processes of viable osteocytes. Interestingly, apoptotic bodies derived from MLO-Y4 cells stimulated osteoclast differentiation in a RANKL-independent manner both *in vitro* and *in vivo*. Apoptotic bodies, isolated from dying osteoblastic cells, were unable to do so, suggesting the existence of one or more osteocyte-specific osteoclastogenic factors (Jilka et al., 2013).

Conditions of severe hypoxia, also directly affect osteoblasts and osteoclasts. In fact reduced oxygen tension is known to act as a direct stimulator of osteoclastogenesis; moreover the inhibition of osteoblastogenesis in hypoxia has shown to be due to decreased

cell proliferation, reduced differentiation of osteoblasts, as demonstrated by the downregulation of the osteoblast transcription factor Runx2 and expression of collagen type I and alkaline phosphatase (Arnett, 2010).

Chen and collaborators showed using an *in vitro* model of murine osteoblasts (MC3T3-E1), that a possible mechanism for hypoxia to inhibit osteoblast proliferation involves hypoxia/HIF-1 α inhibition of Wnt pathway, and they found an upregulation in Sost (gene encoding for sclerostin) RNA level after 48 hours of severe hypoxia (1% O₂), suggesting that hypoxia activates Sost gene expression (Chen et al., 2013; Chen et al., 2012).

These findings suggest that hypoxia associated regulation pathways of osteocytes could be of fundamental importance in the biology and pathology of bone tissues.

Chapter 3. AIMS OF THE PROJECT

The loss of bone observed in disuse models was the result of osteocyte hypoxia, caused by deprivation of loading induced oxygen transport (Stevens et al., 2006).

It has been hypothesized that oxygen deprivation, due to decreased mechanical loading, could have an effect on osteocytes apoptosis (Noble et al., 2003; Plotkin et al., 2005) and that it could lead to osteoclast recruitment and bone resorption (Aguirre et al., 2006). Since it is well known that osteocytes orchestrate bone homeostasis by regulating both bone-forming osteoblasts and bone-resorbing osteoclasts (Nakashima et al., 2011), this *in vitro* study proposed to understand the molecular mechanism exerted by hypoxia in osteocytes using the MOL-Y4 osteocyte-like cell line (Bonewald, 1999; Kato et al., 1997) as cellular model.

Considering that hypoxia is a physiological condition for osteocytes, and a concentration of approximately 5% O₂ is more likely physiological for osteocytes than a concentration of 20% O₂, and that 1% of oxygen can be considered a severe hypoxia condition occurring during disuse (Ritter et al., 1992; Xiong and O'Brien, 2012; Hirao et al., 2007; Arnett, 2010), we chose to test two hypoxia conditions :

- 1% O₂ (SEVERE)
- 5% O₂ (PHYSIOLOGICAL)

together with 20% O₂ considered as the NORMOXIC condition (representing the atmospheric oxygen level); although it is well known that a pO₂ of 20% actually corresponds to hyperoxia (Arnett, 2010).

Purposes of the study were:

- to investigate the **osteocyte response** to the hypoxic condition; specifically we investigated

- the **trigger of hypoxia** condition,
 - the osteocyte **viability** and **proliferation**,
 - the presence of **apoptosis**;
 - the expression of a protein probably involved in the osteocyte adaptation to oxygen deprivation called 150-kDa oxygen-regulated protein (**ORP150**).
- Therefore, we have attempted to delineate the pivotal functional role of osteocytes in regulation of bone remodelling under hypoxia conditions and whether the **RANKL/OPG** signalling axis is the relevant mechanism in hypoxic osteocyte regulation of osteoclastogenesis, by using murine monocyte/macrophage cell line (RAW 264.7) as *in vitro* model for osteoclasts.
 - Moreover, we aimed to investigate if hypoxic osteocytes, through the release of soluble factors, can also regulate osteoblasts viability and activity (**Alkaline Phosphatase activity –ALP activity-** and **β-actin mRNA**), in order to lay the basis to understand the intricate mechanism involved in the regulation of bone homeostasis orchestrated by severely oxygen deprived osteocytes.
 - Furthermore we investigated if **sclerostin** and **prostaglandin 2 (PGE₂)** released by osteocytes in hypoxia/normoxia conditions, exerted an effect on regulation of osteoblasts activity.

Chapter 4. *MATERIALS and METHODS*

4.1 Cell culture

Murine long bone osteocyte Y4 (MLO-Y4) cell line was used and cultured as previously described (Bonewald, 1999). This cell line was derived from a transgenic mouse in which the immortalizing T-antigen was expressed under control of the osteocalcin promoter. MLO-Y4 cells exhibit properties of osteocytes including high expression of osteocalcin, connexin 43, the antigen E11/gp38. In contrast, expression of the osteoblast marker, alkaline phosphatase, is low. In addition, the dendritic morphology of MLO-Y4 cells is similar to that of primary osteocytes (Kato et al., 1997).

Briefly, the cells were cultured on collagen - coated (rat tail type I collagen, Becton Dickson Bioscience, MA, USA) plastic ware and grown at 37°C, 5% CO₂, 95% air using α -MEM containing ribonucleosides, deoxyribonucleosides, and L-glutamine, (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 2.5% fetal bovine serum (FBS) (PAA The Cell Culture Company, NJ, USA), 2.5% bovine calf serum (CS) (HyClone, Utah, USA) and penicillin/streptomycin at 100 U/ml (Invitrogen Corporation, Carlsbad, CA, USA). In order to define the best culture condition for hypoxic experiments, five different MLO-Y4 cell densities, 2000, 3000, 4000, 5000, 6000 and 7000 cells/cm² and two different total serum (FBS +CS) concentration 5% and 1% (v/v) were tested. For these experiments the cells were cultured for 24, 48 and 72 hours in standard conditions, 37°C in an atmosphere of 5% CO₂ and controlled humidity.

Pre-osteoblast cell line MC3T3-E1 Subclone 4, obtained from ATCC cell bank (Manassas, VA, USA), was used as model of osteoblasts; they were well-characterized murine osteoblasts, which express osteoblast-specific proteins including alkaline phosphatase, collagen, osteocalcin and they are capable of mineralization (Wang et al., 1999). MC3T3-

E1 were cultured in α MEM containing ribonucleosides, deoxyribonucleosides, and L-glutamine, 10% FBS and 100U/ml penicillin/streptomycin.

Murine monocyte/macrophage cell line Raw 264.7 obtained from ATCC cell bank (Manassas, VA, USA), was used as model of osteoclastogenesis (Collin-Osdoby et al., 2003); they were established from a tumor induced by Abelson murine leukemia virus. Raw 264.7 were cultured as suggested by the supplier, in Dulbecco's Modified Eagle's Medium high glucose (ATCC Manassas, VA, USA), 10% FBS and 100U/ml penicillin/streptomycin.

4.2 Hypoxia conditions

For the hypoxia experiments, 5000 cells/cm² (MLO-Y4 and MC3T3-E1) were seeded onto collagen-coated multi-well dishes, incubated in α MEM w/o phenol red, 2.5% FBS + 2.5% CS and 100U/ml penicillin/streptomycin. The time 24 hours post seeding, was the designated time 0, when the cells were culture under normal conditions (normoxia 20% O₂), for 8, 16, 24, 48 and 72 hours or cultured under hypoxia conditions (hypoxia 1% O₂ and 5%O₂).

For hypoxic conditions the cells were placed inside a Billups-Rothenberg Chamber (San Diego CA, USA) (figure 5), where a mixture of gas (95% N₂ and 5% CO₂) was injected resulting in 1% and 5% O₂; the oxygen percentage was controlled by the use of an Oxygen Analyzer/Monitor (Vascular Technology, Incorporated).



Figure 5. Billups-Rothenberg hypoxia Chamber

Pimonidazole hydrochloride (HypoxyprobeTM-1, Chemicon, Temecula, CA, USA) (Raleigh et al., 2001) was used to evaluate hypoxia of osteocytes. Pimonidazole Hydrochloride is a substance with low-molecular weight that binds only the cells that have an oxygen tension of 10 mm Hg or lower at 37°C, equivalent to oxygen concentration less than 14 μ M ($pO_2 \sim 1,4\%$) (Genetos et al., 2010).

A monoclonal antibody IgG1 (HypoxyprobeTM_1Mab1), provided by the kit, that recognizes pimonidazole hydrochloride bound to the cells, has been used to detect the hypoxic cells, according to the manufacturer's protocol.

Following having supply to the cells culture, pimonidazole hydrochloride reaches all the cells and it is reductively activated only in the hypoxic ones; the activated intermediate forms stable covalent adducts with thiol (sulphydryl) groups in proteins, peptides and amino acids only in those cells that have an oxygen concentration less than 14 micromolar, equivalent to a partial pressure $pO_2 = 10$ mm Hg at 37°C.

For each cell culture the assay done in quadruplicate, was performed.

4.3 Conditioning of Osteoblasts and Osteoclasts

The culture media of the MLO-Y4 grown in both the hypoxic conditions (1% and 5% of oxygen) and normoxic condition (20%) were collected at all the time setting points. The culture media were centrifuge at 2 g for 7 minutes at +4°C and the supernatant was collected; therefore, only soluble factors secreted by hypoxic and normoxic osteocytes were present in the conditioned media (CMs). 1 ml of each CMs was stored at - 20°C for ELISA assay, the remaining CM were collected in cryovials, rapidly frozen in liquid nitrogen and stored at -80°C.

4.3.1 RAW 264.7 conditioning

To induce osteoclast formation, RAW264.7 cells were seeded onto tissue culture plates and cultured at 2500 cells/cm² density in presence of 20 ng/ml Mouse RANK-Ligand soluble factor (sRANKL) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) in serum-supplemented DMEM. Three days after the seeding, hypoxic 1% O₂ and 5% O₂ conditioned medium (1% O₂-CM and 5% O₂-CM) and normoxic conditioned media (N-CM) collected at all the time points (time 0, 8, 16, 24, 48 and 72 hours (h)) were added to the cells in a 1:1 ratio with 20 ng/ml sRANKL in serum-supplemented DMEM. This concentration of sRANKL was the optimized minimum stimulus to induced osteoclast formation for supplementing but without masking the effect of CM. The conditioning was performed for 7 days and the culture media, mixed 1:1 with CM, was changed every two days.

4.3.2 MC3T3-E1 conditioning

The cells were seeded onto tissue culture plates and cultured at the density of 5000 cells/cm² and supplied of normal growth media, before mentioned. After 24 hours hypoxic

1% O₂ and 5% O₂ conditioned medium (1% O₂-CM and 5% O₂-CM) and normoxic conditioned media (N-CM) collected at all the time points (time 0, 8, 16, 24, 48 and 72 hours (h)) were added to the cells in a 1:1 ratio with normal growth media. The conditioning was performed for 7 days and the culture media, mixed 1:1 with CM, was change every two days.

4.4 Evaluation of Cell Viability and Apoptosis

Osteocytes viability and total cells number were determined by trypan blue staining (Ahuja et al., 2003; Jilka et al., 1998). Only blue-stained cells were considered dead, because the Trypan blue is a vital stain used to selectively color dead cells in blue; living cells with intact cell membranes appeared not colored. All 9 big squares of the hemocytometer chamber were used for cell counting. The results were obtained by counting the number of positive cells relative to the total number of cells.

Three experiments performed were done in quadruplicate.

Cells viability and total cells number with trypan blue method:

- collect all the supernatant in a tube
- wash in PBS and collected the solution
- add 400µl of trypsin and incubated at 37°C for 2-3 minutes
- add 1ml Calf serum
- centrifuge at 500 g for 5 minutes
- discard the supernatant
- resuspend the cells in 100µl of PBS +0.1% of BSA
- 10 µl of tripan blue + 30 µl of cells suspension
- count in a hemocytometer = total number in all the field of the chamber and the cells dead.

Osteoblast viability was assessed by MTT assay.

The MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was prepared at 5 mg/ml in 1x PBS. Cells were incubated with the MTT reagent 1:10 for 2 h at 37°C. Medium was collected and cells incubated with 1 ml of Dimethyl sulfoxide for 15 min.

In this assay, the metabolically active cells react with the tetrazolium salt in the MTT reagent to produce a formazan dye that can be observed at 570 nm, using a Multiskan FC Microplate Photometer (Thermo Scientific). This absorbance is directly proportional to the number of metabolically active cells. Mean values of absorbance of 5 replicates were determined.

Osteocytes apoptosis was assessed, after DAPI nuclear staining, by counting cells exhibiting chromatin condensation or nuclear blebbing (Plotkin et al., 1999). Positive cells were quantified by visualizing random fields of view per sample under 20X magnification and counting the number of positive cells versus the total number of cells per image.

Apoptosis assessment using nuclear fragmentation:

- wash the cells in PBS
- fix in PAF 2% in PBS for 5 minutes at +4°C
- wash in PBS
- stain with 100 µl (per well) DAPI (1:250 in distilled water) for 5 minutes at R.T. in gentle agitation
- wash in PBS

4.5 TRAP staining of conditioned RAW 264.7

The TRAP positive cells detection is a method to determine the presence of osteoclasts. After 7 days of conditioning with 1% O₂-CM, 5% O₂-CM and n-CM collected at all the time setting-points of hypoxia and normoxa experiments, the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) following the Kit (Sigma- Aldrich, St Louis, MO, USA) protocol instruction.

Briefly, cells were fixed in a solution containing formalin (37% v/v), acetone (99.5% v/v) and citrate solution (27 mM) for 1 minute at room temperature (RT), and rinsed thoroughly in deionized water. Then the cells were incubated for 1 hour in 37°C in controlled humidity and protected from light, in a solution containing Naphthol AS-BI phosphoric acid, 12.5 mg/ml, Fast garnet GBC base, 7.0 mg/ml, in 0.4 mol/l hydrochloric acid with stabilizer, Acetate buffer, 2.5 mol/l, pH 5.2, Sodium nitrite, 0.1 mol/l and L(+)-Tartrate buffer, 0.335 mol/l, pH 4.9. The cells were then rinsed thoroughly in deionized water and counterstained for 2 minutes in Hematoxylin Solution. Osteoclasts were quantified by imaging five fields of view under 20x magnification and counting the number of TRAP-positive cells with three or more nuclei.

Osteoclast area was also manually traced and quantitated using Adobe Photoshop software, following the criteria of TRAP positivity and multinuclearity (Huang et al., 2003).

4.6 Alkaline phosphatase assay of conditioned MC3T3-E1

After 7 days of conditioning with 1% O₂-CM, 5% O₂-CM and n-CM collected at all the time setting-points of hypoxia and normoxia experiments, cell Alkaline Phosphatase (ALP) activity was quantified using an enzymatic assay based on the hydrolysis of p-nitrophenyl phosphate (pNP-PO₄) to p-nitrophenol (pNP) (Teixeira et al., 1995).

Briefly, 25 µl of cell lysate, obtained after cell incubation with 150 µl 1x PBS with 0.1% (v/v) Triton-X, were added to pNP-PO₄ solution and allowed to react at 37 °C. Absorbance was read at 0, 30, 60, 90 min and 24 hours at λ_{max} of 405 nm, using a microplate reader (Tecan, Research Triangle Park, NC) and ALP activity calculated by cross-reference to a standard curve of nanomoles of p-nitrophenol liberated.

4.6 Dosage of soluble factors RANKL, OPG , PGE₂ and Sclerostin

(ELISA assay)

For all the dosages the experiments were performed in duplicate. One hundred microliters of conditioned media were coated onto each the respective high-binding ELISA plates at 4°C overnight. Culture media collected from hypoxic 1% and 5% O₂) and normoxic osteocytes at all the time points tested were used for the dosage of the soluble factors. Briefly, the enzyme-linked immunosorbent assay (ELISA) is a common method used to quantified the presence of soluble factors, and it is based on capacity of specific antibodies of recognise selectively a sequence of the factor detected. The presence of the factor is detected by using a secondary antibody peroxidase conjugated; the adding of the enzymatic substrate (OPD) leads to the production of a visible signal, detected with spectrophotometry, which is directly proportionated with the quantity of factor in the sample. The absorbance was read at 450 nm. The total amounts of soluble factors production were converted to picograms per milliliter using the standard curve (4-PL). RANKL was detected by using Mouse Trance/RANKL Immunoassay (R&D, Minneapolis 55413, USA). OPG was detected by using Mouse OPG Immunoassay (R&D, Minneapolis 55413, USA). PGE₂ was investigated by using High Sensitivity PGE₂ enzyme immunoassay (Arbor Assay, Eisenhower PI Ann Arbor, MI 48108, USA).

Sclerostin was detected by using Mouse Sclerostin, SOST ELISA Kit (Wuhan EIAab Science Co, Ltd Wuhan 430079, China).

4.7 Quantitative Real-Time PCR

RNA isolation has been performed following the method of TRI Reagent-RNA/DNA/Protein isolation Reagent, Molecular Research Center, Inc. The amount and purity of the isolated RNA were then determined using a Nano Drop spectrophotometer.

Total RNA (500 ng) was reverse transcribed (RT-PCR) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a 20 μ L reaction volume:

Revers Transcription cycles:

Step 1: 25°C for 10'

Step 2: 37°C for 2 hr

Step 3: 85°C for 5''

Step 4: 4°C forever

The amplification was performed using TaqMan Assay probes (Applied Biosystems) and specific primers included in a StepOne Plus Real Time PCR System (Applied Biosystems) with the 40 cycle amplification protocol (Stage 2):

q-PCR cycles:

STAGE 1:

Step1: 50°C for 2'

Step2: 95°C for 10'

STAGE 2:

Step3: 95°C for 15''

Step4: 60°C for 1'

Primers (Applied Biosystems): target gene ORP 150 (Mn00491279) and β -catenin (Mm00499427); housekeeping gene β 2macroglobulin (Mn00437762) and GAPDH (Mm99999915).

All 5 experiments for the quantification of ORP 150 mRNA were done in triplicate, using β 2 macroglobulin as an internal reference (housekeeping gene). All 2 experiments for the quantification of β -catenin mRNA were done in triplicate, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference (housekeeping gene). The threshold cycle (Ct) value was determined by the default settings by using StepOne Software v.2.1. We calculated the relative expression of ORP 150 in hypoxic MLO-Y4 cells and compared this expression to the expression of MLO-Y4 cells grown for 8 h under normoxic conditions and calculated the $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

4.8 Western blotting

Cells cultured under hypoxia or normoxia were lysed in a RIPA Buffer complete with protease inhibitor cocktail (Biomol International, Pennsylvania, USA). Four experiments on MLO-Y4 were performed to compare the ORP 150 expression in hypoxia and normoxia conditions at all different time points. Three different experiments were conducted to compare ORP 150 expression in normoxic osteocytes and pre-osteoblasts. Protein concentration was determined in each cell lysate supernatant by a colorimetric assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein (17 μ g) were used for the analysis. Briefly, western blot methods consist in four different phases, Gel electrophoresis, Transfer, Blocking and Immunodetection. 1) Gel electrophoretic separation of protein by molecular weight was performed using 10% polyacrylamide gels and buffers loaded with sodium dodecyl sulphate (SDS-PAGE), the electrophoresis was done at 100 V for 2 hours at room temperature; 2) to make the proteins accessible to antibody detection the separated

proteins were transferred to a nitrocellulose membrane through a "blotting" process; the transfer was done at 34 V at +4°C overnight. 3) The membranes were blocked with 2,5% non-fat dry milk (Bio-Rad, Hercules, CA, USA) in PBS 1X at room temperature for 30 minutes and 4) incubated 1 hour and 30 minutes at room temperature with primary antibodies Anti-ORP 150 (Epitomics, California, USA) and Anti- β Actin (AbFrontier, Seoul, Korea) as internal control. A horseradish peroxidase-linked secondary antibody was used to cleave a chemiluminescent agent, and the reaction product provides luminescence in proportion to the amount of protein. A sensitive sheet of photographic film is placed on the membrane, and the exposure to the light from the reaction creates an image of the antibodies bound to the blot.

4.9 Statistical analysis

Data were analyzed for normal distribution using Kolmogorov–Smirnov test. Differences between the two oxygen conditions were assessed using Mann–Whitney U-test, and differences between the experimental times were assessed using Kruskal-Wallis test for non-normally distributed data. A p value of ≤ 0.05 was considered significant. Differences between the data obtained by western blot analysis for the comparison of ORP150 expression in MC3T3-E1 to MLO-Y4 cells were assessed using T-Test.

All the statistical analyses were performed by using SPSS v. 14.0 software (SPSS Inc., IBM, Chicago, IL, USA).

Chapter 5. RESULTS

5.1 Definition of the best culture conditions for MLO-Y4 cells

Toluidine blue staining and transmission electron microscopy (TEM) evaluation showed the characteristic morphology of the MLO-Y4 cell line (Fig. 6).

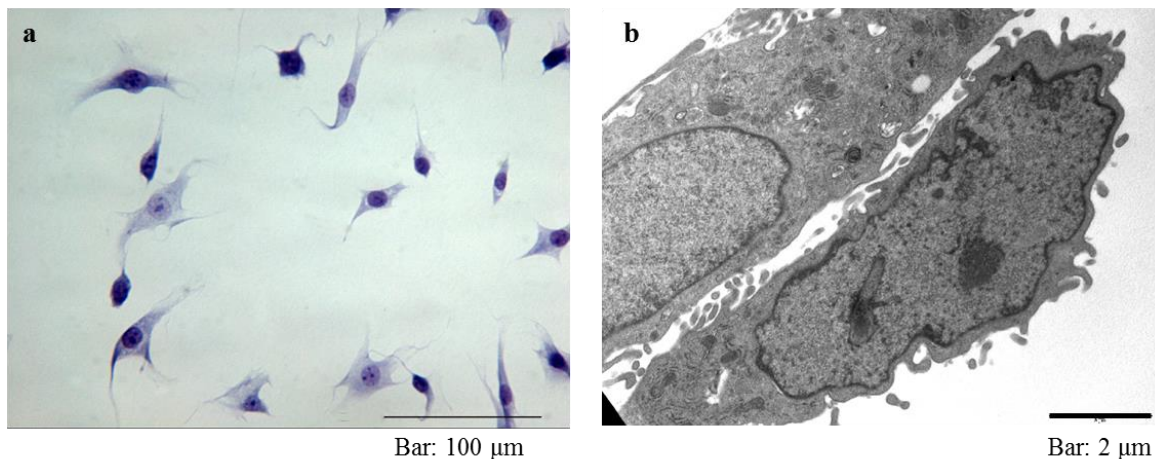


Figure 6. Figure a) shows an optical microscope image of MLO-Y4 after toluidine blue staining; figure b) shows the morphological detail obtained by TEM analysis.

The morphological analysis (Fig. 7) showed that the cells maintained their characteristic dendrites and cellular body morphology only in presence of 5% of total serum. The cells, grown in 1% of total serum, showed a very different morphology: decrease in cell body dimensions, decrease in dendrites number and extensions; in addition, the pictures of the cells grown in 1% of total serum showed a higher number of dead cells compared to cells grown in 5% of total serum. Moreover, it was evident that a cells density of 5000 cells/cm² was the optimal seeding density to guarantee the maintenance of cell morphology and avoidance of over-confluences (> 70%) for all experimental time points.

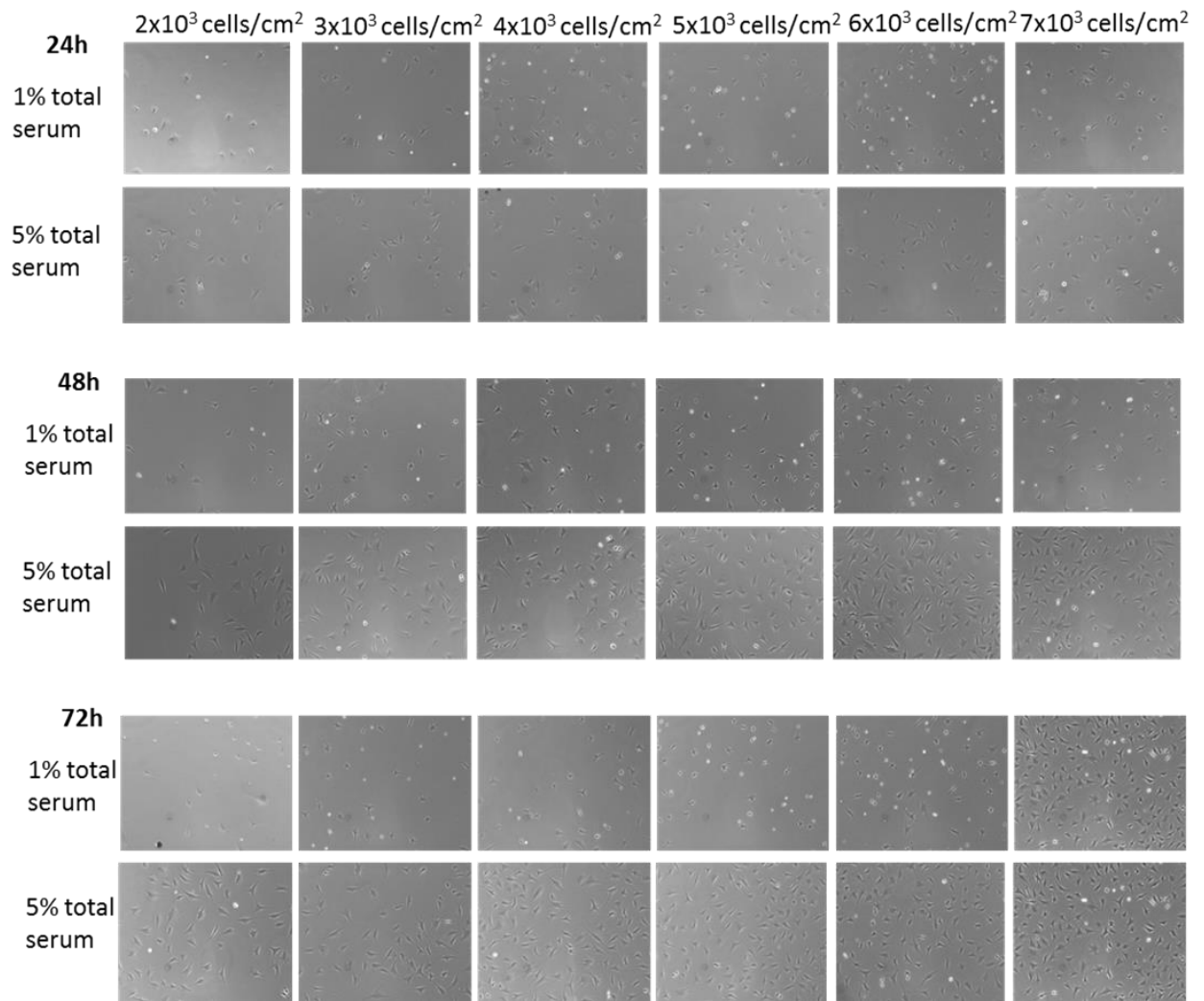


Figure 7. Morphological analysis to determinate the best culture condition for the execution of the experiments.

5.2 Hypoxia induction

MLO-Y4 cells cultured at 1% O₂ showed a significant increase in Hypoxyprobe staining compared to cells cultured at normoxia condition at 16, 24, 48, and 72 hours, while the cells grown at 5% O₂ showed a significant increase in Hypoxyprobe staining compared to cells cultured at normoxia condition at 24 hours and 48 hours, and at 16 hours compared to 1% O₂ (Fig. 8). The number of Hypoxyprobe positive cells was significantly increased with the time of culture under both the hypoxic conditions and also under normoxic conditions suggesting that the cells became more sensitive to hypoxia (Table 1).

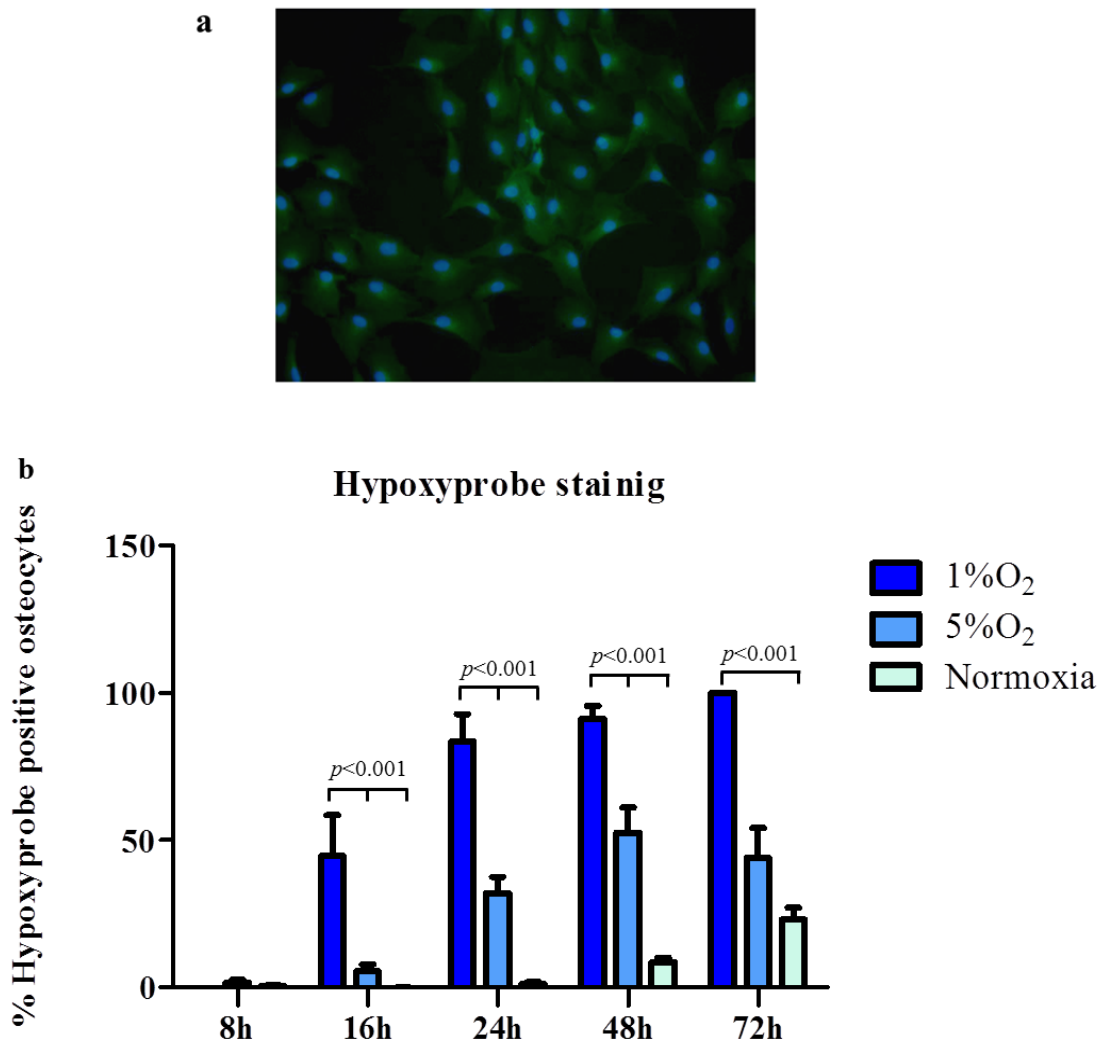


Figure 8. Effect of oxygen deprivation on MLO-Y4 detected by Hypoxyprobe staining.

Image **a**. Representative image of Hypoxyprobe staining of (%) hypoxic MLO-Y4; pimonidazole in green stains hypoxic cells and DAPI in blue stains cell nuclei. Graph **b** shows the mean and standard error of percentage of Hypoxyprobe positive MOL-Y4 cells at different time points cultured in both hypoxia and normoxia conditions.

Table 1. Hypoxyprobe staining. Statistical differences existing over the times for each oxygen condition. P value obtained with Kruskal- Wallis Post Hoc test.

Times	1%O ₂		5%O ₂			Normoxia	
	48h	72h	24h	48h	72h	48h	72h
8h	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
16h	-	-	-	p<0.001	p<0.001	p<0.001	p<0.001
24h	-	-	-	-	-	p<0.001	p<0.001

5.3 Osteocyte proliferation, viability and apoptosis

Counting of cells showed an increase in cellular **proliferation** with duration of the culture (Table 2), but the presence of 1% and 5% of oxygen significantly decreased the proliferation of MLO-Y4 after 8, 24 and 48 hours compared to the same time points of normoxic condition (Fig. 9 a).

Osteocyte **viability**, as determined by trypan blue staining, was compromised by severe oxygen deprivation (1% O₂) and resulted in a significant increase in the number of dead cells at 24 and 72 hours (Fig. 9 b). In both the normoxia and 1% O₂ hypoxic conditions, the increase in cell death over the time of culture showed a clear trend, with a significant difference between 16 and 72 hours of culture (p value= 0.012 in normoxia and p value= 0.032 in 1% O₂). No difference exists comparing 5% oxygen and the other conditions; moreover, no difference in cell death was seen in that condition over the time.

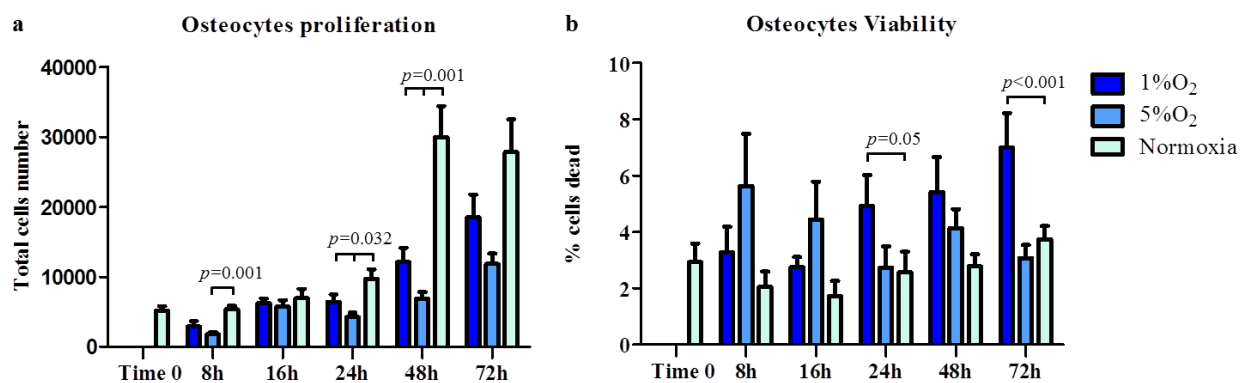


Figure 9. Effect of oxygen deprivation on MLO-Y4 proliferation and viability.

Graph **a** shows the proliferation by counting the total cells number grown in our experimental conditions. Graph **b** shows the quantification of MOL-Y4 dead cells at different time points cultured in hypoxia and normoxia conditions. For both the graphs, mean and standard error are indicated.

Table 2. Osteocyte proliferation. Statistical differences existing over the time for each oxygen conditions. P value obtained with Kruskal- Wallis Post Hoc test

Times	1%O ₂		5%O ₂		Normoxia	
	48h	72h	48h	72h	48h	72h
8h	p<0.004	p<0.004	p<0.001	p<0.001	p<0.001	p<0.001
16h	-	-	-	-	p<0.001	-
24h	-	-	-	p<0.001	p<0.001	-

The number of **apoptotic** osteocytes, as determined by nuclear fragmentation (Fig. 10 a, b and c), was increased by both the hypoxic conditions compared to the normoxic one and this increase was found to be statistically different at 16, 24 and 48 hours. Besides, significant increase in apoptotic MLO-Y4 cells was found in 5% O₂ compared to 1% O₂ at 24 hours. Surprising, at 72 hours, the number of apoptotic cells cultured under 1% O₂ hypoxic conditions was decreased compared to normoxia, but this difference was found to be not significant.

This decrease in apoptotic cell number at 72 hours was found to be also lower than at 48 hours, although, this difference was not statistically significant (Fig. 10 d).

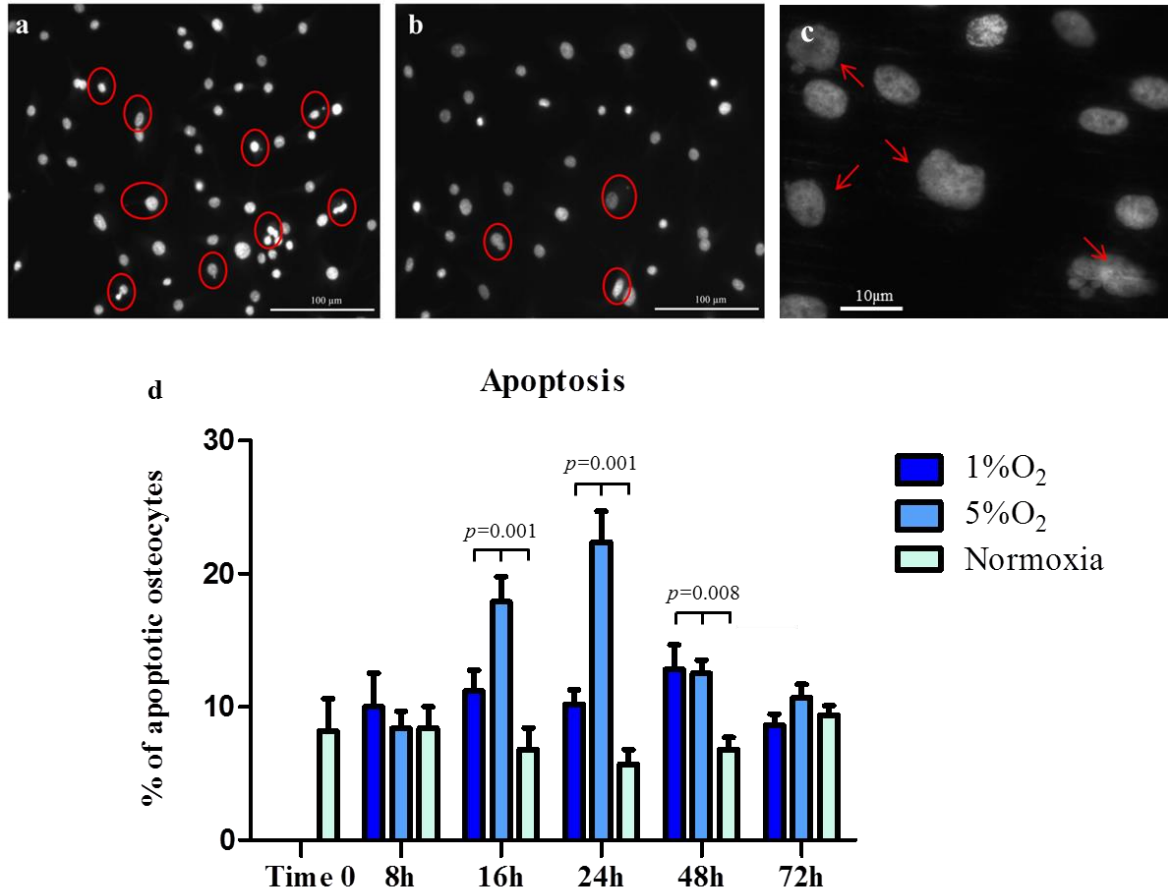


Figure 10. Evaluation of Apoptosis by nuclear fragmentation.

Images of DAPI stained MLO-Y4 cells under hypoxia (%) (a) and normoxia (b) conditions after 48 hours of culture. Image c shows a detail of nuclear fragmentation at higher magnification. Arrows indicate apoptotic nuclei.

Graph c. shows the average and standard error of the means of apoptotic cells grown in hypoxia and normoxia conditions.

5.4 Expression of ORP150

No significant difference was found in MLO-Y4 ORP 150 mRNA expression over the time in all the tested conditions. No significant changes were observed in mRNA levels under

the tested conditions, 1% O₂ and 5% O₂ and normoxia (Fig. 11 a). Nevertheless, it was possible to observe a trend that 5% O₂ induced the expression of ORP150.

Western blot analysis, conducted on MLO-Y4 grown in 1%, 5% of oxygen and normoxic conditions, showed no statistically significant differences in ORP 150 expression at protein level at all the time points, confirming the results of the ORP 150 at mRNA level (Fig. 11 b).

Moreover, western blot analysis showed that the expression of ORP150 was significantly lower in normoxic MC3T3 (ORP150/ACT ratio=0.531±0.03 average ± standard deviation) compared to normoxic MLOY4 (ORP150/ACT ratio=0.845±0.06 average ± standard deviation), with a p-value= 0.001, as reported in literature (Guo et al., 2010).

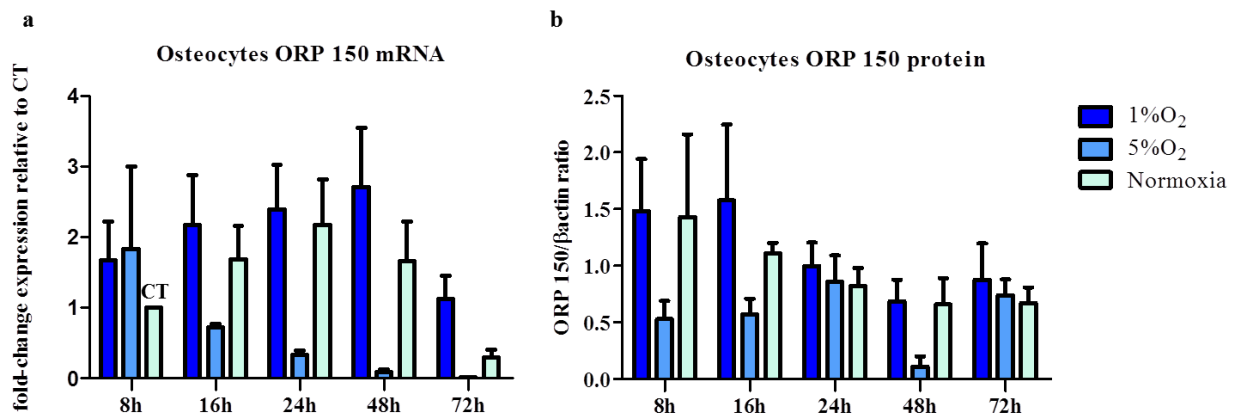


Figure 11. Effect of oxygen deprivation on ORP 150 expression in osteocytes.

Graph **a**. shows the fold-change expression of ORP 150 mRNA compared to the control (CT), analysed by the $2^{-\Delta\Delta C_t}$ methods. Graph **b** shows the level of ORP 150 protein normalized for β -Actin. For both the graphs, mean and standard error are indicated.

5.5 Dosage of soluble factors PGE₂, RANKL, OPG, and Sclerostin

Although no significant difference exists in the level of each soluble factor measured in the hypoxic and normoxic osteocytes culture media, it is possible to identify a trend of expression.

PGE₂ seems to be released at higher levels by osteocytes grown at 1% of oxygen compared to the other conditions, especially after 24, 48 and 72 hours of culture (Fig. 12 a).

The level of RANKL in our experimental conditions, becomes detectable only after 48 hours of culture; otherwise, an increase in OPG release over the time of culture was detected, without any difference among the oxygen conditions (Fig. 12 b and c). Resulting in an overall decrease in the RANKL/OPG ratio as shown in Table 3.

The levels of Sclerostin, released in all the experimental conditions, were lower respect to the limit of detection of the kit assay.

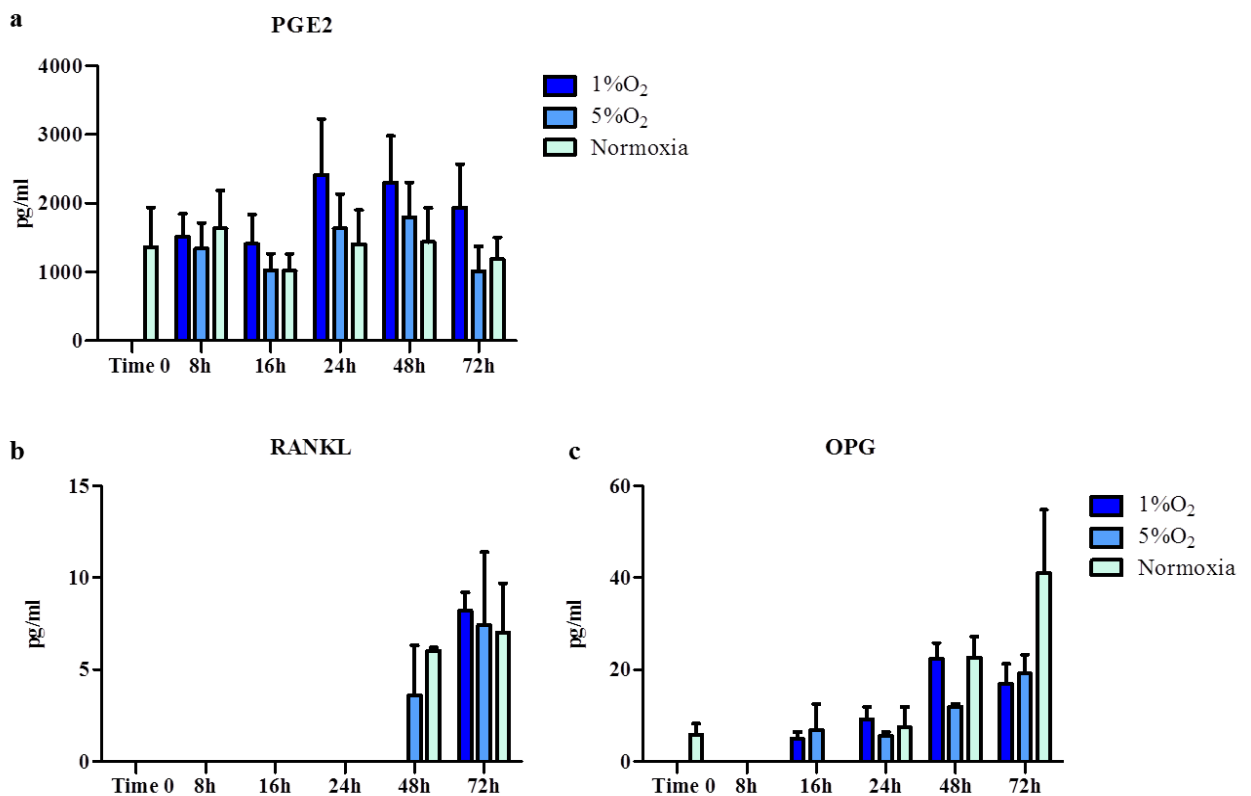


Figure 12. Effect of oxygen deprivation on MLO-Y4 release of the selected soluble factors.

The graph shows the mean and standard error of the soluble factors quantified by ELISA (pg/ml). PGE₂ (a), RANKL (b) and OPG (c).

Table 3. RANKL/OPG ratio in the culture media of hypoxic and normoxic MLO-Y4

RANKL/OPG			
	1%O₂	5%O₂	Normoxia
Time 0	-	-	-
8h	-	-	-
16h	-	-	-
24h	-	-	-
48h	-	0,298319	0,264589
72h	0,486702	0,385098	0,172001

5.6 Trap staining and induction of osteoclastogenesis

After culturing Raw 264.7 cells for 7 days with conditioned media (CM) collected from osteocytes grown in all our experimental conditions (1% O₂-CM, 5% O₂-CM and N-CM at T0, 8, 16, 24, 48 and 72 hours), the detection of TRAP positive cells was performed (Fig. 13) following two different methods:

- 1) by counting the multinucleated Trap positive cells (MNC TRAP⁺ cells) and
- 2) by evaluating the pixel area covered from Trap positive cells, as described by Huang and collaborators (Huang et al., 2003) .

I chose to describe the results obtained from the two analyses separately because, although the results showed the same trend, some differences in the statistical analysis were identified.

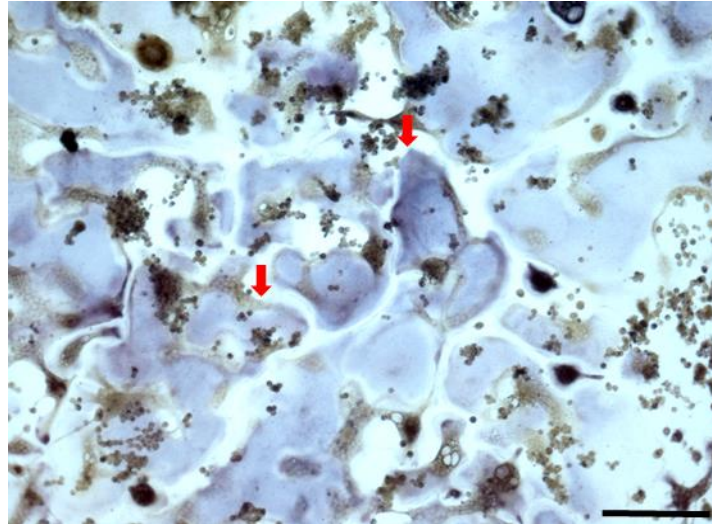


Figure 13. Representative image of multinucleated TRAP positive cells, specific markers of osteoclast (red arrows). Scale bar: 50 μ m.

5.6.1 Multinucleated Trap positive cells (MNC TRAP+ cells) results

Looking separately at each conditions, the analysis showed a significant decrease in MNC Trap positive cells in the culture conditioned for 72h with 1% O₂-CM compared to the 8, 16 and 48 hours 1% O₂-CM, and the same results were found in N-CM (Table 4). Regarding the difference in Trap positive cells induced by the three CMs derived from our oxygen conditions, I found that the conditioning of RAW cells for 48h with 1% and 5% O₂-CM induced a significant increasing of MNC Trap+ cells compared to 48h N-CM. While 72h 1% O₂-CM lead to asignificantly increased osteoclastogenesis compared to 72H N-CM (Fig. 14).

Table 4. Multinucleated TRAP positive cells. Statistical differences existing in the different times CMs. P value obtained with Mann Whitney Post Hoc test.

	1%O ₂ -CM		5%O ₂ -CM			N-CM
Times CMs	8h	48h	8h	24h	48h	16h
72h	p=0.001	p p=0.001	p=0.001	p=0.001	p=0.001	p=0.012

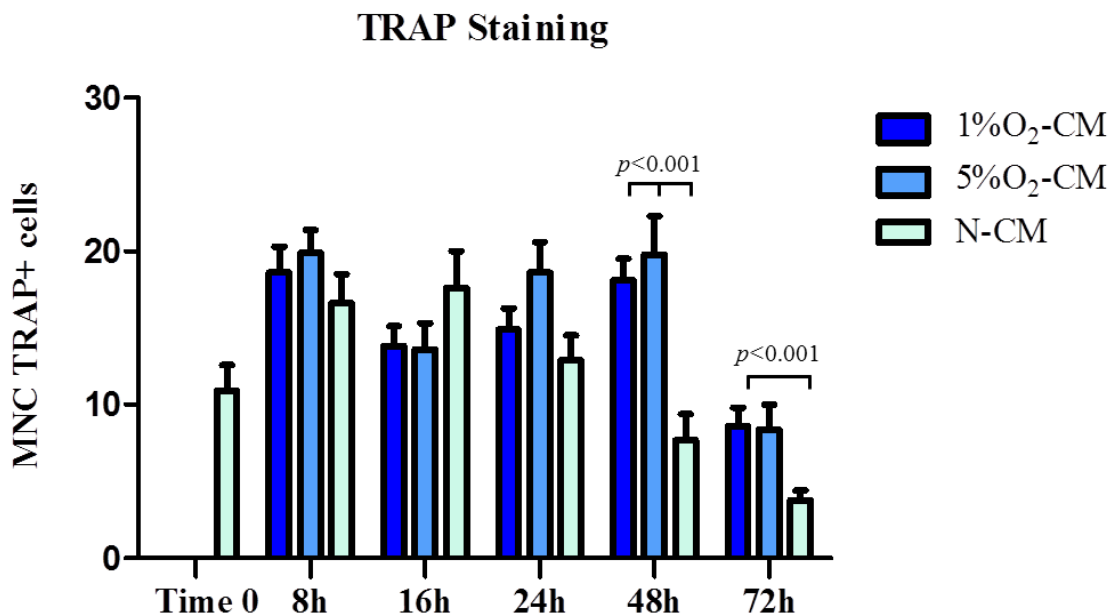


Figure 14. Effect of the CMs (from hypoxic/normoxic osteocytes) on the osteoclastogenesis.

The graph shows the mean and standard error of the number of MCN Trap positive cells induced by the conditioning with the CMs tested.

5.6.2 Pixel area covered by Trap positive cells

The results showed a decrease of the area covered by osteoclast related to the time of conditioning, in detail all the 72h CMs tested (1% O₂-CM, 5% O₂-CM and N-CM) had

lower inductive effect on osteoclastogenesis compared to the effect exerted by the CMs collected at the other time points (Table 5).

However, CMs collected after 24, 48 and 72 hours showed a significant difference in inducing osteoclast formation; in particular the 24, 48 and 72h 1% O₂-CM significantly increased the osteoclasts formation compared to 24, 48 and 72h N-CM; while only 24h 5% O₂-CM was found significantly different compared to 24h N-CM (Fig. 15).

Table 5. Multinucleated TRAP positive cells. Statistical differences existing in the different times CMs. P value obtained with Mann Whitney Post Hoc test.

Times CMs	1%O ₂ -CM			5%O ₂ -CM			N-CM		
	8h	24h	48h	8h	16h	24h	8h	16h	24h
16h	-	-	-	p<0.001	-	-	-	-	-
48h	-	-	-	p<0.001	-	-	p<0.001	-	-
72h	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001

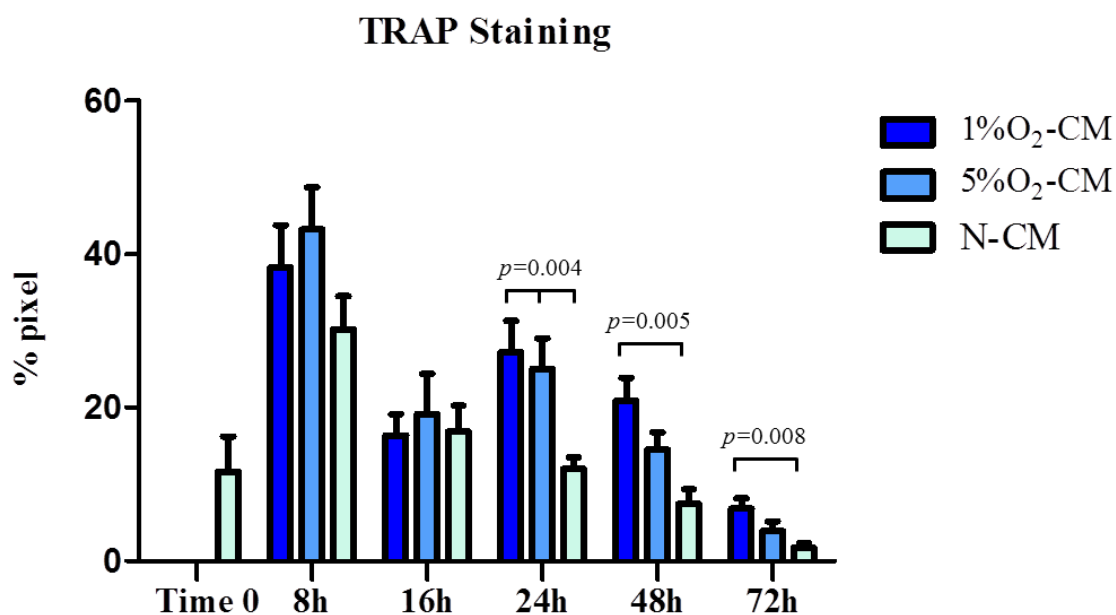


Figure 15. Effect of the CMs (from hypoxic/normoxic osteocytes) on the osteoclastogenesis.

The graph shows the quantification of osteoclast formation by counting of pixel area covered by MCN Trap positive cells induced by the conditioning with the CMs tested. Mean and standard are indicated.

5.7 Osteoblasts response to the hypoxic osteocytes conditioning media

After conditioning of MC3T3-E1 cells for 7 days with CM collected from osteocytes grown at either 1% O₂, 5% O₂ and normoxia at T0, 8, 16, 24, 48 and 72 hours, osteoblast viability, ALP activity and the quantification of β -catenin mRNA were performed.

5.7.1 Osteoblast viability (MTT assay)

The results showed that the conditioning for 7 days with all the CMs tested did not compromise the MC3T3-E1 viability, compared to time point 0. Looking separately at the 1% O₂-CM, 5% O₂-CM and N-CM, I saw a significant decrease in cell viability between 16h N-CM and 72h N-CM (p value= 0.026), while a significant increase was observed

between 48h 1% O₂-CM and 72h 1% O₂-CM (p value= 0.031). It is also interesting that 72h 1% O₂-CM showed a significant higher cell viability compared to 72h 5% O₂-CM and N-CM, while 24h 5% O₂-CM was found to be different compared to 24h 1% O₂-CM and N-CM (Fig. 16).

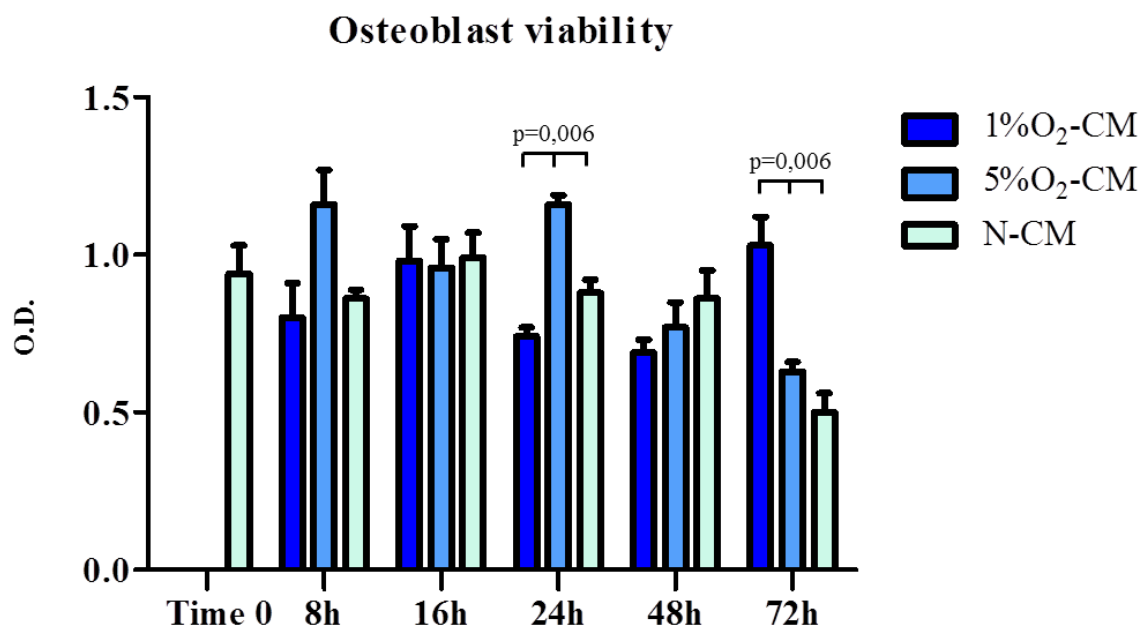


Figure 16. Effect of the CMs (from hypoxic/normoxic osteocytes) on the Osteoblast viability. The graph shows the quantification of osteoblasts (MC3T3-E1 cell line) viability assessed by MTT. Mean and standard error are indicated.

5.7.2 Alkaline Phosphatase (ALP activity) and β -catenin mRNA expression

For both ALP activity and β -catenin mRNA expression, our results indicated that the conditioning with both the hypoxic CMs seemed to positively regulate these two tested markers of osteoblasts activation, compared with the effect exerted by the normoxic CMs. In detail, the ALP activity seems to be not induced by the conditioning with all the N-CMs tested except for 48h and 72h N-CM that showed a significant increase of the enzyme compared to time point 0 N-CM (Table 5). Moreover, both the 1% O₂-CMs and 5% O₂-CMs induced an increase in ALP activity compared to N-CM. This increase was found to

be statistically significant when MC3T3-E1 cells were conditioned with 8h, 16h, 24h and 72h hypoxic CMs (Fig. 17).

Despite the inductive effect exerted by the both hypoxic CMs compared to the normoxic CM, it is necessary underline that this inductions decrease over the time of the CMs. Significant decrease was found among 16h and 48h 1% O₂-CM compared to 72h 1% O₂-CM, and the same decrease was seen between 8h and 24h 5% O₂-CM (Table 6).

The same trend was found in the mRNA expression of **β-catenin**; even if no significant difference was found, it was possible to indicate that both the hypoxic CMs seemed to be involved in an upregulation of this gene, especially compared to the gene expression level of time point 0, used as internal control of the analysis (Fig. 18).

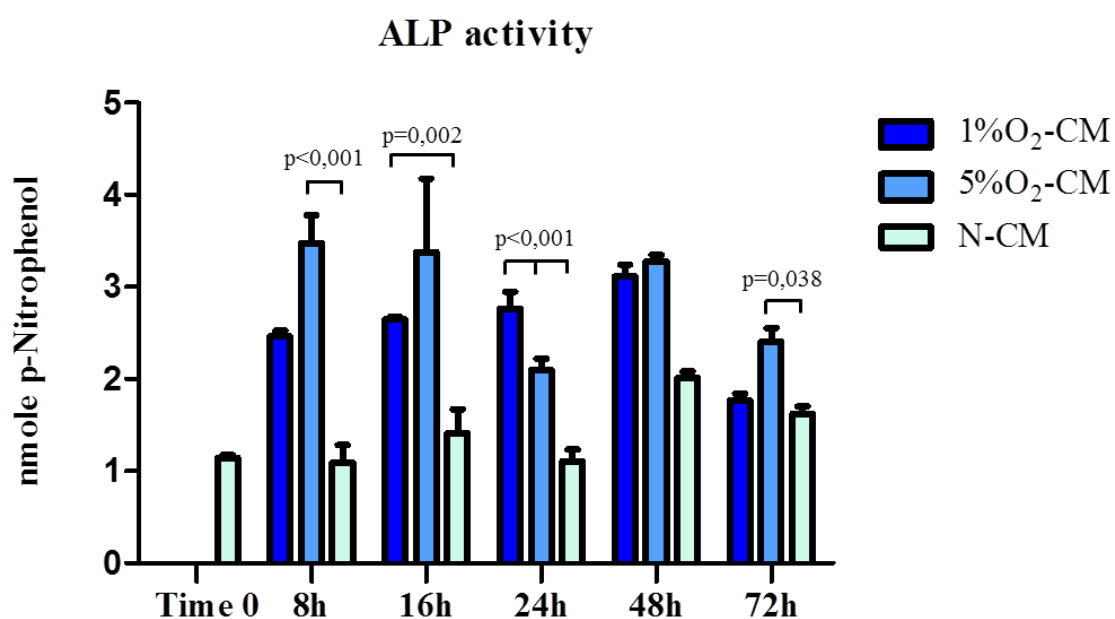


Figure 17. Effect of the CMs (from hypoxic/normoxic osteocytes) on the ALP activity

The graph shows the quantification of enzymatic activation of ALP, indicate in the graph as nmole p-Nitrophenol, the enzymatic products of ALP, in osteoblasts (MC3T3-E1 cell line) grown with the different CMs tested. Mean and standard error are indicated.

Table 5. ALP activity. Statistical differences existing in the different times CMs. P value obtained with Kruskal-Wallis Post Hoc test.

Times CMs	1%O ₂ -CM		5%O ₂ -CM	N-CM		
	16h	48h	8h	Time 0	8h	24h
24h	-	-	p=0.018	-	-	-
48h	-	-	-	p=0.001	p=0.001	p=0.001
72h	p=0.001	p=0.001	-	p=0.001	-	-

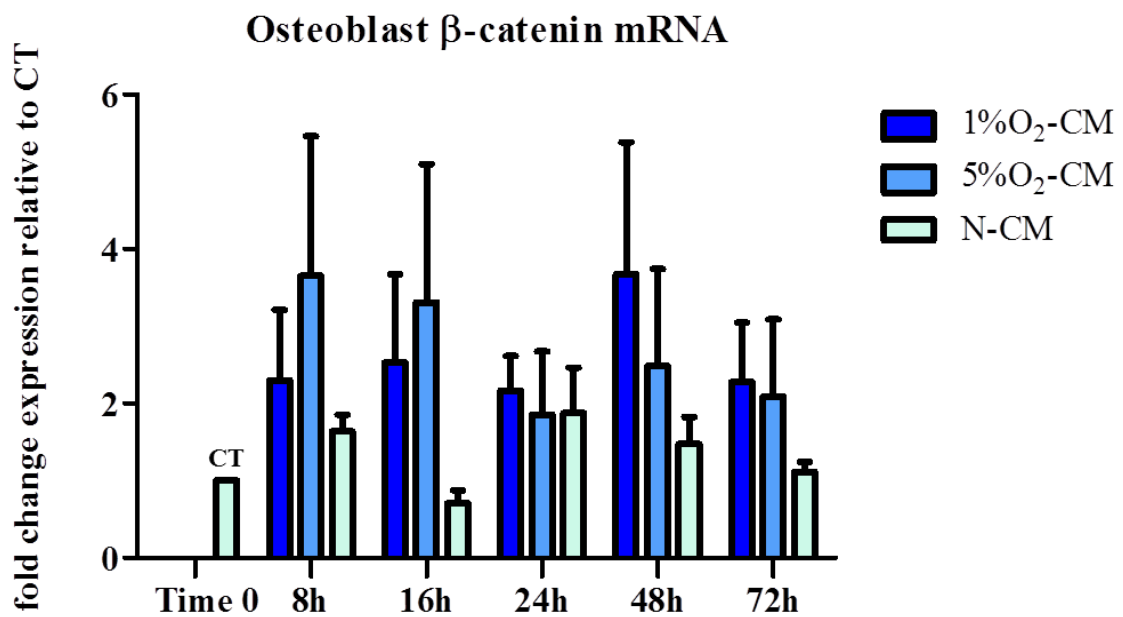


Figure 18. Effect of the CMs (from hypoxic/normoxic osteocytes) on the β -Catenin mRNA

The graph shows the fold-change expression of β -Catenin compared to the control (CT), in osteoblasts (MC3T3-E1 cell line) grown with the different CMs tested. The analysis of the data was done follow the $2^{-\Delta\Delta C_t}$ methods. Mean and standard error are indicated.

Chapter 6. *DISCUSSION*

The objectives of this study were to determine the response of osteocytes to the hypoxia, and to delineate the functional role of osteocytes in regulation of bone remodelling under hypoxia conditions, exploring the signalling from osteocytes to osteoclasts and osteoblasts. Three cells line has been used in this study as *in vitro* model of the bone cells; murine long bone osteocyte Y4 (MLO-Y4), murine pre-osteoblast cells line (MC3T3-E1) and murine monocyte/macrophage cell line (Raw 264.7) as osteoclasts model, in order to assess the intracellular cross talk that occurs during skeletal unloading leading to severe hypoxia conditions.

MOL-Y4 cells represents the unique mature osteocyte-like cell line established and characterized and they are widely used by the scientific community as *in vitro* model.

These cells expressed the characteristic markers of osteocytes, CD44, connexin 43, osteocalcin, osteopontin and low ALP expression; but the principal characteristic to identify this type of cells, was still considered the exhibition of characteristics dendritic process phenotype. It has been shown that MLO-Y4 cells can maintain this homogeneous morphology up to 45 passages in culture, if maintained in appropriated culture conditions and if not exceed 60-70% of confluence (Bonewald, 1999; Kato et al., 1997). For these reasons it was very important to strictly define the culture conditions in term of cell seeding density and concentration of serum supplementing media used during the hypoxic experiments (Fig. 7). Moreover, the passage number used in the experiments was relatively low (27-30), in order to avoid altering protein expression or morphological profile by time in culture.

Besides, it is important to point out that *in vivo* hypoxia is a physiological condition for osteocytes and a concentration of approximately 5% O₂ is more likely physiological for osteocytes than a concentration of 20% O₂, and that 1% of oxygen can be considered a

severe hypoxia condition occurring only during disuse or some pathological conditions (Arnett, 2010; Hirao et al., 2007).

However, for the intrinsic limitation derived from the use of cells line *in vitro*, in our study the two different percentage of oxygen used (1% and 5% O₂) should to be respectively considered hypoxic conditions, while the atmospheric oxygen concentration (approximately 20% O₂), in which normally cells line were established and maintained, was considered as the normal control of the study (Normoxia).

6.1 Osteocyte response to Hypoxia condition

MLO-Y4 cells showed to be sensitive to the decrease of oxygen concentration, as indicated by the positive Hypoxiprobe staining observed in hypoxia compared to normoxia condition. This staining detects and accurately measures hypoxia and oxygen gradients at the cellular level through the pimonidazole hydrochloride molecule, considered the most useful immunochemical hypoxia marker presently available. The obtained hypoxiprobe staining results indicated that different oxygen gradients occurred in the three conditions of partial pressure of oxygen used in this study (Fig. 8).

This study showed also that oxygen deprivation compromised osteocytes proliferation and viability (Fig. 9); although the expected increase in cellular proliferation over the time of culture was observed also under hypoxia conditions. The results indicate also that the conditions of 1% and 5% of oxygen decreased the cellular proliferative potential. However, only at some time points of severe hypoxia condition (1% O₂) I observed an increase of cell death compared to normoxia, indicating the very strong effect exerted by severe oxygen deprivation on the cell life.

These results also demonstrated that oxygen deprivation plays a role in inducing apoptosis in MLO-Y4 cells. Significant inductions of apoptosis were detected in both hypoxic conditions compared to normoxia at certain time points. No relation between the trigger of apoptosis and the time of culture could be found (Fig. 10). These findings support the evidence, already postulated in literature (Plotkin et al., 2005), of the role played by the oxygen deprivation in inducing osteocytes apoptosis.

The molecular response to hypoxia was investigated by looking at the expression of ORP 150, anovel endoplasmic-reticulum-associated chaperone in the osteocyte adaptation of hypoxia (Guo et al., 2010; Tamatani et al., 2001). No significant differences were observed in ORP 150 mRNA and protein expressions in the conditions investigated (Fig. 11). My results suggest that ORP150 mRNA expression was delayed in respect to the measurable induction of hypoxia via hypoxyprobe staining. Unlike other cell types (Cechowska-Pasko et al., 2006; Ozawa et al., 1999), such as neuronal cells (Kitano et al., 2004; Ogawa, 2003), renal cells (Arrington and Schnellmann, 2008) and cardiomyocytes (Aleshin et al., 2005), ORP 150 in MLO-Y4 cells didn't seem to be regulated by hypoxia at early time periods and didn't seem to be involved in the prevention of osteocytes apoptosis induced by oxygen deprivation. The western blot analysis showed that ORP 150 protein levels were higher in MLO-Y4 osteocytes compared to MC3T3-E1 pre-osteoblasts grown in normal oxygen condition. These findings were in accordance with the literature that demonstrated low oxygen tension playing an important role in differentiation of osteoblasts into osteocytes (Dallas and Bonewald, 2010; Hirao et al., 2007). Guo and collaborators showed a higher amount of hypoxia related protein in osteocytes, among which ORP 150, compared to the osteoblasts (Guo et al., 2010).

Although the marker chosen in this study for hypoxia susceptibility, seemed not involved in the process of protection during the time from hypoxia, the higher level of ORP 150

protein expressed by osteocytes compared to osteoblasts could be a possible explanation for the higher osteocyte's capacity to adapt to a hypoxic environment in comparison to osteoblasts.

6.2 Hypoxic osteocytes regulation on bone homeostasis

It is well known that osteocytes respond to the oxygen deprivation, as well as to the lack of loading, by secreting soluble signals that might modulate the formation and activity of the other bone cells (Bonewald, 2011).

One of the aims of this study was to investigate whether hypoxia condition altered the RANKL/OPG signalling axis and whether it exerted an effect on the Sclerostin and PGE₂ expression by osteocytes. Furthermore, the effects of soluble factors released by osteocytes on osteoclastogenesis and osteoblast activity were studied using the condition medium collected from hypoxic (1% and 5% O₂) and normoxic MLO-Y4 cells.

Although, it has been shown that MLO-Y4 cells support osteoclast formation through RANKL expression and that the ratio of RANKL to OPG mRNA was higher in MLO-Y4 cells than in any other cell types examined (Zhao et al., 2002), the quantification of RANKL, but also of OPG, in both hypoxic and normoxic MLO-Y4 showed very low level of these factors released in all the conditions tested (Fig. 12). There are controversial studies concerning the release of OPG by MLO-Y4 cells. Lau and co-worker and Al-Dujaili and collaborators showed that OPG protein levels were not detectable in MLO-Y4 culture media using an ELISA immunoassay (Al-Dujaili et al., 2011; Lau et al., 2010). Otherwise, Mulcahy and collaborators showed that MLO-Y4 grown in 3D collagen scaffold was able to produce about 170 pg/ml of OPG without any stimuli (Mulcahy et al., 2011); but it has been also detected the OPG in MLO-Y4 cultured in normal 2D condition by another group (You et al., 2008). Nevertheless, the data of RANKL and OPG obtained

in this study showed that the release of these factors increased with the time of culture; indeed the RANKL became detectable only after 48 hours of culture with no difference among the oxygen conditions, but the RANKL/OPG ratio remained low in all the oxygen condition tested (Table 3). These results could explain the decrease in MNC TRAP positive cells found in the different times of CMs. In fact, these showed that the inductions of osteoclastogenesis decreased over the time of the CMs collected in all the oxygen conditions (Table 4 and 5); although the decreasing observed over the times of the CMs was found significant in all the oxygen conditions, the hypoxia CMs collected at the late time setting points (24, 48 and 72 hours) showed a significant different increasing on MNCs TRAP positive cells respect to the same time of normoxic CMs (Fig. 14).

The significant differences in increase of pixel area covered by osteoclast observed in the three oxygen conditions, gave a clear indication of the effect exerted on osteoclast activation in particular by the CMs obtained from MLO-Y4 grown in severe hypoxia condition (Fig. 15). As large osteoclasts are associated with higher resorbing activity (Lees and Heersche, 2000), the data suggested that MLO-Y4 cells grown under severe hypoxia condition for 24 hours and longer, secreted soluble molecules that positively regulate the formation of osteoclasts, and potentially the amount of bone resorption.

Unfortunately, the levels of Sclerostin in all the CMs from MLO-Y4 cells were below the detection range of the ELISA kit used.

Sclerostin is considered a potent negative regulator of bone formation for its effect of inhibition of canonical Wnt and Wnt-induced BMP signalling by binding to the Wnt coreceptors LRP5/6 (Kogawa et al., 2013). The lack of Sclerostin in both hypoxic CM could be related to the stimulatory effect of hypoxia upon bone formation via reductions in sclerostin expression, to thereby mitigate its inhibitory influence upon bone formation, as previously hypothesized by Genetos and collaborators in 2010 (Genetos et al., 2010).

The activation of osteoblasts exerted by hypoxic CMs, was demonstrated by an increase of ALP activity and of mRNA β -catenin expression (Fig. 17 and 18), and potentially a lack of sclerostin. However, the reason for the absence of sclerostin release also in normoxic osteocytes remains unclear. In the literature controversial findings are reported about the absence or presence of sclerostin released from MLO-Y4 cells cultured under normal conditions. Yang and collaborators reported that MLO-Y4 cells expressed low levels of sclerostin mRNA; Papanicolaou et al. in 2009 shown that no sclerostin expression was detected in mature MLO-Y4 cells (Papanicolaou et al., 2009; Yang et al., 2009). In contrast, other studies reported that sclerostin was induced in response to different stimuli in the MLO-Y4 cell line. It has been also shown that the expression of sclerostin by MLO-Y4 cells tends to disappear after several *in vitro* passages (Baek et al., 2014; Kim et al., 2013; Mabileau et al., 2010; Vincent et al., 2009). One possible explanation for this controversy could be the differentiation stage of MLO-Y4 at which the expression of sclerostin was investigated.

The data concerning PGE₂ level demonstrated that hypoxic MLO-Y4 osteocytic cells released higher amounts of this factor (Fig. 12); although weakly, at 24, 48 and 72 hours of culture, the release of PGE₂ seemed to be increased especially in 1% O₂ of oxygen compared to normoxia condition. The observed osteoblasts activation by hypoxic CM, demonstrated viability, ALP activity and β -catenin mRNA (Fig. 16, 17 and 18), could be related to PGE₂ released by hypoxic MLO-Y4. In fact, it was reported that MLO-Y4s exposed to some type of stress increased the production of PGE₂ and this soluble factor is able to crosstalk and positively regulate the β -catenin signalling, the principal pathway of osteoblast activation (Castellone et al., 2005; Kamel et al., 2010).

Chapter 7. CONCLUSIONS

This project aimed to better understand the mechanisms in which osteocytes are involved when exposed to hypoxia, postulating that oxygen flux was decreased by bone disuse/unloading.

This study has produced many results, although not always positive or in the expected direction. However, it lays the basis to increase the knowledge of the complex pathways involved in the osteocytes response to deprivation of oxygen.

Firstly, it was demonstrated that hypoxia compromised osteocyte viability and induced apoptosis.

ORP150, was suggested in the literature as protective factor against hypoxia and oxidative stress. Yet, according to the findings presented here this seems not be the case in MLO-Y4 cells grown short term under severe oxygen deprivation. In fact, ORP 150 was highly expressed in osteocytes as compared to osteoblasts but it was not elevated with hypoxia compared to normoxia condition up to 72 hours of culture. It could be hypothesized that the amount of ORP 150 in MLO-Y4 was already enough at baseline to protect the cells from hypoxia for up to 72 hours, while, probably, a longer exposure to oxygen deprivation could lead to an upregulation of ORP150 expression.

Moreover, this study strengthens the role of osteocytes as principal orchestrator of bone homeostasis. In fact, severe osteocytes hypoxia lead to increased osteoclastogenesis with a mechanism probably not strongly dependent of RANKL/OPG pathway. It is possible to hypothesize that the increase of osteocytes apoptosis induced by oxygen deprivation, could regulate the osteoclasts formation through a RANKL/OPG independent mechanism, such as by release of apoptotic bodies (Jilka et al., 2013). Furthermore, this study has shown that hypoxic osteocytes positively regulate the activity of osteoblasts with a mechanism

probably related to the PGE₂ crosstalk with β -catenin, while Sclerostin seemed to be not involved in the regulation of bone homeostasis exerted by hypoxic osteocytes.

In summary my results suggest that, differences existed between the 1% and 5% of oxygen concentration; with 1% O₂ being the severe hypoxia state showing the strongest regulatory effects.

Our study presents several limitations:

I) It was based on the postulation that bone unloading/disuse necessary caused a low supply of oxygen up to osteocytes lacunae, although this condition was demonstrated only at the bone marrow level (Stevens et al., 2006; Zhang et al., 2007); up to the present, it is still not available a technology to detect the actual percentage of oxygen occurring inside the osteocyte lacunae, due to their small dimensions ;

II) In some cases the data leaves room for speculation. Yet, we firmly think that any part of the complex pathway involved in the cross talk existing among bone cells in our study conditions, can increase the global knowledge of this field.

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