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Bone texture modifications and osteocyte cell-signaling changes occurring during bone regeneration in response to treatment with Teriparatide

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ABSTRACT

The purpose of this thesis is to analyze the structural bone tissue modifications and the alteration of osteocyte cell-signaling during the repair of experimentally induced bone lesions (*in vivo*) and in an immortalized cell model (*in vitro*) under the treatment of the osteo-protective drug Teriparatide. Teriparatide is the active fragment (1-34) of the endogenous human parathyroid hormone (PTH). The physiological effect of PTH is to induce hypercalcemia: this effect is partially achieved by increasing both the calcium intestinal absorption and the renal tubular reabsorption; however, under non-physiological conditions the hypercalcemia is mainly resulted by the increase of bone resorption. Many studies showed that chronic administration of high doses of PTH results in decreased bone mass while intermittent exposure to PTH activates osteoblast bone deposition. In particular, it has been shown that intermittent exposure to PTH increases bone density by stimulating osteogenesis; whereas continued exposure to the same hormone causes strong bone reabsorption. Most of the structural studies published so far about the effect of Teriparatide focused their attention on the amount of the newly-deposited bone without investigating the quality of the newly-formed bone tissue; moreover most of the papers enhance the attention to the osteoblast's cellular and molecular involvement during the process of bone repair, ignoring the pivotal role that the osteocytes play in the bone environment by means of the modulation of their signaling. In many previously published papers, it has been shown that osteocytes are the main actors of the communication among bone cells; in particular, they are in close contacts with osteoblasts through cytoplasmic extensions and specialized gap-junctions, indicative of their guide role. Bone regeneration retraces the sequence of events occurring in membranous ossification: after a

preliminary formation of fibrous tissue and the successive vascular proliferation, two different types of osteogenesis follow: firstly the process of Static Osteogenesis (SO), which produces preliminary bony trabeculae with woven texture, under factors/cytokines of vascular origin, and later the process of Dynamic Osteogenesis (DO), in which osteoblasts, driven by osteocytes, produce a more ordered (mainly lamellar) bone, more valid by the mechanical viewpoint. At the present state of knowledge, there are no scientific references focused on the functional relationships among osteocytes and osteoblasts during the bone repair process.

This doctoral work focused, at first, on the morphological analysis of the qualitative repair of bone lesions experimentally induced in diaphysis of rats femurs and then on the study of the osteocyte cell-signaling, both in response to the treatment with Teriparatide in order to deepen the knowledge of morphological and molecular events that occur in bone regeneration, with particular attention to the osteocyte signaling. The results obtained showed that intermittent administration of Teriparatide in the animal model anticipates the beginning of Dynamic Osteogenesis, which is characterized by the production of a more resistant bone. The molecular biology evaluations were performed on an osteocyte immortalized cellular model (MLO-Y4) treated with the drug at different time points. Microarray analysis showed different expression of genes involved in osteocyte signaling compared with the control condition. These results have encouraged us to plan new and more focused analysis of proteomics and to use a new bone cell line (IDG-SW3). This cell line is capable of going through a complete differentiation from pre-osteoblast to late osteocyte, allowing us to better understand the signaling modifications that may occur during the treatment with Teriparatide.

INTRODUCTION

Bone tissue

The bone tissue is a specialized connective tissue formed by mesenchyme-derived cells and calcified intercellular matrix. It is the mostly represented tissue in skeletal segments, highly vascularized rigid organs, having multiple functions: support for muscles, locomotion, protection of vital organs and soft tissues; moreover, they represent the biggest reserve of calcium in the body, thus contributing to the mineral homeostasis (i.e. the roughly constant balance of serum calcium and phosphate ions).

The fibrillary component of the organic intercellular matrix of bone tissue, called osteoid, is mainly made up of Type 1 collagen fibers, that represent about the 30% of the weight of the total bone tissue. In a smaller fraction, even type III and V collagen can be found in adult bone. Many authors (Beraudi et al. , 2010; Bou-Gharios & De Crombrugghe, 2008; G. Marotti et al., 1995; Nair et al., 2013) already demonstrated that the organization of collagen fibers is essential for the bone strength and mechanical properties. Other proteins such as: glycoproteins, glycosaminoglycan, fibronectin, proteoglycans, osteopontin, osteocalcin, sclerostin, RANK, alkaline phosphatase, bone sialoprotein, dentin matrix protein 1 and parathyroid hormone receptors are highly expressed in different bone cells and are involved in many different bone processes such as: bone growth, regulation of bone matrix organization, regulation of bone resorption or deposition, cell attachment and proliferation, various molecular pathways, cellular signaling, mediation of hydroxyapatite deposition and regulation of mineralization, as widely explained by Ritter et al. (1992) and Gorski (2011). The

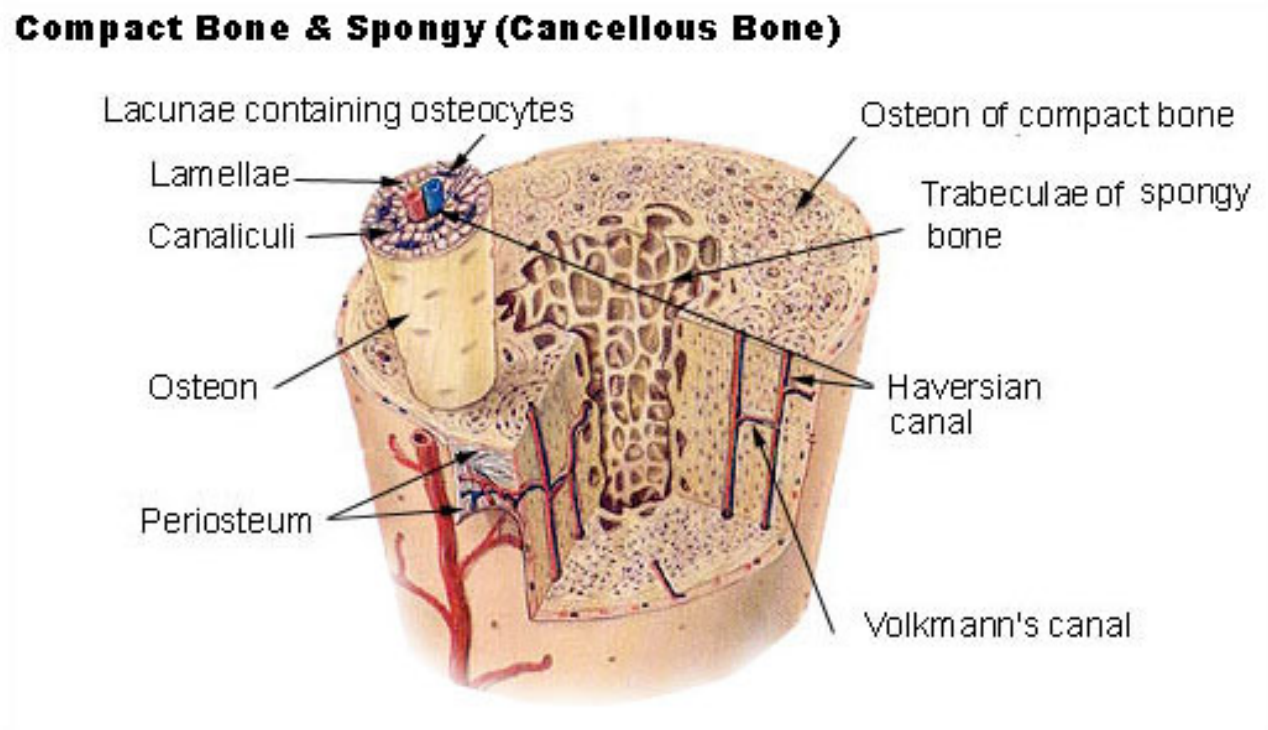
remaining 70% of the total bone weight is characterized by inorganic tissue, particularly calcium and phosphorus, which combined in crystalline precipitations form hydroxyapatite.

In bone segments, two kinds of architectures are macroscopically identifiable: cortical and trabecular bone. Cortical bone, also known as compact bone, is extremely dense, hard, with low porosity and constitutes mainly the diaphysis of long bones. Trabecular bone, also known as cancellous bone, has various degree of high porosity and mainly constitute the epiphysis of long bones as well as flat and isodiametric bones.

By the microscopic point of view, in adult subjects, the bone tissue consist of cylindric systems, the osteons also named Haversian systems. Each osteon is composed of concentric lamellae, about 3 to 7 μm of thickness, made up of collagen fibers that regularly embed hydroxyapatite and other mineral crystals. The center of each system is path longitudinally from a channel containing one or more vessels perpendicular to smaller channels called Volkmann canals; the interconnection of those two type of channels accommodate small arteries, arterioles, capillaries, and venules of the microcirculation system (Buckwalter & Cooper, 1987). Concerning the cancellous bone, it has wider vascular surfaces with respect the compact one, and the lamellar systems are less organized inside the trabeculae (Kragstrup & Melsen, 1983) containing less osteons and rare Volkman channels. Cancellous bone constitutes a three-dimensional, interconnected network of trabecular rods and plates filling the epiphysis and metaphysis of long bones as well as flat and isodiametric bones, like sternum and vertebrae, respectively.

The bone tissue, forming the skeleton, is a dynamic tissue that is constantly being remodeled in response to alterations in physical activity, dietary calcium levels, hormonal changes, and local paracrine signals within the bone microenvironment. As reported by Sims & Walsh (2012), bone remodeling is a fundamental mechanism of removing and replacing bone tissue during adaptation of

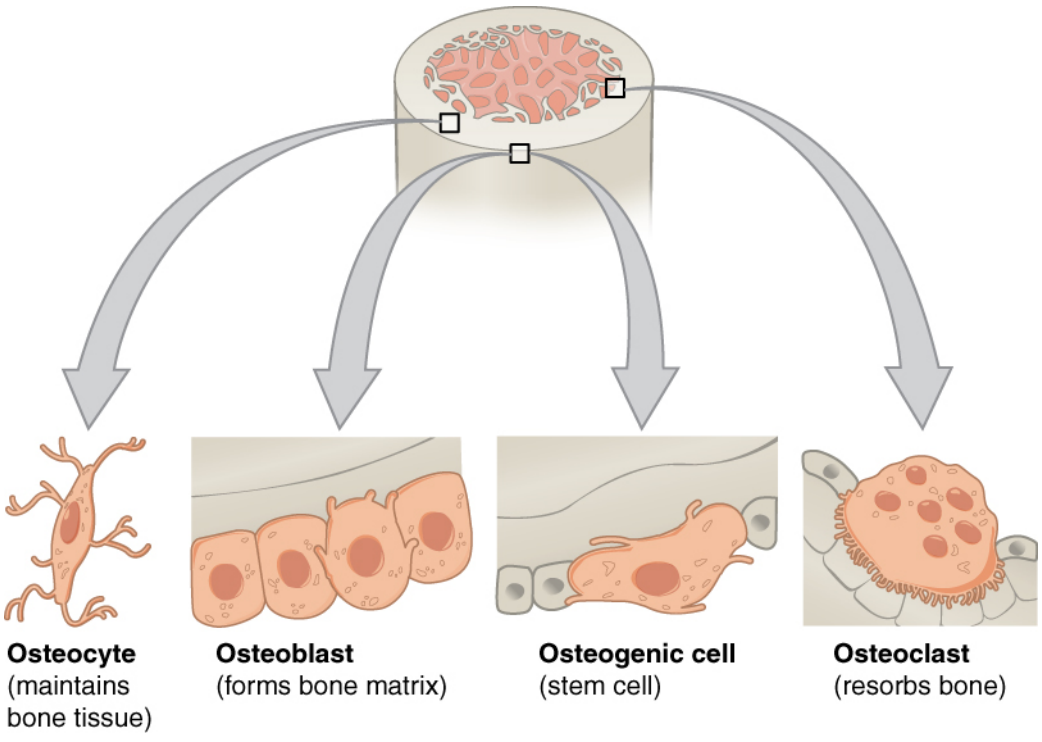
the skeleton that occurs through all the time life in maintaining the mechanical and mineral homeostatis, and this homeostasis is maintained via a balance between bone resorption and bone formation.



Compact & Spongy bone - SEER - U.S. National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) Program

Bone cells

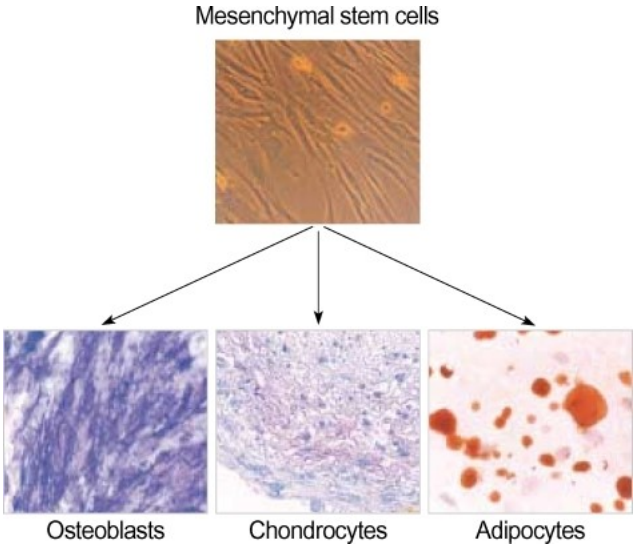
The bone cell population consists of two distinct lineages: the osteogenic lineage (including stromal cells, pre-osteoblasts, osteoblast, osteocytes and bone lining cells) and the osteoclastic lineage (represented by osteoclasts). The maintenance of normal bone structure is driven by the intercellular cross-talk that occurs among bone cells. As proposed by many authors (Dodds et al., 1993; Marotti & Palumbo, 2007; Pead et al., 1988; Skerry et al., 1989) and largely demonstrated by Bonewald (2011) and Nakashima et al. (2011), osteocytes are considered the mechanosensors of bone; they are actively involved in the orchestration of both bone-forming osteoblasts and bone-resorbing osteoclasts. Osteoblasts derive from mesenchymal stem cells and, probably based on a predetermined fate, can become osteocytes or lining cells.



Bone cells - Anatomy & Physiology, Connexions Web site

An overview on Mesenchymal Stem Cells and Osteoblasts

According to the most recent paperwork, mesenchymal stem cells (MSC), derived from both the bone marrow (BMSC) or the small fraction derived from adipose tissue (ASC), have the capability to differentiate into three specific cell lines: osteogenic, adipogenic, and neurogenic. This differentiation results to be modulated by numerous factors.



Co-transplantation of human mesenchymal stem cells promotes human CD34+ cells engraftment in a dose-dependent fashion in NOD/SCID mice, Park et al. 2007

It has been known for a long time that Bone Morphogenetic Proteins (BMP), a pool of more than 15 proteins (in humans) that belongs to the TGF-β superfamily, play an important role in stem cells proliferation and differentiation, but, more importantly, different studies showed that BMP play an important role in osteoblastic differentiation and bone formation. Kang et al. (2009) showed that the activity of alkaline phosphatase (ALP) can be improved by overexpressing BMP2, 4, 6, 7, and 9 in a murine BMSC cell line. Chuang et al. (2007) showed that the early gene introduction of BMP2

promoted the ALP activity and mineralization in human BMSCs. A similar result was found in a study on the gene introduction of BMP2 to Dental Pulp Stem Cells (DPSCs) by Li et al. (2010) which investigate the repair of a critical size damage in a jaw of a rabbit animal model using gene-introduced stem cells. The animals were divided into three groups: the first group was implanted onto the nano-hydroxyapatite/polyamide (nHA/PA) scaffold with BMP7-introduced BMSCs; the second group were implanted with the scaffold containing only BMSCs and the third was the control group where only the scaffold was implanted. Excellent-quality of the bone tissue by the mechanical viewpoint has been showed in the first group after both 4 weeks and 8 weeks compared with the other two groups; those differences disappeared after 16 weeks, proving that the introduction of BMP7 could promote the bone formation in early treatments after bone damage. In many ongoing research studies, other genes have been introduced with BMP for the modulation of osteoblastic differentiation for effective bone regeneration. Some reports showed that in order to promote the osteoblastic differentiation and bone regeneration not only needs the gene introduction of different BMPs such as BMP6/7, but also other factors like VEGF and angiogenesis.

Related with BMPs differentiation, Liu et al. (2011) demonstrated that overexpression of Angiopoietin1 (Ang1) in BMSCs could stimulate their osteogenic differentiation increasing the Alkaline Phosphatase (ALP) Activity. Already Machein et al. (2004) found Ang1 to be a very effective growth factor which promotes the regeneration of capillary vasculature.

Another pivotal protein in bone differentiation and regeneration is Runx2, a major transcription factor that is essential in osteogenic development and modulation of differentiation as demonstrated by Pan et al. (2010), which transfected a full-length Runx2 and a mutant, non-functional, Runx2(M) in BMSC showing a high differential expression of genes related to the differentiation of osteoblast between the two experimental groups. Among all the genes it is important to underscore that

Runx2(M) group showed high expression of osteocalcin (OC), osteopontin (OPN), collagen I (Col I), and cementum protein 23 (CP23) compared to the Runx2 group; all those genes are strictly connected with the cell differentiation. Since Runx2 is regulated via BMP signaling, its over-expression in murine BMSCs increased osteogenic differentiation during BMP-modulated osteoblastic differentiation, *vice versa* mice with the mutant Runx2 reduced the bone formation.

Aquaporins are integral membrane proteins from a larger family of major intrinsic proteins (MIP) that form pores in the cell membrane, often called water channels. There are 13 known types of Aquaporins and among them Aquaporin 1 to 4 are the most studied, however recently Aquaporin-5 has been associated with many different roles in a multitude of tissues. Yi et al. (2012) showed an increased differentiation capability in BMSCs when Aquaporin-5 was silenced observing a significant difference in bone mineralization, ALP activity, in vivo bone healing, gene and protein expression of different bone differentiation markers (such as CBFA1 the protein encoded by Runx2 gene).

FGFs are a family of grow factors implied in various endocrine signaling pathways in many different cell lines and tissues. Among all the proteins of this family, one of the first factor to be underlined is the Basic fibroblast growth factor (also known as FGF2) that has been showed to promote the healing of acute and chronic injuries and promote the differentiation of BMSCs into osteoblasts. The receptor of this family is FGFR and its bind with the related proteins seems to activate and promote many different signaling pathways including those involved in the proliferation and differentiation of osteoblasts. One of the most explicative experiment involving FGF family, and more important FGF2, was conducted by Oh et al. (2012) which cultured BMSC with a medium containing recombinant FGF2 and performed comparisons with control cells. The result was an increase of expression of those factors connected with the osteoblast differentiation

such as Col I, OPN, BSP, and OCN in BMSCs cultured with FGF2 and an increase in mineralization if compared with controls.

Tumor Necrosis Factor (or TNF-α) is a cytokine mainly produced by activated macrophages involved in systemic inflammation, cell differentiation, proliferation, and as already been demonstrated (Cho et al., 2010; Hesset al., 2009; Huang et al., 2011; Paula-Silva et al., 2009) is involved in osteoporosis pathology. Moreover, as a factor in charge of catabolism, it promotes the activity of osteoclasts but counteracts the activity of osteoblasts. Up to now, the effect of TNF-α on MSC has been largely studied, however papers published so far report a different behavior with a very wide panel of results of this molecule depending on the time and the manner of administration. For example Li et al. (2007) reported that TNF-α suppressed the osteogenic differentiation of mesenchymal stem cells, meanwhile Hess et al. (2009) claimed that the osteogenic differentiation of human BMSCs was promoted by TNF-α. Those authors proposed two different mechanisms on which TNF-α was acting. Finally, Huang et al. (2011) demonstrated that, depending on the concentration, the effect of TNF-α could absolutely reverse; they also demonstrated that the effect of the stimulation with TNF-α changes overtime. In particular, when TNF-α is added for a short time there is osteoblastic differentiation of MSC but the effect is the opposite when TNF-α is added for long time.

Some other paracrine factors produced by osteoblasts that has been showed to influence positively osteoclast formation are ParaThyroid Hormone related Protein (PTHrP), Interleukin (IL)-1beta and IL-6.

Transforming growth factor beta (or TGF-β) is a secreted protein that controls proliferation, cellular differentiation, and other functions in many cell lines, including macrophages, but its effect is localized mostly in the bone matrix. This protein may play two opposite roles as well as TNF-α:

promoting early osteoblastic differentiation and obstructing late osteoblastic differentiation and mineralization. As showed by de Gorter et al. (2011) when TGF- β receptor is silenced in murine MSCs the BMP-6-induced ALP activity decreases. They also showed that after 16 days of cell treatment with TGF- β, depending on which kind of treatment used (continuously or intermittent), the effect could be either to promote the differentiation or to block it. This suggests that TGF-β can promote or suppress osteogenic differentiation according to the culturing condition, period, and duration.

A protein often associated to bone metabolism is Leptin, which mostly is known to play the role of adjusting food intake. What is less known about this protein is that it is encoded in the Ob gene and it is secreted by osteoblasts during bone formation with the effect of suppressing osteoclast activity, which lead to a reduction of bone resorption and a promotion of bone mineralization (Ducy et al., 2000; Umet al., 2011; Zhang et al., 2009).

Recently, many other experiments identified several factors that induce osteoblast differentiation, such as Wnt, or inhibit osteoblast differentiation, like Dlk1/Pref-1 or Noggin. An emerging concept is that some genes such as dlk1/FA1 could modulate the expression of several pro-inflammatory cytokines, which could lead to a differentiation via controlling the composition of the micro-environmental ‘‘niche”.

Among the Wnt family, the focus should be given to Wnt5a that seems to have been implicated in oncogenesis and in several developmental processes, including regulation of cell fate and patterning during embryogenesis (Peng et al., 2010). Recently, the journal Nature published an article of Okamoto et al. (2014) aimed to demonstrate that Wnt5a-deficient mice exhibited reduced Wnt/β-catenin signaling, which impaired osteoblast differentiation and enhanced adipocyte differentiation. Later, Yu et al. (2015) showed that TRIB3 seems to modulate the sustained

expression of Wnt5a in osteoblasts stimulated by some lipopolysaccharides found in periodontal and periapical diseases, meanwhile other reports have described that TRIB3 is critically involved in a novel negative feedback control pathway of NF-κB-induced gene expression (Duggan et al., 2010).

The role of Pleiotropin (PTN), also called osteoblast-specific factor 1, has been studied for over 70 years. Recently Lamprou et al. (2014) published a review that summarize the existing evidence on the role of PTN in bone repair going through over 200 published articles. Apparently, PTN plays a complex role in bone repair; it seems to promote endothelial cells proliferation, to down-regulate the stimulatory effect of VEGF, to stimulate in vivo angiogenesis and to have chemotactic effects on both human osteoblastic and endothelial cells. Moreover, it was been shown that PTN serum levels are related with fracture healing. In a recent clinical study on 530 postmenopausal women, with and without hip fractures (Mencej-Bedra et al. 2011), a PTN gene promoter polymorphism could contribute to the genetic background of osteoporosis, affecting bone mass density values in the lumbar spine and in the femoral neck. The relevance of PTN implication in skeletal homeostasis and diseases is currently under investigation. PTN is thought to affect osteoblast rather than osteoclast functions; however, the mechanisms involved are not yet fully elucidated.

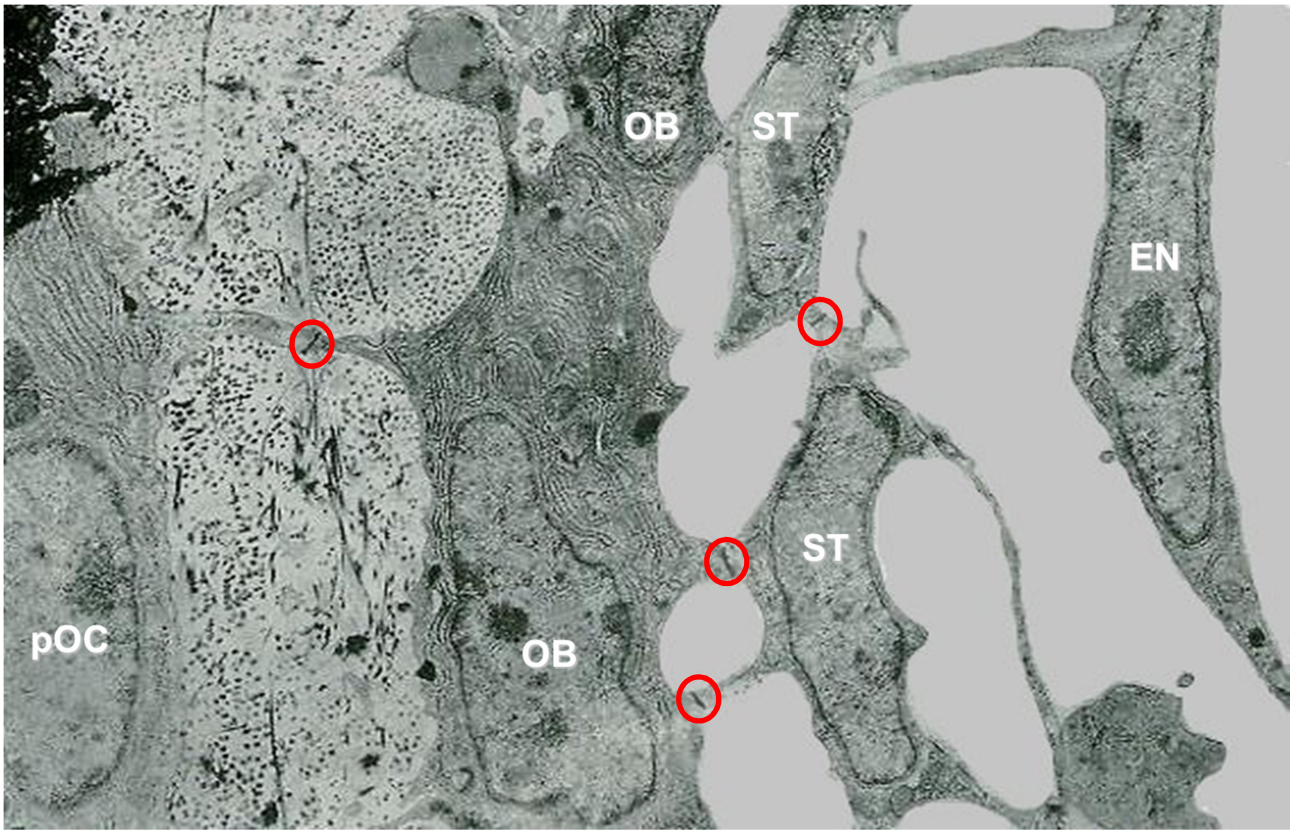
Plasminogen Activator Inhibitor-1 (PAI-1 or Serpine1) is the principal inhibitor of plasminogen activator; Daci et al. (2000) reported that PAI-1 deficiency partially protects against bone loss in estrogen-deficient mice, meanwhile Tamura et al. (2013) demonstrated that PAI-1 deficiency protects against diabetic bone loss in female mice; finally Mao et al. (2014) showed that PAI-1 is involved in the impaired bone repair process induced by the diabetic state in part through a decrease in the number of ALP-positive cells, and that PAI-1 deficiency restores bone formation by promoting the differentiation of mesenchymal stem cells into osteoblasts instead of adipocytes.

Connective Tissue Grow Factor (CTGF or CCN2) is part of the CCN protein family and it has important roles in many biological processes, including cell adhesion, migration, proliferation, angiogenesis and skeletal development as already demonstrated by Wang et al. (2009). Recently, Aoyama et al. (2015) in a complete and well described article found a novel role of CCN2 as an OPG inhibitor; as the authors show in their article, CTGF has the ability to bind RANK enhancing his effect, as already suggested by some other authors (Shuiet al., 2002; Takigawa, 2013), but at the same time to block directly OPG, enhancing a RANKL-induced osteoclast differentiation. Aoyama et al. (2015) proposed CTGF as a candidate of the fourth key molecule working in RANK/RANKL/OPG signaling.

Another gene of the CCN family recently related with bone differentiation is Wisp2 (or CCN5) which encodes for the WNT1-inducible-signaling pathway protein 2. In a study on dentinogenesis, Sagomonyants & Mina (2014) showed that continuous exposure of primary dental pulp cultures to FGF2 during the proliferation phase were associated with increased expression of the components of the Wnt (Wisp2, Wnt10a) and BMP (Bmp2, Dlx5, Msx2, Osx) pathways, and decreased the expression of an inhibitor of the Wnt signaling, Nkd2; the paper brought new evidences about the relation of Wisp2 with the differentiation of primary dental pulp cultures into odontoblasts and the correlation with the FGF2 stimulation.

The Osteocyte

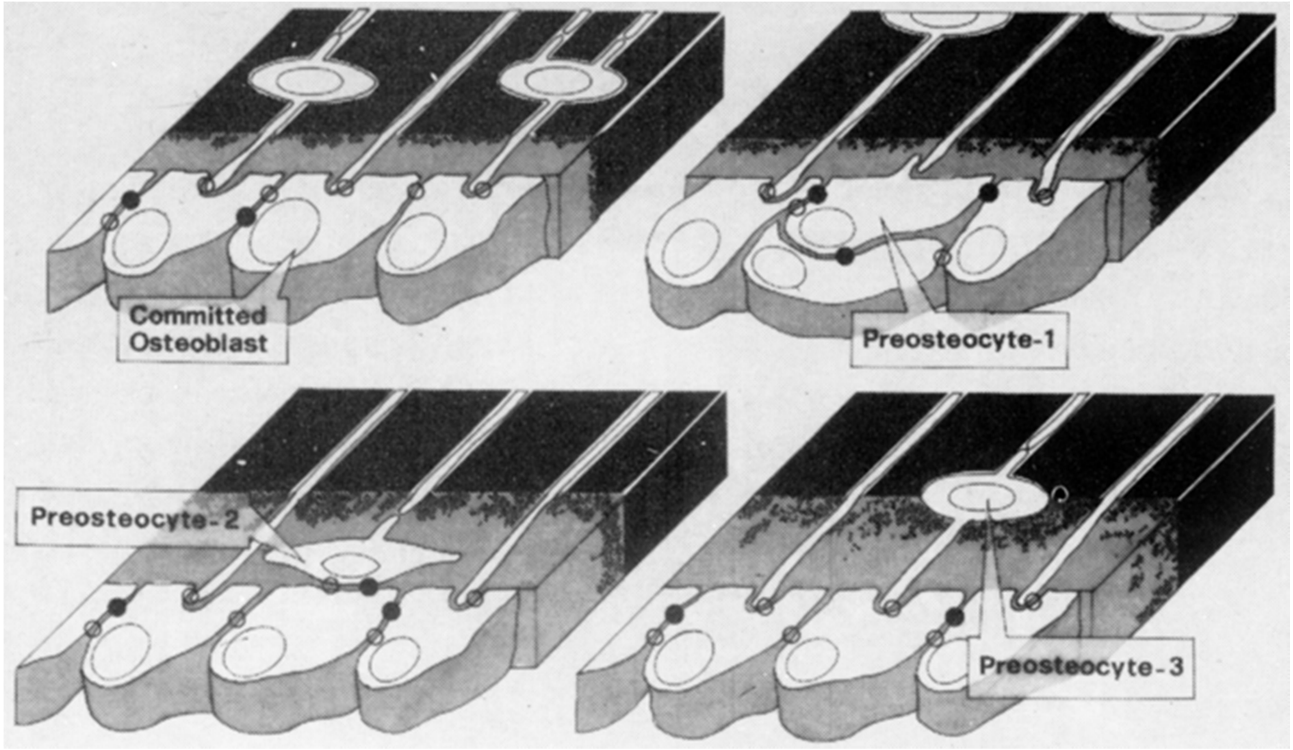
Osteocytes are the permanent and most numerous cells in the bone tissue, with a life span up to 25 years. They came from osteoblasts, which derive from a specific differentiation of MSC. Osteocytes are dendritic cells easy to identify in a bone section since they are embedded in the bone matrix inside spaces called *lacunae*. According quantitative estimation, their cell surface could be 400-fold larger than the total Haversian and Volkmann system, and more than 100-fold larger than the trabecular bone surface. Each osteocyte possesses up to 50 long and branched cytoplasmic processes that extend throughout the bone inside a network of interconnecting *canaliculi* that put in communication adjacent osteocyte lacunae; inside canaliculi the cytoplasmic processes can communicate each other by means of gap junctions (Marotti, 2000; C. Palumbo et al., 1990) in order to exchange molecular signals. Another interesting communication path is achieved by the interstitial fluid (Marotti & Palumbo, 2007) that flows through the osteocytes canaliculi. This network is essential in coordinating the response of bone to mechanical and biological signals.



Connections through gap junctions (red circles) among bone cells (pOC = pre Osteocyte, OB = Osteoblast, ST = Stromal cell, EN = Endotelial cell)

The embedding in which the osteocytes are buried makes them really difficult to be isolated and to be studied; this is one of the reasons why for a long time researchers preferred to focus their attention on the more accessible osteoblasts, located on the bone surfaces. Moreover, for a long time osteocytes were considered “sleeping cells” without any active role in bone physiology. Thanks to the development of new technologies, such as electron microscopy, new animal models, cell lines, molecular imaging and bio-informatic approaches, we are now able to perform new studies which may show us much more about these amazing cells.

In a first and old review, Franz-Odendaal et al. (2006) indicate how osteocytes could originate from osteoblasts. In their review, they were able to propose five recognizable stages of osteocyte differentiation: type I preosteocyte, type II preosteocyte, type III preosteocyte, young osteocyte and old osteocyte. Those different stages were identifiable thanks to the morphological analysis of the ultrastructure of the osteocyte during bone formation, in which an osteoblast subpopulation starts its differentiation route to become functionally osteocytes. Palumbo et al. (1986 and 1990b) clearly described the successive steps of osteoblast-to–osteocyte transformation: the first step of the osteocyte differentiation is characterized by its location inside the osteoblastic lamina (type I preosteocyte); then the body is completely encased in the osteoid seam (type II preosteocyte). Once the cell body is totally detached from the osteoblastic lamina the osteocyte starts to reduce its size, which consequently shows higher nucleus-to-cytoplasm ratio; the cytoplasmic processes start to emerge from the cell body according the spreading of osteoid mineralization. At this stage, the endoplasmic reticulum and Golgi apparatus are reduced whit a consequent decrease in protein synthesis and secretion. This latter differentiation stage could be further divided in 2 steps, the first when osteocyte matrix is not completely mineralized (type III preosteocyte) and the second when the matrix is mineralized and the young osteocyte is formed.



Representation of the 3 different types of Preosteocytes – Structure-function relationship in the osteocyte, Marotti et al., 1990

At the stage of mature osteocyte, many of the previously expressed osteoblast markers, including OCN, BSPII, collagen type I and ALP are downregulated or switched off, while osteocyte markers, such as dentine matrix protein 1 (DMP1) and Sost are highly expressed. Studies on osteoblast-to-osteocyte differentiation were extensively performed during last decades and many new information were added to our knowledge of bone cells, such as that the amount of time required for a complete differentiation *in vivo* is about three days or the fact that the synthesis of the extracellular matrix triplicate its own volume during this time. In the most recent years many studies have shown that, according to the type of bone formed, the newly embedded osteocytes may be different in size and shape if compared with older, more mature osteocytes already embedded in bone matrix. As an

example, in primary bone, which is formed rapidly with randomly oriented collagen fibers, the osteocytes show spherical-like shape, while in the lamellar bone osteocytes are ellipsoidal and polarized towards the vascular surfaces. Our group of research to further investigate this topic performed many experiments demonstrating that the two different types of bone texture (woven and lamellar) derived from two different steps of osteogenesis, occurring in sequence during bone formation: firstly Static Osteogenesis (SO) and later Dynamic Osteogenesis (DO) (Ferretti et al., 2002) which will be described below in a specific section. Once the osteocyte achieves maturation and is completely embedded inside the bone mineralized matrix it starts to communicate with other cells by gap junctions, as previously described. Their shape, distribution and spatial organization are consistent with their sensing and signaling functions. Thanks to the many studies published in the last decade, it has been accepted the concept that osteocytes are involved in the transduction of mechanical stimuli into biochemical signals and can orchestrate bone formation and bone resorption acting on osteoblast and osteoclast recruitment depending on different mechanical and metabolic conditions (Pead et al., 1988). However, the role of osteocyte in osteoblast regulation under physiologic conditions is controversial and not fully characterized. Some evidences indicates that osteocytes negatively regulate osteoblast differentiation and bone tissue. Many investigations demonstrated that *in vivo* target ablation of osteocytes severely reduces bone formation (Tatsumi et al., 2007) and many recent studies correlated the survival of the osteocytes with the success of prosthesis osseo-integration increasing the adhesion with the bone surface and reducing the resorption of the adjacent bone (Albertini et al., 2015).

One of the most important markers of the osteocyte seems to be E11 (also known as podoplanin), which seems to regulate the morphology of the late osteocyte (Schulze et al., 1999; Wetterwald et

al., 1996). E11 was firstly detected on the cell surface of osteocytes in rat bone and odontoblasts in rat teeth (Zhang et al. 2006; Schwab et al. 1999). Studies tried to correlate the effect of E11 with the formation of new dendrites; in fact, blocking E11 on osteocyte-like MLO-Y4 cells it results a reduction in growth of dendrites. *In vivo* studies showed that the conditional deletion of the protein in bone cells resulted in decreased canaliculi and increased trabecular bone (Guo et al. 2009)

In addition to E11, organized expression of tubulin, vimentin, and actin in osteocyte cell bodies and dendrites is crucial to maintain their dendritic morphology (Tanaka-Kamioka et al., 1998).

Differences in distribution of fimbrin, villin, filamin, and spectrin have been described to be expressed during the differentiation from osteoblast to osteocyte (Kamioka et al., 2004), as well as CapG and destrin, molecules necessary for cytoskeletal rearrangement (Guo et al., 2010).

One of the proteins that better follows the differentiation from osteoblast to osteocyte is Alkaline Phosphatase (ALP), which is reduced during the process; totally opposite behavior has the casein kinase II which increases during differentiation likewise osteocalcin (Mikuni-Takagaki et al., 1995). Additional markers are expressed on osteocytes, including phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX), matrix extracellular phosphoglycoprotein (MEPE), dentin matrix protein 1 (DMP-1), fibroblast growth factor 23, (FGF-23), sclerostin, and ORP150 (a factor that is thought to be protector against hypoxia).

Surprisingly, osteocytes can also express markers of osteoclasts, such as acid phosphatase and cathepsin K, under certain conditions such as lactation to remodel their perilacunar matrix (Zhao et al., 2002).

RANK ligand (RANKL) has been shown to be expressed on dendritic processes. The expression of this factor lead to the conclusion that osteocytes may play a role in orchestrating bone resorption

through osteoclast activation (Wijenayaka et al., 2011). Conditioned medium from the MLO-Y4 cells also supports osteoblast differentiation and, surprisingly, mesenchymal stem cell differentiation (Zhao et al., 2002), supporting the theory that osteocytes could be orchestrators of bone remodeling.

Another important aspect of osteocytes is their capability to stimulate the bone remodeling even during their death process. Dying osteocytes are in fact able to recruit osteoclast and to produce both pro-apoptotic and anti-apoptotic molecules (Kogianni et al., 2008). When an osteocyte starts the apoptotic process, it releases apoptotic bodies expressing RANKL in order to recruit osteoclasts. However, few is known about necrosis and differences that may occur between those two processes. In fact, many of the papers published in available literature use the term of cell death to describe the process of osteocyte ablation. Regardless of the kind of cell death, many studies demonstrated that ablation of osteocyte, caused either by toxin injection (Tatsumi et al., 2007) or by deletion of b-catenin in a mouse model in vivo (Kramer et al., 2010) – in which animal's osteocyte death cause a phenotype with a ''moth-eaten appearance''-, leads to an increase of osteoclast recruitment with a consequent generation of a porous bone phenotype followed by an increased skeletal fragility. Osteocyte cell death can occur in association with pathologic conditions as osteoporosis and osteoarthritis or with menopause, leading to an increased skeletal fragility characterized by micro-fractures. Such fragility is to be considered due to loss of the capability to sense micro-damage by the osteocyte and/or to perform signaling for bone repair.

A not commonly associated gene to osteoporosis is SCARA3 which in a study conducted by Balla et al. (2009) on osteoporotic and not-osteoporotic bone segments showed a differential expression between the two groups, proposing SCARA3 as a novel gene for the study of osteoporosis.

SCARA3 is a scavenger gene that may bind exogenous ligands, e.g. peptide nano-complexes (Ezzat et al., 2012) and protect against oxidative stress (Han et al. 1998).

A commonly used method to induce osteocyte apoptosis is the administration of glucocorticoids. Recent studies demonstrated how mechanical loading can block the glucocorticoid-induced apoptosis through the release of prostaglandin, which activated the Wnt/b-catenin pathway (Kitase et al., 2010). Other studies hypothesized that glucocorticoids may have an opposite effect to mechanical loading because of the effect that mechanical loading and prostaglandins have on the family of focal adhesion kinases (FAKs) and the proline-rich tyrosine kinase 2 (Pyk2) (L I Plotkin et al., 2005; Lillian et al., 2007).

In the last two decades, various researchers pointed out the physiological effect of autophagy as a mechanism of self-preservation in many different cell types. On bone field, and specifically on osteocyte, few has been described about this mechanism; however, autophagy seems reasonably to be a valid mechanism that may preserve the cell survival, maintaining bone homeostasis and integrity (Xia, et al., 2010).

Negative regulators of the Wnt/b-catenin pathway such as Dkk1 and sclerostin are highly expressed in osteocytes. Dkk1 is expressed throughout the body, but sclerostin is expressed mainly in osteocytes.

Sclerostin, a protein encoded by the SOST gene, is one of the most typical markers of mature osteocyte because of its specificity for this cell type. Among with Dkk1, SOST gene seems to regulate the Wnt pathway. In a first time Sclerostin was believed to be an antagonist of BMP proteins family but recently was demonstrated that is actually an antagonist against lipoprotein receptor 5 (LRP5), a positive regulator of bone mass (Li et al., 2005; van Bezooijen et al., 2004);

that's why mutations of SOST gene causes high bone mass in humans (Collette et al., 2012). Interestingly, it has been reported that mechanical loading reduce sclerostin expression, as well as parathyroid hormone (PTH), which may account for some of the anabolic effects of PTH on bone formation (Bellido et al., 2005; Keller & Kneissel, 2005; Robling et al., 2008).

To better understand the topic, MLO-Y4 cells were mechanically loaded using the fluid-flow shear stress technique. The effect of this mechanical loading was to protect the cells against dexamethasone-induced apoptosis, and the mechanism for this protective effect appeared to be mediated through prostaglandin E2 (PGE2) crosstalk with b-catenin signaling. The mechanism of action was described as follows: shear stress, through PGE2 release, activates both PI3K/Akt and cAMP-PKA signaling, which converge to inactivate GSK-3, leading to the increase in nuclear accumulation of b-catenin (Bonewald & Johnson, 2008; Kamel et al., 2010). Moreover, b-Catenin has been showed to bind the Cx43 promoter, stimulating Cx43 expression and functional gap junctions between osteocytes (Xia et al., 2010); this fact ascribes to this protein another important role in osteocyte communication. Surprisingly, it has been showed that osteocytes express RANKL and OPG at levels comparable with or exceeding those of osteoblasts. These data support previous findings that osteocytes recruit osteoclasts and that osteocytes are key regulators of bone homeostasis.

Osteocytes appear also to regulate phosphate and mineralization through molecules such as PHEX, DMP-1, MEPE, and FGF-23 which are all highly expressed in osteocyte (Bonewald 2007; Thompson et al. 2002; Nampei et al. 2004; Liu et al. 2006). Other suggestions about the activity of the osteocyte in bone were given by many different scientists in their studies. In 1967, Belanger and coworkers coin for the first time the term "osteocytic osteolysis" suggesting that osteocyte was able to remodel the lacunar wall lysing its peri-lacunar matrix under the stimuli either of PTH or low-

calcium diet. Another evidence in support of the osteocyte-mediated osteolysis, was obtained from the histomorphometric analysis of human iliac crest bone biopsies of patients affected by hyperparathyroidism *versus* patients affected by hyperthyroidism. This analysis revealed that PTH, but not thyroid hormones stimulated periosteocytic osteolysis. Later, both Baylink et al. (1971) and Zambonin-Zallone (1980) suggested that mature osteocytes have also the ability to form bone inside the lacuna.

A deep gene analysis of osteocyte during lactation in mammals revealed a surprisingly elevate expression of some genes normally associates to osteoclast activity during bone erosion. Among those genes, it is important to underscore the presence of tartrate-resistant acid phosphatase and cathepsin K. Therefore, the osteocyte is a peculiar cell expressing genes of both the mesenchymal and hemapoietic lineages. These observations suggest that the healthy osteocyte can both add and remove mineral from its lacunae and canaliculi. Such remodeling would be mediated through the PTH type 1 receptor because (since in lactating animals this gene was lacking), osteocytes failed to remodel their perilacunar matrix. Activation of PTH type 1 receptor in osteocytes results in elevated bone remodeling and elevated bone mass (O'Brien et al., 2008). This fact suggest that PTH type 1 receptor could play an important role is osteocyte viability and function.

The importance of aging is fundamental. The dimension of the lacunae reduces with age due of a hyper-mineralization of its perilacunar matrix which leads to cell death and higher bone fragility (Augat & Schorlemmer, 2006; Schnitzler, 1993). Another hypothesis about the reason why during age the mechanical resistance of the bone and the response to mechanical stimuli is reduced could be linked to this reduction of the dimension of the lacunae that could affect the dynamics of bone fluid flow through the osteocyte lacunar-canalicular network.

Little is known about the bone fluid that flows through the osteocyte lacunar-canalicular system; it is quite easy to identify by means of a single injection of a fluorescent dye into the tail vein of the mouse or in the sub-cutaneous tissue, that would result in a complete diffusion throughout the lacunar-canalicular system. However, there are still many unanswered questions about the magnitude, frequency, and type of flow (such as pulsatile or oscillatory), to which the osteocyte may be subjected. MLO-Y4 osteocyte-like cells, as previously mentioned, were extensively used in this field of research to study osteocyte function; those cells are particularly sensitive to fluid-flow shear stress which induces the release of prostaglandin from them; but shear stress has many effects on MLO-Y4 cells. A wide range of effects includes: the release of nitric oxide, adenosine triphosphate (ATP), and prostaglandins; the opening of hemi-channels and the activation of gap junctions; the promotion of dendrite elongation; the prevention of apoptosis; the triggering of signaling pathways such as the Wnt/bcatenin, protein kinase A (PKA), and other signaling pathways; the induction of b-catenin translocation to the nucleus; the activation of some gene transcription and translation, and so on.

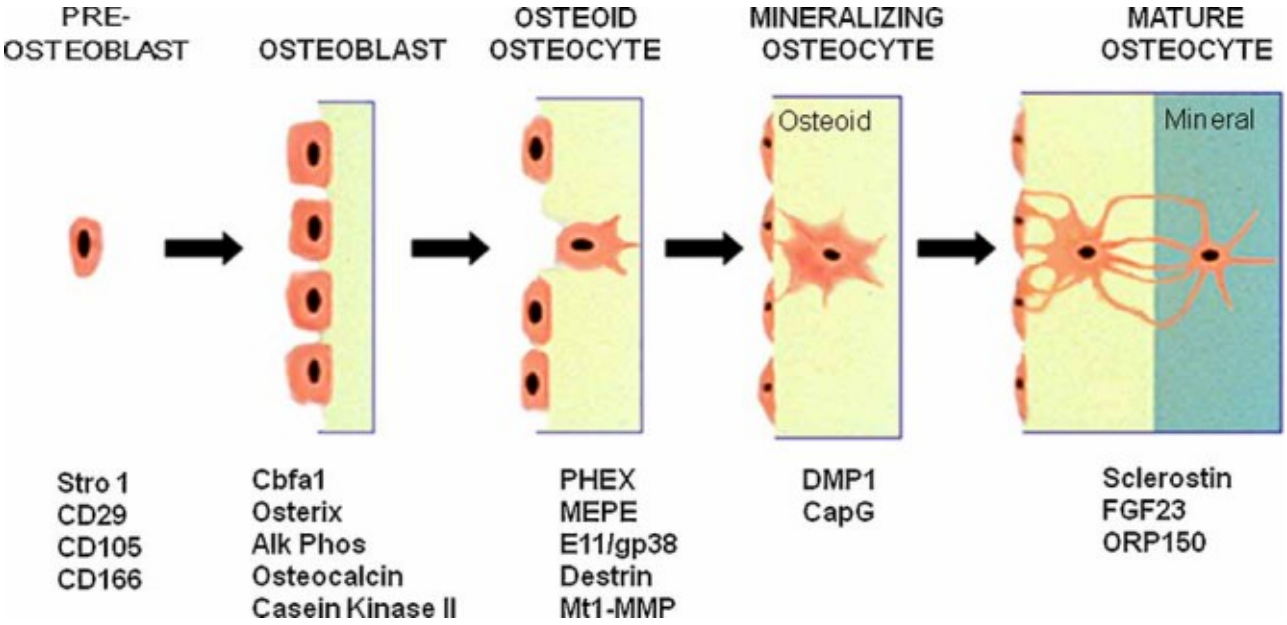
Recently the mechano-transduction of the osteocyte has been questioned and some authors showed how just the dendritic processes, but not the cell body, would play a role in mechano-sensing through the glycocalyces on the surfaces (Burra et al., 2010; Han et al., 2004). It has also been noticed that osteocyte possesses some single flagellar-like structures called cilia that may been use to release prostaglandin in response to sense different stimuli (Cherian et al., 2005; Forwood, 1996).

Other not canonical osteocyte-related proteins that have been studied in recent years were found to be involved with bone metabolism, bone resorption and bone growth; for example, lack of polycystin 1 (PC-1) seems to be directly related with osteopenia caused by a downregulation during

bone formation (Xiao et al., 2006). This shows that PC-1 in osteocytes is essential for the bone anabolic response to load.

Nitric oxide (NO) is a mechanical mediator that is released by dendrites of osteocytes almost simultaneously to PGE2. Its effect it is to inhibit resorption and promote bone formation. Both osteoblasts and osteocytes release NO in response to mechanical strain (Bakker et al., 2001; Zaman et al., 1999). ATP and intracellular calcium also can be released from osteocytes in response to extracellular calcium or mechanical stimulation (Genetos et al., 2005; H Kamioka et al., 1995).

P2X7 is a nucleotide receptor (an ATP-gated ion channel) that may play a role in mechano-sensation because its deletion resulted in 70% reduction of bone anabolic response in mice (Li et al., 2005). Inhibitors of P2X7 receptors suppress prostaglandin release, whereas agonists enhance the release in bone cells, suggesting that the P2X7 receptor is necessary for release of prostaglandin in response to mechanical load. Accordingly to Adamczyk et al. (2015), P2X7 receptor activation regulates the externalization of transglutaminase-2 (TGM2), which is a an enzyme with a pivotal role in stabilizing extracellular matrices and modulating cell-matrix interactions in tissue repair. Yanik et al. (2011) already demonstrated that a forced adipogenesis differentiation of BMSCs leads to an increase of the expressions of TGM2. Since adipocyte differentiation in bone marrow is potentially deleterious to both bone integrity and lymphopoiesis, it could be supposed that the expression of TGM2 by bone cells could be a negative signal of aging or pathological condition; however, really few has been described and is known about this protein, and its molecular mechanisms are still unclear.



Some of the expression changes that occurs during differentiation - The amazing osteocyte,

Bonewald, 2010

Static vs Dynamic Osteogenesis

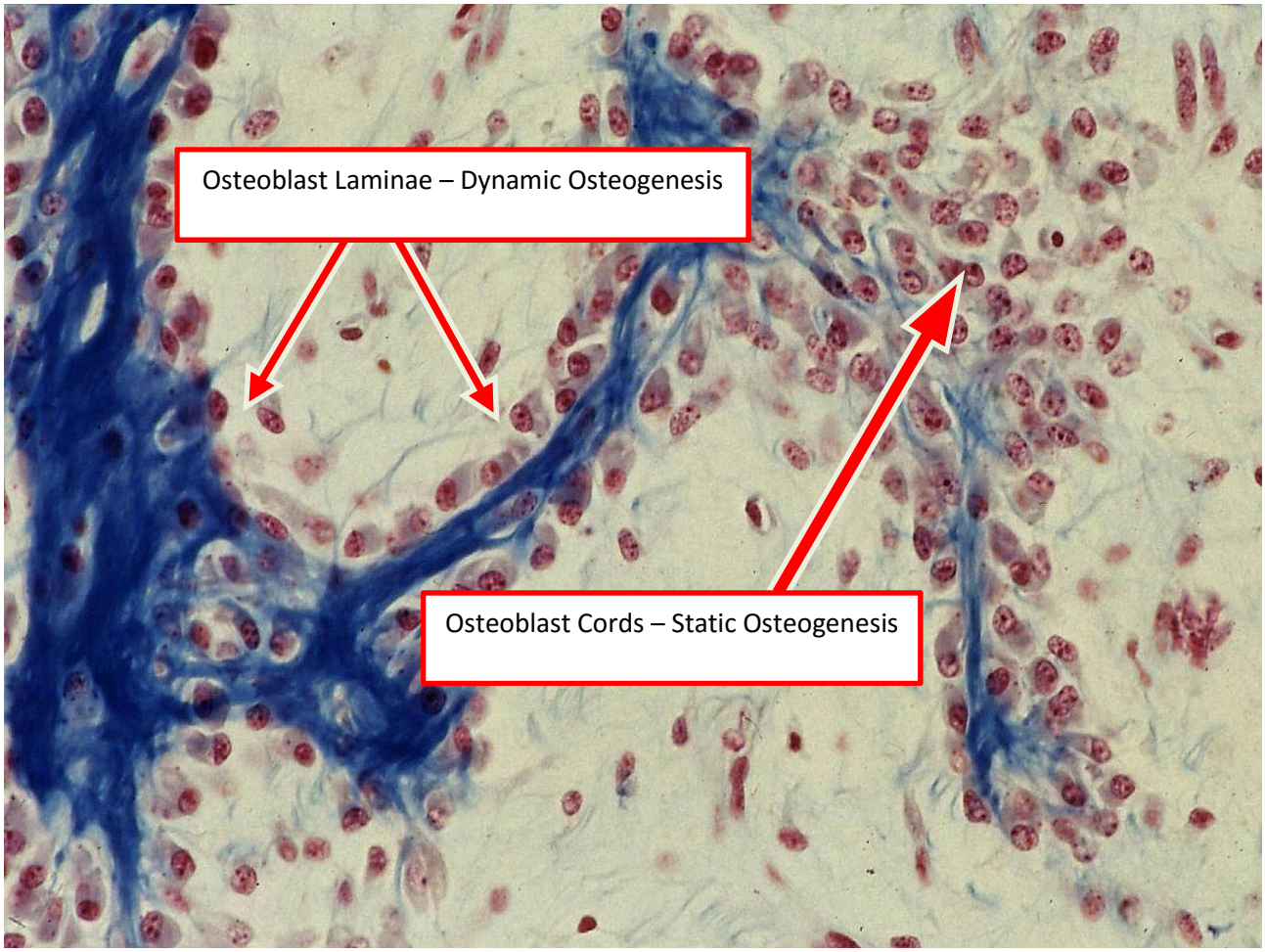
As postulated long time ago by Jeansonne et al. (1979) and then deepen by Palumbo et al. (1990a,b), the bone matrix deposition during osteon formation, as previously and briefly described, depends on the secretory activity of monostratified osteoblastic laminae connected each other by gap junction. Those cells appear to be polarized towards the osteogenic surface. During this process some selected osteoblasts proceed to transform into osteocytes remaining entrapped in the newly formed matrix as Marotti et al. (1992) and Palumbo et al. (1990a,b) clearly showed. Meanwhile, the osteoblast not committed to transform into osteocyte later transform into bone lining cells covering the bone. Indeed, the process of bone formation occurring at the onset of bone histogenesis (as during intramembranous ossification) has been showed to occur by means of two distinct successive phases (Ferretti et al. 2001). The first phase was named Static Osteogenesis (SO) since it is due to the immovable stationary osteoblasts that transform into osteocytes at the same site where they had differentiated. The second phase is the canonical and well known osteogenesis occurring during bone compaction described by Marotti et al. in 1990 and later named Dynamic Osteogenesis (Ferretti et al. 2001) since it is due to the classical movable osteoblastic laminae.

To better understand those two different types of osteogenesis we have to give a closer look to the events that occurs during intramembranous ossification, which is the process that we normally find during the bone regeneration.

The mesenchyme, where bone formation starts, is a highly cellular and vascularized tissue full of elongated and flattened cells arranged in layers inside the perichondrium/periostium of the preliminary cartilaginous shaft. Stimulated by oxygen tension as well as by different factors released by the abundant vessels, like grow factors and endothelial-cell-derived cytokines, some of

the MSCs (that were previously described) start to differentiate into osteoblasts. Apparently, pre-osteoblasts "sense" the position of the vessels. Those newly formed osteoblasts are arranged in cords, have not a polarized organization and never form the typical monostratified osteogenic laminae. These osteoblast, called "stationary" because of their direct transformation into osteocytes at the same site where they had differentiated without migration, secrete a matrix that soon undergoes mineralization and start radiating cytoplasmic processes. At the end of the mineralization, tiny trabeculae containing 2-3 layers of osteocytes enclosing blood vessels are formed. Those small trabeculae with their supporting function (as much as those made up of calcified cartilage in endochondral ossification) are necessary for the next step of osteogenesis, in fact movable osteoblasts, all polarized in the same direction, differentiate along the surface of the trabeculae allowing the onset of the dynamic osteogenesis. These movable osteoblastic laminae lay down concentric layers of bone, thus filling the primary haversian spaces with primary haversian systems (or osteons). It seems that static osteogenesis is mainly devoted to create a rigid scaffold at the onset of the ossification center activation and to introduce the bone mechanosensor (i.e. osteocyte), whereas dynamic osteogenesis is mainly involved in bone compaction or, at least, in thickening the primitive trabeculae. The mechanism used during static osteogenesis by osteoblast to become osteocyte is commonly called "self-burial", meanwhile in dynamic osteogenesis the osteoblasts committed to transform into osteocytes are embedded within the bone by the secretory activity of the adjacent movable osteoblasts. This fact explains why clusters of osteocytes within "confluent lacunae" can only form during static osteogenesis.

The main difference between the two types of osteogenesis is the distribution, arrangement and polarization of the osteoblasts, that during static osteogenesis cannot be driven by pre-existent osteocytes.



Dynamic Osteogenesis (DO – characterized by osteoblast laminae) and Static Osteogenesis (SO – characterized by osteoblast cords) in the same histological section: Blue = newly formed bone, Red = nuclei

Parathyroid hormone

Parathyroid hormone (PTH) is a hormone secreted by the cells of the parathyroid glands with the aim to increase the concentration of serum calcium. The secretion of parathyroid hormone is controlled by calcium-sensing receptors located on parathyroid cells sensible to serum calcium concentration through negative feedback (Coburn et al., 1999).

PTH essentially acts upon the parathyroid hormone 1 receptor, which is present at high levels in bone (Vilardaga et al., 2011).

PTH half-life is approximately 4 minutes in human bloodstream (Bieglmayer et al., 2002). In bone, it stimulates the release of calcium from the large reservoir contained in bones through the bone resorption performed by osteoclasts, which are indirectly stimulated by the hormone (Poole & Reeve, 2005). Stimulation is indirect since osteoclasts do not have a receptor for PTH, but it has been widely demonstrated that PTH receptors are present on osteoblasts, the cells responsible for producing bone, that by means of the "reversal phase" can trigger the osteoclast formation (Datta & Abou-Samra, 2009). In particular, by the molecular point of view, PTH has been demonstrated to act stimulating osteoblasts to increase their expression of RANKL and inhibit their expression of Osteoprotegerin (OPG). OPG compete with RANKL from interacting with RANK (the receptor for RANK-Lingand). If RANKL binds to RANK osteoclast precursors are stimulate to fuse, forming new osteoclasts, thus enhancing bone resorption (Silva & Branco, 2011). We previously mentioned that PTH receptor is also expressed on osteocyte, notwithstanding this relation and the possible effects of PTH on osteocytes are still unclear.

Teriparatide

PTH(1-34), also known as Teriparatide, is an FDA approved anabolic drug for osteoporosis treatment (Riek & Towler, 2011); it's effect is to enhance the bone growth and reduce the risk of fractures (Saag, 2007). However, it has been demonstrated that, notwithstanding the net effect of its intermittent daily administration is to increase the bone mass, PTH(1-34) under some conditions can also stimulate bone resorption. In particular, the effect of this drug seems to be strictly dependent on the timing of administration. Several studies reported that the intermittent administration of PTH(1-34) leads to bone deposition and bone growth (Neer et al., 2001) meanwhile continuous administration of PTH(1-34) produces a catabolic effect characterized by osteoclast activation and increased bone resorption (Guimarães et al., 2013; Poole & Reeve, 2005).

In 2008 Cherian et al. showed the importance of the timing of administration of PTH(1-34) on mineralization using the osteoblast-like cell line MLO-A5 that rapidly mineralizes in culture. They suggest that the overall expression of Cx43 (the protein that leads to the formation of gap junctions), and its presence on the cell surface are differentially regulated by intermittent or continuous PTH(1-34), implying the involvement of Cx43 and Cx43-forming channels in mediating the effects of PTH on bone formation.

The paper of Iida-Klein et al. (2005) was one of the first and few studies reporting the catabolic effect of continuous treatment of PTH(1-34) in mice and showing a significant decrease in the density of trabecular connectivity after 2 weeks of treatment.

Keller & Kneissel (2005) studied the effect of PTH on SOST regulation showing a reduced expression of Sost gene in 8-months-old mice in calvaria and femurs when treated intermittently

with PTH; they also confirmed their experiments with an *in vitro* model of osteoblasts, suggesting that SOST regulation may play a role in mediating PTH action in bone.

An absolutely opposite report was published by Bellido et al. (2005) showing that chronic administration of PTH in mice dramatically decreases the expression of Sost in osteocytes, suggesting that the PTH-induced suppression of Sost secretion by osteocytes unleashes the actions of pro-osteoblastogenic cytokines that cause the commitment of multipotential mesenchymal progenitors towards the osteoblast lineage. They also showed that, in contrast to the effects of continuous PTH elevation, intermittent PTH only transiently affected Sost mRNA levels, concluding that decreased sclerostin expression does not substantially contribute to the increase in osteoblast number resulting from daily injections of PTH.

PTH(1-34) can be used to improve fracture healing although this may be dependent on severity and location of bone injury as well as on individual's overall bone health.

PTH(1-34) was widely used in osteoporotic patients and bone lesions repair in humans, as shown by many papers on clinical studies (Borba & Mañas, 2010; Zanchetta et al., 2003; L. Zhang et al., 2012); this notwithstanding, a better knowledge on some aspects of PTH(1-34) action on bone tissue could be argued mostly studying the sequence of the events occurring during bone healing in experimental lesions on animal models under PTH (1-34) treatment. Up to now, most investigations on transcortical bone lesions reported in literature aimed at quantifying bone mass formed during bone repair, rather than analyzing the quality of the newly-secreted bone in relation to the type of osteogenesis (static or dynamic) that is occurring in the meantime.

Really few is known about the molecular mechanisms occurring in osteocytes during treatment with PTH; many reports published so far are often in contrast each other and the activity of PTH on

osteocytes has not been elucidated yet; moreover, the effect of osteocytes and their guidance over the other bone cells, the bone cell communication and the osteocyte-signaling have never been deepened, thus erroneously mostly enhancing the only the role played by the osteoblasts in bone regeneration.

AIM OF THE STUDY

The quantification of changes in bone deposition and bone density in subjects under intermittent administration of Teriparatide (PTH 1-34) has been widely described in both human and mice. Much less is known about the quality of the newly-formed bone; indeed, it is already well known that higher amount of bone doesn't necessarily correspond with higher resistance to strength. Many factors concur to determine the resistance of the bone such as collagen organization, mineralization and osteocyte survival. This aspect acquires particular importance in the problem of fracture healing during which, as normally occurs during bone histogenesis, static and dynamic osteogenesis are temporally correlated and follow each other.

PTH (1-34) is the only anabolic agent clinically approved for the treatment of osteoporosis and more sporadically it is used for fracture healing. The molecular mechanism whereby PTH mediates these anabolic effects has been studied for years mainly focusing on the effects that (PTH 1-34) exerts on osteoblasts; less is known about the effects on osteocytes and the role that those cells may play in cell-signaling during bone healing and regeneration. However, high doses of PTH continuously administered are known to increase the bone resorption, diminishing the bone mass and enhancing the bone fracture risks. The catabolic actions of PTH has been recognized in patients with hyperparathyroidism, or with acute infusion of the Teriparatide (PTH 1-34). Contrary to the anabolic actions of daily injection with PTH that have been well studied in both humans and mice, the catabolic actions of PTH on human and murine bone remain to be defined.

As previously mentioned, some studies already described the molecular response of osteoblasts to Teriparatide, however very few is known about the effect of Teriparatide on osteocytes; moreover, nobody ever concerned to look for molecular signaling changes that occurs when osteocytes are

treated with Teriparatide at different time points (simulating a long continuous treatment –anabolic effect- *versus* a short and intermittent effect –catabolic effect-).

To better understand and deepen some of the aspects that has not been enlighten yet about the effect of Teriparatide on bone we set up a double experiment.

The first part of the study was focused on investigations on time and manner of bone healing in rat femur, after drilling transcortical holes, with/without administration of Teriparatide. The novelty and the importance of this investigation is the evaluation of the drug effect during the sequential occurrence of the two types of osteogenesis (static *versus* dynamic) on:

1. the time in which it is mostly effective
2. the type of microscopic structure of the newly-formed bone tissue, lamellar or woven bone, more or less suitable to loading, respectively.

The final goal is to identify the phase of osteogenesis (static *versus* dynamic) in which Teriparatide can mostly exert its effect, to determine how and when using the drug with the maximum efficacy in improving bone lesion repair strategies.

In the second part of the study we investigate the effect that Teriparatide exerts on osteocytes; in particular, we focused on the effect that the drug shows at different time points of treatment, to simulate the effect of the two types of *in vivo* treatment (continuous vs intermittent); moreover, it has been investigated how the osteocyte gene expression would be consequently modified by the different administration (continuous *versus* intermittent) of the drug. We used, as cellular model, the MLO-Y4 osteocyte-like cell line described by Bonewald (1999) and Kato et al. (1997) and, as cellular treatment, the same Teriparatide used in the *in vivo* experiments adjusted for cell culture.

The goal of the experiment was to evaluate:

1. Eventual gene expression differences among the time points selected.

2. Identification of those gene that may be up- or down-regulated, by means of protein analysis.

MATERIAL AND METHODS

In vivo model

Experimental Animals and Treatment

Twenty-eight Sprague -Dawley male rats of 3 months of age were divided into 6 groups (after 7 days of acclimation to housing conditions) in which transcortical holes, simulating bone fractures, were drilled by means of a dental bur (1.5mm in diameter) at the mid-diaphysis of the lateral aspect of right femurs. All rats were housed individually in single cages and maintained under laboratory controlled conditions (22 ± 1°C, 55-60% humidity, 12-h light:12-h dark). Since the day after the experimental bone lesion, some animals were also treated with PTH(1-34), supplied by Eli Lilly and Company (U.S.A.), solubilised in saline (40 µg/ml) and subcutaneously injected in a volume of 100 µl/100gr body weight per rat, according to the following scheme:

GROUP 1 (n=5): animals fractured (as fractured control) and sacrificed after 10 days.
GROUP 2 (n=5): animals fractured and treated with PTH(1-34) for 10 days and then sacrificed.
GROUP 3 (n=5): animals fractured (as fractured control) and sacrificed after 28 days.
GROUP 4 (n=5): animals fractured and treated with PTH (1-34) for 28 days and then sacrificed.
GROUP 5 (n=4): animals fractured (as fractured control) and sacrificed after 45 days.
GROUP 6 (n=4): animals fractured and treated with PTH (1-34) for 45 days and then sacrificed.

The animals of groups 3, 4, 5 and 6 underwent a subcutaneous injection of 15mg/kg calcein (Fluka, St Louis, MO, USA) at day 10 after hole-drilling, to highlight the actual newly-formed bone.

All experiments were carried out according to the Bioethical Committee of the Italian National Institute of Health and authorized with Decrees of the Italian Ministry of Health Animal care, maintenance, and surgery were conducted in accordance with Italian law (D.L. no. 116/1992) and European legislation (EEC no. 86/609).

Histology and Histomorphometry

Soon after euthanasia, femora were harvested, fixed in 4% buffered paraformhaldeide, dehydrated in a graded series of ethanol and embedded in methylmetacrylate resin (Sigma Aldrich, Milan, Italy). Transversal sections (5µm thick) of the femora in the region containing the hole were obtained by means of a Reichert-Jung 1150/Autocut microtome (Walden, Austria) and stained with a Masson trichrome solution. Some sections were also observed under Transmitted Polarized Light (TPL). Sections for fluorescence microscopy were not stained. The sections were photographed using a NIKON Eclipse 90i microscope equipped with a DS-Fi1 Nikon digital camera and driven by the Nikon ACT-2U software; histomorphometry was performed by means of the software Image-J (NIH, Bethesda, USA). The amount of bone deposited within the hole area was evaluated in all experimental groups and expressed as BV/TV in percentage value. In those animals sacrificed at 10 days, the surface of prismatic osteoblast laminae with respect to the bone surface of healing hole (Ob.S/BS expressed in percentage value) was measured, to evaluate the amount of dynamic osteogenesis. In those animals sacrificed at 28 days both the values of calcein labelled bone area (L B Ar) and the amount of cortical compact bone (Ct B Ar) were measured with respect to the hole area (hole Ar), both expressed in percentage values. The fluorescent calcein labels were selected with the threshold function of Image-J and then measured as area fraction within the hole area.

Micro-hardness

The micro-hardness (HV) of the newly-formed bone inside the holes was evaluated in 28 and 45 days in all control and treated animals by means of a Leitz-Durimet micro-hardness tester (Germany) equipped with a Vickers indenter. After obtaining the histological sections (see above), the remaining part of the specimens were polished with Alumina Yellow Solution; then, six imprints were performed under a load of 50 grams for each specimen in the newly-formed bone, and the length of the diagonals of each imprint was measured by means of a screw micrometric eye-piece at an enlargement of 400X. The micro-hardness number (expressed in Kg/mm^2) was computed according to the formula HV=1854.4*L/d2 where (L) is the load in grams and (d) is the mean length of the diagonals of the imprint measured in micron.

Scanning Electron Microscopy - SEM

To visualize the collagen texture of the newly-formed bone, some sections (previously used for micro-hardness evaluation) were observed with the ESEM Quanta-200 Scanning Electron Microscope under low vacuum condition and in backscattered mode.

Serum Biochemical Analysis

After death, blood samples were collected by cardiac puncture and serum was immediately separated by centrifugation (4°C) at 1,500 g for 15 min, and then aliquoted into small volumes and stored at -20°C for successive analyses. The levels of total calcium (Ca) and inorganic phosphorus (P) in serum were determined using the high performance Beckman Coulter analyzer AU680 Chemistry System. The immune-metric assays for the determination of levels of Osteoprotegerin (OPG), specific bone alkaline phosphatase (BALP), CTX (Beta CrossLaps) and Bioactive-intact-

PTH (1-84) in rat serum were provided by Pantec s.r.l. (Italy); all kits are intended for research use only. In particular, Rat-OPG and Rat-BALP are two ELISA kits produced by SunRed Hotecnology Company (China); RatLaps is an EIA kit produced by Immunodiagnostic Systems Ltd, (UK); Rat Bioactive-intact-PTH is an ELISA method produced by Immunotopics Inc. (USA). The small amounts of reagents supplied in the kits prevented the possibility to perform automated procedures on laboratory analytical platform. To minimize the variables influencing the test, all good laboratory practice principles were applied: immediate storage of samples after serum separation at -20°C; manual execution of tests in close agreement to the manufacturer's instructions; execution of all tests over two consecutive days in order to avoid repeated freeze/thaw cycles of samples.

Statistical Analysis

Student's T test, using the Software STATA 11.0 (StataCorp, Texas, USA), was performed to compare means of treated and control groups, according to the time of sacrifice. Values of $p<0.05$ were considered significant.

In vitro model

Cell line

Murine long bone osteocyte Y4 (MLO-Y4) cell line was used in order to perform the in vitro test on osteocyte response to Teriparatide. 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, GIBCO - Thermofisher, MA, USA) plastic ware and grown at 37°C, 5% CO2, 95% air using DMEM (Euroclone, Italy) containing ribonucleosides, deoxyribonucleosides, and L-glutamine, supplemented with 5% fetal bovine serum (FBS), 5% bovine calf serum (CS) and penicillin/streptomycin at 100 U/ml. Cells were initially defrosted and plated at a concentration of 150.000 cells/ware then splitted once they reached the 80% of confluence.

Experimental conditions

For these experiments, the cells were cultured for 6, 9 and 12 hours with and without Teriparatide at 37°C in atmosphere of 5% CO2 and controlled humidity. We previously tested the Teriparatide and decided to use a concentration of 10^{-8} M. The drug was added into the media; the same amount of PBS was added in the media used for control samples.

Samples were then collected and processed for the Microarray analysis (3 treated samples for 6 hours, 3 samples as controls for the 6 hours treatment, 3 treated samples for 9 hours treatment and 3 samples as controls for the 9 hours treatment) or the Western Blot analysis (3 treated samples for 6, 9 and 12 hours and 3 samples as controls for each time point).

Gene expression

Total cellular RNA was isolated form the samples previously collected using RNeasy RNA isolation kit (Qiagen, USA) following the manufacturer's recommendations. Disposable RNA chips (Agilent RNA 6000 Nano LabChip kit) were used to determine the concentration and purity/integrity of RNA samples using Agilent 2100 bioanalyzer. cDNA synthesis, biotin-labeled target synthesis, GeneChip® Mouse Genome 430 2.0 Array (Affymetrix, USA) arrays hybridization, staining and scanning were performed according to the standard protocol supplied by Affymetrix. The amount of a transcript mRNA (signal) was determined by the MAS 5.0 absolute analysis algorithm. All expression values for the genes in the MAS 5.0 absolute analyses were determined by using the global scaling option. Alternatively, probe level data were converted to expression values using robust multi-array average (RMA) procedure. Perfect match values were background adjusted, normalized using invariant set normalization and log transformed. The RMA-generated data were uploaded onto GeneSpring software version 7.2 using the log 2 transformation procedure. A 'per gene' normalization was achieved by dividing each signal by the median of its values in all samples. A low-level filter in GeneSpring filtered out all those probe-sets called 'Present' in less than 10% of samples or whose normalized expression levels were always between 0.5 and 2 across all of the samples. Affymetrix MAS 5.0 comparison algorithm was used for

supervised analysis selecting those transcripts showing a change call 'I' or 'D' in at least 90% of the pairwise comparisons. Supervised analyses were performed using an analysis of variance (one-way ANOVA) test (Welch ANOVA at a confidence level of 0.01) with the Benjamini and Hochberg correction of the false discovery rate, and using dChip Compare Sample procedure. Briefly, the comparison criteria utilized in dChip require the fold change and the absolute difference between two groups means to exceed user-defined thresholds (in the present study, 3 and 200, respectively). The "use lower 90% confidence bound" has been selected to use the lower confidence bound of fold changes for filtering. The lower confidence bound is intended as a conservative estimate of the real underlying fold change. False discovery rate (FDR) has been used to adjust P-value for multiple comparisons by 100 random permutations of the group labels. The GeneSpring advanced filtering options were used to combine gene lists generated from different analyses. Hierarchical agglomerative clustering of the selected probe list was performed using GeneSpring. Class prediction analyses were performed using the Support Vector machine algorithm as implemented in GeneSpring (Polynomial Dot Product (Order 1) Kernel Function. Diagonal Scaling Factor: 0).

Sample preparation and SDS-PAGE

Samples previously collected were followed frozen-thaw cycles in order to break the cell membranes; then, an additional lysis was performed with a Lysis Buffer. Proteins extracted were quantified with the Bradford method using BSA as standard and rehydration buffer as blank.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli's procedure. In brief, 5 μg of total proteins for each sample were mixed with the Laemmli sample buffer with the addition of β-mercaptoethanol as reducing agent. Samples were then boiled at 95 °C for 5 min and subsequently loaded onto 12 % SDS polyacrylamide gel. To

ensure optimal band resolution, the electrophoretic run was carried out in a minigel slab apparatus (Bio-Rad), using TGS running buffer (25 mM Tris–HCl, 192 mM glycine, 0.1% SDS, pH 8.3). Electrophoresis was run firstly at 100 V for 30 min, followed by an increase up to 200 V, until the dye front reached the bottom of the gel.

Western Blot

A total of 5 μg of proteins were separated on 12 % SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were stained with Rosso Ponceau to verify the correct migration of the proteins from the gel to the membranes that were then blocked with 5 % non-fat milk and subsequently incubated overnight at 4 °C with the following primary antibodies (all from Santa Cruz Biotechnology, USA): anti-Pleiotropin (rabbit polyclonal, 1:250); anti-Connective Tissue Growth Factor (rabbit polyclonal, 1:250); anti-WNT1 Inducible Signaling Pathway Protein 2 (rabbit monoclonal, 1:250); anti-Serpine1 (rabbit polyclonal, 1:250), anti-cathepsin K (rabbit polyclonal, 1:250). Then, the membranes were incubated with a solution containing 1:2000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (DakoCytomation, Denmark). Target bands were visualized using a mix of peroxidase solution plus a luminol enhancer solution (WesternSure™ PREMIUM Chemiluminescent substrate). Results acquisition and band densitometric analysis (represented by arbitrary units, AU) were performed using the C-DiGit® Blot Scanner (LI-COR Biosciences, NE, USA) and the Image Studio Lite 5.0 software (LI-COR Biosciences, NE, USA).

RESULTS

In vivo model

The histological observations performed in the rat model during hole repair have showed, going from the periosteal side towards the endosteal one, the same successive phases occurring during fracture healing: after the hematoma and inflammatory stages, (*i*) the gap is filled with highly vascularized connective tissue; afterward, (*ii*) cords of stationary osteoblasts differentiate in between the blood capillaries, giving origin to a trabecular bony framework laid down by SO; (*iii*) typical laminae of movable osteoblasts differentiate successively on the surfaces of such SO-trabeculae and thicken them by DO (Fig. 1).

10 days after drilling

After 10 days from hole drilling, in both untreated and treated animals, the bone gap is filled by trabecular bone, mostly laid down by means of static osteogenesis (Fig. 2). The total amount of newly-formed trabecular bone (expressed as BV/TV%) recorded in the areas of healing hole doesn't vary in the two animal groups (Table 1). Notwithstanding the same amount of newly-formed bone, different histological features are detectable in control and treated groups. In control animals, within the fibrous tissue separating the forming bony trabeculae, cords of pluristratified stationary osteoblasts are abundant and well visible (circles in Fig. 2A). In treated animals, instead, close to the surfaces of trabecular bone deposited by static osteogenesis, well developed laminae of movable osteoblasts (typical feature of dynamic osteogenesis) were clearly observed (arrows in Fig. 2B).

Concerning the amount of dynamic osteogenesis, the surface of prismatic osteoblast laminae with respect to the bone surface of healing hole (Ob.S/BS%) is significantly higher in treated animals (group 2) than in untreated ones (group 1) (Fig. 3).

28 days after drilling

Within the same amount of bone tissue that completely fills the trans-cortical hole in both animal groups (Table 1), a higher amount of compact bone (at the periosteal side) compared to trabecular bone (at the endosteal side) is present in treated animals with respect to control ones (Fig. 4); in fact, histomorphometric parameter Ct B Ar is about 70% in treated animals (group 4), about 53% in untreated animals (group 3), though without statistical significance (Fig. 5). The percentage value of the total amount of calcein-labeled newly-formed bone (L B Ar) does not show significant differences between treated and control animals (about 9% in both groups), but the areas of calcein-label in the treated animals appear to be developed in length with linear shape (in accordance with the typical arrangement in laminae of movable osteoblasts acting during dynamic osteogenesis); otherwise, in untreated animals, the areas of labeled bone appear to be developed in thickness with irregular profile (in accordance with the typical organization in cords of stationary osteoblasts acting during static osteogenesis) (Fig. 6). Concerning micro-hardness evaluation, slight differences were observed in the newly-formed bone inside the holes between control and treated groups: the micro-hardness was higher (64 Kg/mm^2) in treated animals *versus* the control ones (59 Kg/mm^2), though the difference does not reach levels of statistical significance.

45 days after drilling

The total amount of bone tissue that fills the trans-cortical hole (BV/TV%) does not vary in both animal groups, as also observed after 10 and 28 days from hole drilling (Table 1). This notwithstanding, the observations of bone sections both under Transmitted Polarized Light and Scanning Electron Microscopy show a birefringence pattern (evident by means of TPL) typical of a ordered-fibered texture (well recognizable also by SEM) well evident in treated animals with respect to the untreated ones, thus indicating a texture made up of more ordered bone tissue, instead of woven bone, in the sites of repaired lesions during administration of PTH(1-34) (Figs. 7 and 8). Concerning micro-hardness evaluation, trivial differences were observed between control and treated groups in the bone filling the holes.

Serum biochemical analysis

In Table 2 are reported the values of parameters from sera collected at the end of the experiment. Mean values of Ca, P, OPG and BALP do not show statistically significant differences between the groups after 10, 28 and 45 days. Concerning the values of CTX and PTH (1-84) it is to be noticed the wide variability among the animals inside each group; also for these parameters, no difference were recorded in the mean values among all groups.

In vitro model

The osteocyte-like cell line selected (MLO-Y4) was selected for the study since it shows almost complete expression of all the classical osteocyte differentiation markers, the typical osteocyte dendritic shape and the capability of responding to specific stress (such as shear-stress) following osteocyte behaviors. In this study, we treated this cell line with the same Teriparatide used for the *in vivo* model, adjusting the concentration and the time exposure for the cell culture. Two time points crucial on the different gene expression of MLO-Y4 under Teriparatide stimulation were firstly identified and confirmed through the Gene Array analysis. Among all the genes differently expressed, we selected by an accurate research on Pubmed the few that have been already clearly know to be involved in different bone processes. Then, we then proceed to validate them with a Western Blot analysis.

Gene Expression profiling of MLO-Y4 under Teriparatide treatment

A genome-wide analysis of gene expression in MLO-Y4 osteocyte-like cell line was carried out using cRNA from cells treated with 10^{-8} Molar Teriparatide (PTH 1-34), hybridized on Affymetrix Mouse Expression Array 430 GeneChip set. This approach allowed the evaluation of the levels of expression of more than 39,000 transcripts following hybridization with approximately 45,000 probe sets. RNA was isolated from a total of 12 samples, 3 samples were treated with Teriparatide for 6 hours and 3 samples were used as controls; 3 samples were treated with Teriparatide for 9 hours and 3 samples were used as controls. The transcriptomes of treated *versus* not treated samples at both selected time points revealed some differences that were not statistically significant, however a comparison among the two different time points selected (Teriparatide-6 hours *vs*

Teriparatide-9 hours) showed and inversion of gene expression for some genes. Genes with a consistent differential gene expression (Teriparatide-6 hours vs Teriparatide-9 hours) of more than 4-fold (log2 ratio <−2.0 or >2.0) were selected. This restriction give us a panel of 40 genes that were differentially regulated among the samples (Table 3). The fold change was kept intentionally high to restrict the field and avoid possible bias. Among those genes, we selected the 13 ones that showed biological/molecular function on bone (reported by Pubmed) and 5 of them were selected for further investigation (Table 4). Gene names and symbols were reported in Table 5.

Protein expression in Western Blot analysis of CTGF, PAI-1, CATK, PTN and WISP-2

In order to validate the results of the gene array and precisely verify the different expression of some factors, we evaluated the levels of CTGF, PAI-1, CATK, PTN and WISP-2 by Western blot. We took in consideration the time that elapses from gene expression and protein synthesis, so we added a 12-hours-time-point fully to evaluate the protein expression in our samples.

As shown in Fig. 9a, all protein levels followed the predicted expression by gene array. Band intensities were converted by Li-cor Software into arbitrary numbers values and then compared.

CTGF showed a drastic reduction of expression after 6 hours of treatment (6380) if compared to control (33000), meanwhile after 9 hours of Treatment the expression lever was comparable to the control (29000); the expression then increased after 12 hours of treatment (40700) if compared to control.

PAI-1 expression was drastically reduced after 6 hours (not determined-ND) if compared to control (2460); after 9 hours of treatment, the intensity of the band was comparable to the control (2380) but after 12 hours of treatment, the intensity value doubled (4990) if compared to control.

CATK presented a double band that we interpreted as a dimerization, so we selected both bands for the comparison. Interestingly, the expression of CATK at different time points was reduced to a value that wasn't determinable (ND) after 6 hours of treatment, meanwhile at 9 hours-time point the intensity was increased to almost twice (8390) if compared to the control (4620); after 12 hours of treatment with Teriparatide the expression level was comparable to the control (4370).

PTN expression was higher in control (13800) compared to all the time points selected. However, it is interesting to observe that even if the expression was reduced after 6 hours of treatment (5620), the expression after 9 hours of treatment was higher (8430) compared to the 6 hours treatment, and the expression after 12 hours of treatment slightly higher (8920) if compared with the 9 hours of treatment.

WISP-2 expression drastically increased after 6 hours of treatment (45300) compared to control (2530). After 9 hours of treatment (6820) protein expression level was decreased compared to the 6 hours of treatment but still higher if compared to the control, meanwhile the band intensity was similar after 12 hours of treatment (2720) compared to the control.

All the western blots run just one time so far. This study is still in development and we are currently repeating every western to have a technical triplicate that could give us a statistical significance.

DISCUSSION

The first point to discuss is the adequacy of the animal model proposed, in which the consecutive phases occurring during the filling of the holes are well recognizable as during bone fracture repair. In the early healing process (10 days), interestingly, the production of the newly-formed trabecular bone occurs in different time and pattern depending on the administration of Teriparatide. The more significant aspect to be underlined is that, unlike the simultaneous beginning of static osteogenesis in treated and control groups, the onset of dynamic osteogenesis starts earlier in treated than in untreated animals. In fact, concerning the differentiation of dynamic osteoblasts, already at 10 days, the number of movable osteoblast laminae is significantly higher in animals with PTH(1-34) administration rather than in untreated ones (Fig. 2). The increased osteoblast number after intermittent administration of parathyroid hormone was also shown by Jilka et al. (2009) in an investigation on murine models. Our observations on osteoblastic laminae, significantly different between treated and untreated animals, fully match with the different morphology of bone areas labeled at 10 days by calcein and observed in treated *versus* untreated animals sacrificed at 28 days after drilling. In particular, in treated animals the labeled bone areas appear to be developed in length with linear shape, thus resembling the typical arrangement of movable osteoblastic laminae acting during dynamic osteogenesis; otherwise, in untreated animals, the areas of labeled bone appear to be developed in thickness with irregular profile, thus resembling the typical organization of stationary osteoblast cords acting during static osteogenesis. This critical observation strongly suggests the mere/crucial effect of Teriparatide, that is to anticipate the formation of bone tissue (by

means of dynamic osteogenesis) with an ordered collagen texture (i.e. lamellar instead of woven-fibered bone), recognized to be more valid by the mechanical viewpoint. Our results at 28 days from drilling, concerning the higher percentage of compact bone in treated animals with respect to trabecular bone is in line with the evaluation of micro-hardness showing higher values in treated animals with respect to the untreated ones; this strengthens our suggestion that Teriparatide anticipate the process of dynamic osteogenesis that produces a mechanically more valid bone tissue. Moreover, 45 days after hole drilling the newly-formed bone of treated animals with PTH(1-34) shows a birefringence pattern typical of a more ordered collagen fiber texture, notwithstanding the micro-hardness is similar in treated and control animals, probably because at diaphyseal level (respect to other skeletal regions) the bone compaction is almost complete. Such more ordered bony texture in treated animals, clearly visible under SEM (Fig. 8), strongly indicates the qualitative (instead of quantitative) effect of PTH(1-34) in improving bone repair. Such observations are in line with those of various authors according to whom PTH(1-34) increases cortical bone mass by stimulating bone formation on bone surfaces (Arita et al., 2004; Jiang et al., 2003; Ejersted, Andreassen, Nilsson, & Oxlund, 1994; Mashiba et al., 2001).

Analyzing in detail the two processes of osteogenesis, it is to be underlined that SO is an osteo-inductive process occurring without pre-existing osteocytes: i.e. without mechano-receptors, that make useless mechanical strains (likely already existing during static osteogenesis) since they cannot be sensed. In fact, SO is responsive to cytokines, as endothelin I and/or vascular growth factors (ECGF) released by the endothelium or by platelets, which are mitogens for osteoprogenitor cells. The primary function of SO is to provide to DO-osteoblastic laminae a rigid scaffold containing osteocytes; therefore, in DO mechanical factors can play a crucial role in transducing mechanical stresses into biological signals. The results here reported strongly suggest that such transduction seems to be amplified by PTH(1-34). These our recent preliminary observations are in

agreement with those of Jilka et al. (2009) showing that intermittent administration of PTH(1-34) indirectly stimulates bone formation on the surface of trabecular bone, enhancing the cell signaling pathways involved in anti-apoptotic phenomena, that in turn bring to the increase of the osteoblast number. Moreover, observing the histologic images in the paper of Jilka & coworkers (2009) and analyzing their results in particular detail, in the light of our evidences on static *versus* dynamic osteogenesis, we strongly highlight that the conclusions obtained by the authors (i.e. <<in periosteal bone where the rate of osteoblast apoptosis is low, PTH must exert pro-differentiating and/or pro-survival effects on post-mitotic pre-osteoblasts>>) refer precisely to the dynamic osteoblastic laminae, standing the fact that PTH(1-34) acts on precursors of osteoblasts lining the SO-bony trabeculae already containing osteocytes. Therefore, once again, the results of Jilka and coworkers (2009) are in line with our suggestion that Teriparatide affects dynamic osteogenesis only.

As already reported by some authors, bone quality strongly depends by the rate of bone remodeling: Chiang et al. (2013) refer that 6-month Teriparatide treatment of patients was associated with increased bone remodeling and partial or complete healing of atypical fractures. In this context, it is important to underline that bone remodeling is exclusively due to dynamic osteogenesis, viz. just the osteogenic process we demonstrate to be enhanced by PTH(1-34) administration. This is also in line with evidences by Ma et al. (2006) showing that Teriparatide promotes the differentiation, activity and lifespan of osteoblasts in both already-occurring and newly-triggered bone remodeling processes.

As far as serum parameters are concerned, it is not surprisingly to observe that Ca and P levels are similar in all groups, as a consequence of the mineral homeostasis in which the skeleton, as well as other systems, is involved. Also BALP does not show differences between control and treated animals, probably since Teriparatide does not affect the quantity of newly-formed bone, but the

quality of bone tissue, which is, as previously mentioned, more orderly laid down by the anticipated process of dynamic osteogenesis.

The hypothesis previously formulated that during DO, under PTH(1-34) treatment, mechanical factors can play a crucial role in transducing mechanical stresses into biological signals it seems to partially agree with the data that we present in the second part of this thesis.

The data obtained by the *in vitro* model, using the osteocyte-like MLO-Y4 cell line, revealed that Teriparatide stimulation may induce a change in the genetic and proteomic expression of several factors involved in bone metabolism. MLO-Y4 cell-line was selected to conduct this experiment for multiple reasons: first of all, the cell line express most of the characteristic markers of fully developed, mature and differentiated osteocytes; moreover, it doesn't express the typical osteoblast markers; finally, it was already widely described its use in mechanical related experiments of shear-stress and its response to induced stresses. Any other cell lines that matched those requirements weren't available when we started the experiments; however, in 2011 Woo & Coworkers described and developed a new innovative cell line. This cell line, IDG-SW3, derives from long bones of mice such as MLO-Y4, but unlikely MLO-Y4 the IDG-SW3 has the capability to go through a complete differentiation from early osteoblast to late osteocyte if cultured with differentiating media. Another interesting feature about this new cell line is that it has the capability to produce extracellular matrix that undergoes mineralization. IDG-SW3 cell line has been started to be used for many porpoises since in the last two years it has replaced MLO-Y4 for many aims. However, MLO-Y4 is still the favorite choice for shear-stress experiments and mechanical-stress related experiments by many authors (Liu et al., 2015; Wu et al., 2015).

In the present investigation we exclusively used MLO-Y4 but our intention is to validate our data in the new IDG-SW3 cell line that seems to be a valid model even for more sophisticated studies.

Even if we didn't perform any shear-stress experiments, we are convinced about the importance to have a cellular model able to respond to mechanical stress; as reported above, Teriparatide seems to play a crucial role during DO, suggesting a possible molecular effect on osteocytes that are constantly under the physiological mechanical stress. We strongly believe that during bone recovery, as already demonstrated by many authors, the physiological mechanical stresses together with Teriparatide are essential for bone health in enhancing the deposition of bone tissue more resistant by the mechanical viewpoint; thus, it is still important to have a cellular model that has been demonstrated to be able to respond to mechanical stress. Once we demonstrated the effect of Teriparatide on the osteogenesis and hypothesized the involvement of osteocytes and the role they may play in this process, we decided to investigate the molecular changes that occurs during the treatment with Teriparatide. To better understand which factors may be involved in the process of osteocyte cell signaling, we took in consideration the differential effect of PTH reported in literature on osteoporotic subjects and on fracture healing in both human and mice, depending on time and manner of administration. Considering that the physiological PTH half-life is approximately 4 minutes in human bloodstream, it can be hypothesized that to exert its anabolic effect the PTH has to act quickly and to be removed quickly as well. In fact, it is already been demonstrated that intermittent administration of PTH(1-34) leads to bone deposition and bone growth, meanwhile continuous administration of PTH(1-34) exerts a catabolic effect characterized by an increased osteoclast activation and bone resorption. Concerning to the *in vitro* model, we selected different time points to study how the exposition to Teriparatide may change the molecular expression on osteocytes over time, mimicking the intermittent *vs* continuous treatment.

The microarray analysis performed on MLO-Y4 showed an alteration of the gene expression whenever the cells were treated with Teriparatide if compared to controls, although with not

statistically significant difference. Indeed, differences become significant if we compare the two different time points selected; many of the genes regulated by Teriparatide showed an inversion of expression if we compared samples treated for 6 hours to samples treated for 9 hours. Apparently, the expression of some genes were inverted when the treatment with Teriparatide was prolonged till 9 hours. In fact, the expression of many genes that were down-regulated under 6 hours of treatment (compared to the control) showed an up-regulation under 9 hours of treatment. *Vice versa*, many other genes showed an up-regulation under 6 hours of treatment but a down-regulation under 9 hours of treatment. To avoid any possible bias, we selected just the genes that showed a fold change higher than 8; this selective restriction give us just the panel of 40 genes that showed this "inversion of expression". Our hypothesis was that among those genes there could be included some genes related with the osteocyte cell-signaling stimulated by Teriparatide.

This particular "inversion of expression" may suggest that those genes could be some of the factors that may regulate the changes occurring when the treatment of Teriparatide changes from intermittent to continuous (i.e. anabolic to catabolic); whereby, our hypothesis is that in our model the treatment for 6 hours with Teriparatide mimics the (short) intermittent administration *in vivo* with Teriparatide, meanwhile under 9 hours of treatment the osteocyte response to Teriparatide is similar to that induced *in vivo* by the continuous administration.

A closer look to those 40 genes revealed that 13 of them already had a well-known biological/molecular function on bone. We started validating (by Western Blot) 5 of those 13 genes (Ctgf, Pai-1, Wisp-2, Ctsk and Ptn) previously selected and, in the meantime, we planned to validate the remaining 8 genes in the next months.

Firstly, we started evaluating the expression of the Connective Tissue Grow Factor (Ctgf o CCN2) recently described by Aoyama et al. (2015) that showed a novel role of CCN2 in inhibiting OPG

besides the capability to bind RANK (enhancing its effect), showing a RANKL-induced osteoclast differentiation. By means of Western Blot analysis we found that the level of expression of Ctgf was drastically reduced after 6 hours of treatment compared to the control (values comparable to controls after 9 hours of treatment), and increased after 12 hours of treatment if compared to the control. If we translate those results to the *in vivo* model (as we previously suggested), it may hint that a short treatment of Teriparatide (6 hours - intermittent) reduces the levels of Ctgf which means a reduction of osteoclast differentiation and, consequently, a less bone resorption, meanwhile a longer exposition to Teriparatide (12 hours – continuous) leads to an increase of the level of Ctgf, in turn enhancing osteoclast differentiation which stimulates the bone resorption.

Another gene evaluation concerned the Plasminogen Activator Inhibitor-1 (Pai-1 or Serpine1); some authors (Canecki-Varžić et al., 2016; Tamura et al., 2013) showed that the loss or reduction of Pai-1 protects against bone loss, in particular Li Mao et al. (2014) demonstrated that the reduction of Pai-1 levels restores bone formation by promoting the differentiation of mesenchymal stem cells into osteoblasts instead towards adipocytes. Once again, our data confirm our suggestion about different effects depending on the time treatment: any appreciable level of Pai-1 was not found after 6 hours of treatment; after 9 hours of treatment with Teriparatide the level of expression of Pai-1 was comparable with the control; meanwhile, after 12 hours its level of expression was drastically increased. This evidences seem to confirm our hypothesis that 6 hours of *in vitro* treatment is comparable to the short-intermittent administration *in vivo* and 9 hours of *in vitro* treatment is comparable to the long-continuous administration of Teriparatide *in vivo*. After 6 hours of treatment (condition comparable to intermittent administration), levels of Pai-1 are not determinable, for the high reduction of its expression; this situation *in vivo* leads to a protection of bone loss through an increase of differentiation of MSC into osteoblasts. *Vice versa,* the levels of Pai-1 are rapidly

recovered after 9 hours of treatment and then drastically increased after 12 hours of treatment (condition comparable to the continuous administration of Teriparatide).

Similarly to the first two proteins, the expression of WNT1-inducible-signaling pathway protein 2 (Wisp-2 or CCN5) confirmed the gene array analysis. Wisp-2 is a member of the CCN family and a factor of the Wnt pathway that positively regulate the osteoblast differentiation. We found an increment of its expression level after 6 hours of treatment of almost 18 times higher compared to the control and more than 6 times higher compared to 9 hours of treatment; just after 12 hours of treatment with Teriparatide its level of expression was similar to the control. Considering this result in the light of the model proposed, we can observe how a short (intermittent) administration of Teriparatide leads to an increase of production of the Wisp-2 protein which *in vivo* stimulates osteoblast differentiation. However, the levels of Wisp-2 protein do not decrease respect to controls in both 9 and 12 hours of treatment with Teriparatide; it seems to occur the gradual decrease until reaching the basal level after 12 hours. This observation may be explained by the hypothesis that: *i)* other factors may concur in the Wnt pathway to prevent the total ablation of the protein which is an important factor for newly-bone formation; *ii)* the decrease of the protein expression wasn't stop after 12 hours of treatment and cells may take longer to remove the protein previously produced. If this second hypothesis would be correct, it may be necessary to investigate the protein expression of Wisp-2 after 12 hours of treatment to verify whether the expression level may decrease.

Interestingly, levels of Cathepsin K (Ctsk) were also altered. Cathepsin K is a typical osteoclast marker which is a protease secreted by differentiated osteoclasts in order to catabolize elastin, collagen, and gelatin. Since osteoclasts are derived from the hematopoietic lineage, it is interesting to be noted not only that osteocytes produce this protein but also that it seems to be able to modify its production under the stimulus of Teriparatide. Our results showed

how Cathepsin K levels were drastically reduced after 6 hours of treatment (no signal was detected by the software) compared to the control (4620); after 9 hours of treatment, the expression level has reached almost twice the value (8390) but after 12 hours of treatment the level of expression (4370) was similar to the control. In this case, it is difficult to understand the trend of the expression; the initial ablation would be easily linked to a reduction of bone resorption caused by the reduction of the enzyme itself as a result of a short interaction with the Teriparatide, meanwhile the increased production of the enzyme (that would cause an increase of *in vivo* bone resorption) probably occurs after a longer exposure of Teriparatide (9 hours), though it is reduced after 12 hours of treatment. A possible explanation of this evidence could be that, since Cathepsin K is not a cell-signaling molecule but an enzyme involved in perilacunar matrix remodeling and resorption, the expression of this protease is not continuously performed by osteocytes and other factors may concur to its regulation. However, the data collected so far are not enough to speculate any further involvement of this protein in bone resorption and more investigations need to better understand the possible involvement of Chatepsin K in bone remodeling triggered by Teriparatide.

Pleiotropin (Ptn) is also known as Osteoblast-specific factor 1; it has been demonstrated being involved in many processes during bone repair, from promoting endothelial cells proliferation to stimulating *in vivo* angiogenesis and it was already demonstrated its chemotactic effects on human osteoblasts and endothelial cells. Our Western blot results don't match with the gene array evaluations which showed a reasonably overexpression after 6 hours of treatment and a downregulation after 9 hours of treatment. It is reasonable to imagine that, in the model we propose, a short and intermittent stimulation with Teriparatide (6 hours) would lead an increase of Ptn which, in turn, stimulates different anabolic processes involved in bone regeneration, meanwhile long treatments (continuous – 9 hours) would lead to a decrease of those levels, as shown by the gene

array. Western blot analysis showed a different trend of the protein expression. After 6 hours of treatment, the protein expression level was reduced more than half compared to control and, even after 9 and 12 hours, protein levels were not restored. Many hypotheses could be made to explain this mismatching of results. If those data would be confirmed, we should consider that Ptn is a protein that plays a wide range of roles in bone regeneration, in particular involved in the preliminary steps such as vascularization and chemotaxis; it could be hypothesized that, just after a quick increase of the gene expression and protein production, some epigenetics factors (such as miRNAs) may drastically reduce the production of the protein, even in early stages. However, probably it's too soon to make any assumption about the role of this protein under the stimulation of Teriparatide.

Among the genes that we didn't start yet to evaluate, however, it is interesting to notice their potential involvement in bone remodeling under the stimulation of Teriparatide. Further eight genes with a known role in bone metabolism were regulated by Teriparatide in our experiment; 6 genes (Aqp5, Scara3, Efemp1, Pdk1, Angpt1 and Col6a1) were found to be upregulated after 6 hours of treatment and downregulated after 9 hours of treatment, meanwhile two of them were found to express the opposite trend (Tgm2 and Trib3). However, really few is known about the real role exerted by those genes in bone metabolisms and most of the papers published so far just started to argue about their involvement in bone processes; moreover, those genes are frequently expressed in many other tissues and are not specific for bone or osteocyte functions. We preferred to focus firstly on those genes that could be more interesting since their role on bone was already been suggested by different authors and papers.

CONCLUSIONS

In conclusion, by means of the animal model here proposed, we have been able to evaluate in which phase of bone healing the Teriparatide administration is crucial and more efficacious to improve the quality of skeletal repair. We demonstrate, for the first time, that the main effect of PTH(1-34) concerns an anticipated occurrence of dynamic osteogenesis, i.e. the deposition of bone more suitable to loading. This finding could be of crucial importance in further translational clinical research in humans to define the best therapeutic strategies to be applied by the orthopedic surgeons in recovering severe skeletal lesions, particularly in terms of time of administration of PTH(1-34).

We also showed an interesting behavior expressed by the *in vitro* osteocyte-like cell line model MLO-Y4 treated with Teriparatide at different time points to mimic the two different types of Teriparatide administration. The model we have proposed is aimed to represent the *in vivo* characteristic effects under the (short) intermittent administration *versus* the (long) continuous administration of Teriparatide. When the MLO-Y4 cell line was exposed for a short period of time to Teriparatide some genes were up- or down-regulated but when the drug exposition was prolonged the gene expression was inverted. We analyzed the genes that showed an "inversion of expression" caused by the Teriparatide treatment at different time points and found that many of them were involved in bone metabolism. Those findings demonstrate that not only the osteocyte-like cell line MLO-Y4 is sensitive to Teriparatide but also that such behavior is in line with the *in vivo* bone response to the drug. These two findings let us to hypothesize that osteocyte-cell-signaling sensitiveness to the PTH(1-34) may be crucial during bone remodeling or bone healing

and motivate us to continue investigating other evidences about the possible modifiable involvement of osteocytes during bone remodeling.

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FIGURES AND TABLES:

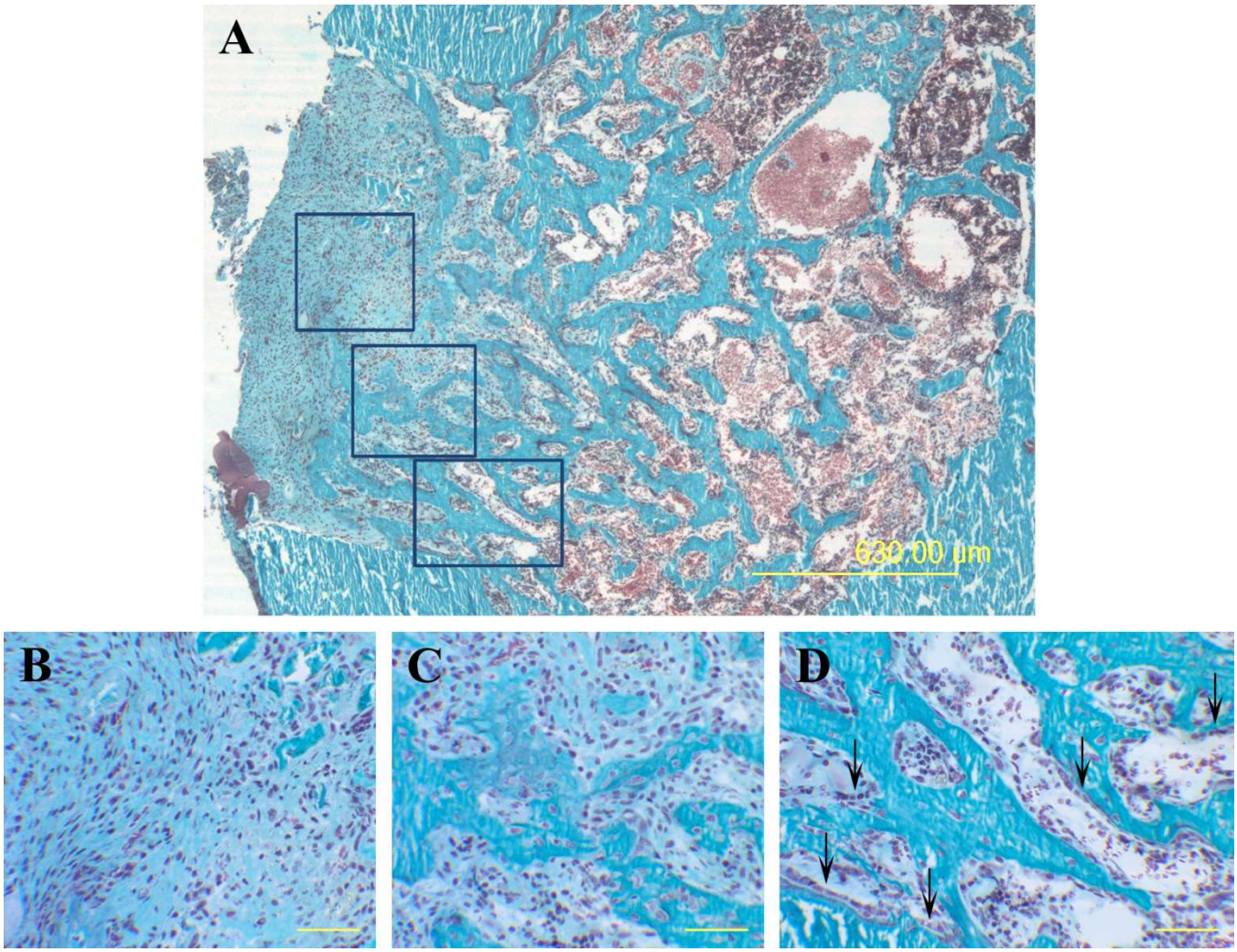

Fig. 1. Representative LM micrographs passing through the hole center, from a control rat 10 days after drilling, showing in A, from periosteal side (left) towards the endosteal one (right): highly vascularized connective tissue (enlarged in B), trabecular bony framework laid down by cords of stationary osteoblasts (enlarged in C), typical laminae of movable osteoblasts (arrows) differentiating on the surfaces of such SO-trabeculae (enlarged in D). B, C, D enlargements of the squared areas in A. Scale bars: A=630µm; B,C,D=50µm.

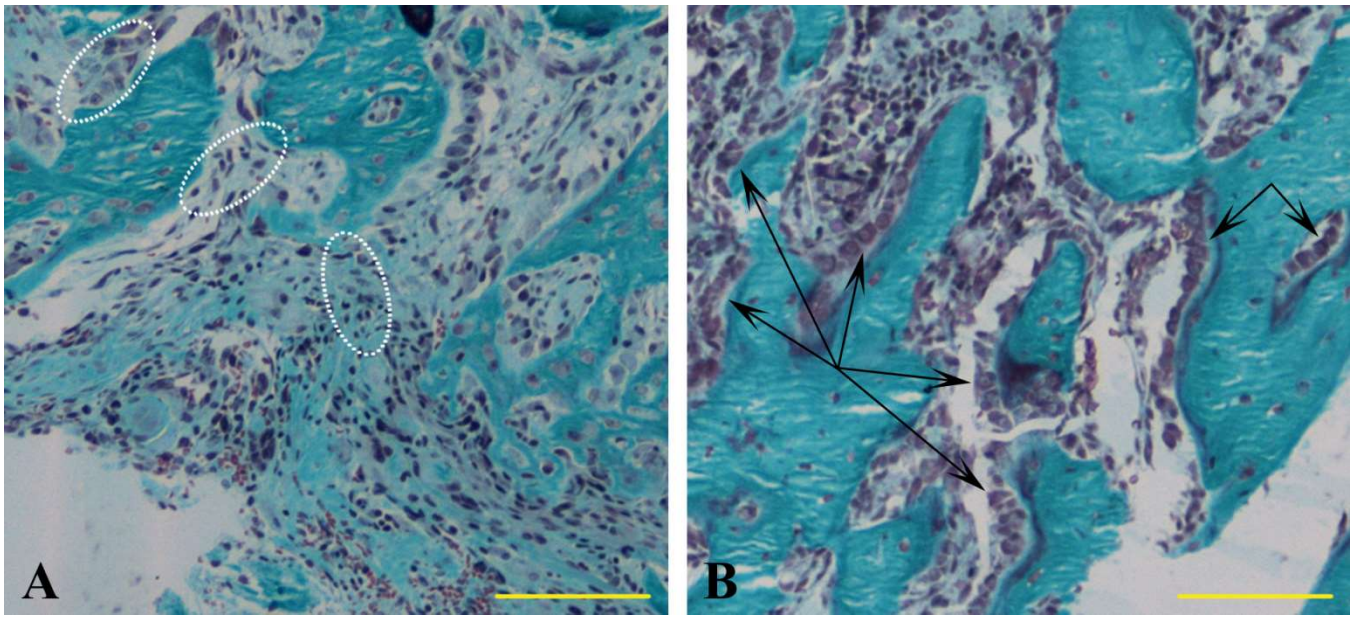

Fig. 2. Micrographs showing new bony trabeculae inside the hole in control (A) and treated (B) animals, 10 days after drilling. The cords of static osteoblasts are encircled by dotted white lines (A); the laminae of movable osteoblasts are pointed by black arrows (B). Scale bars: A,B=100µm.

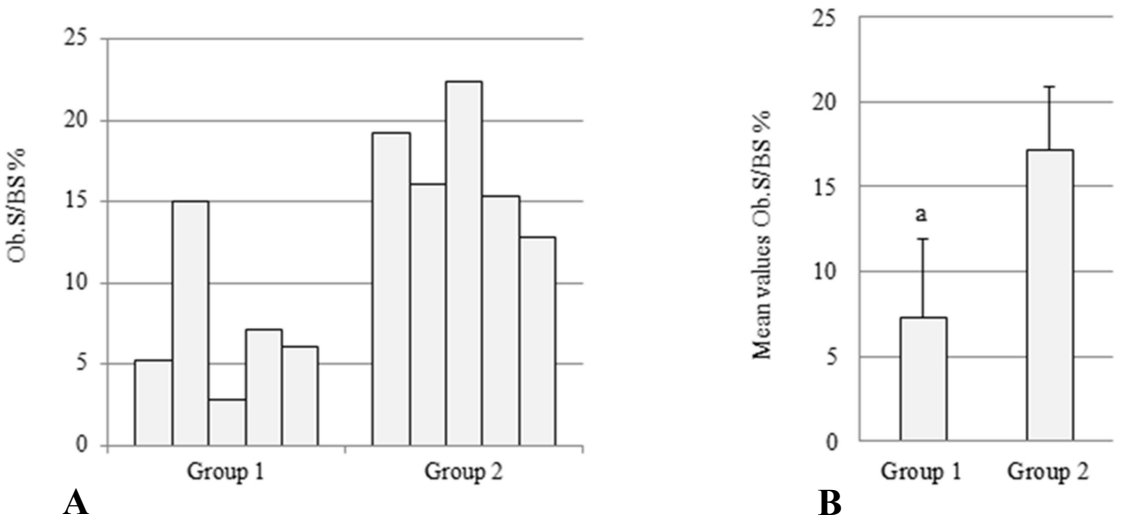

Fig. 3. Histogram (A) reporting the values of the extent of the surface of prismatic osteoblast laminae with respect to the bone surface (Ob.S/BS) in the drilled control (group 1) and the drilled/treated with PTH(1-34) (group 2), at 10 days from drilling. Histogram (B) reporting mean values for the same parameter; a=$p<0.05$

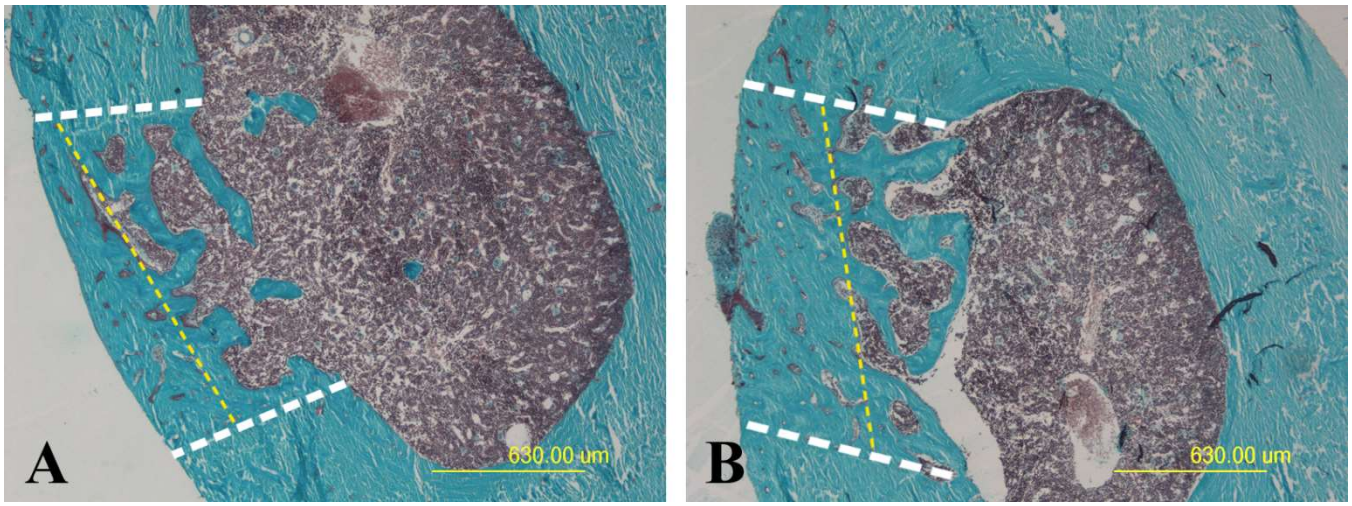

Fig. 4. Micrographs of transverse sections of the rat femurs passing through the center of the hole in drilled control (A) and drilled/treated with PTH(1-34) (B), at 28 days after drilling. The dotted yellow lines in A and B point the limit between compact (left) and trabecular (right) bone. The repaired holes are included between the dotted white lines. Note the greater amount of compact bone in drilled/treated with PTH(1-34) (B) with respect to drilled control (A). Scale bars: A,B=630µm.

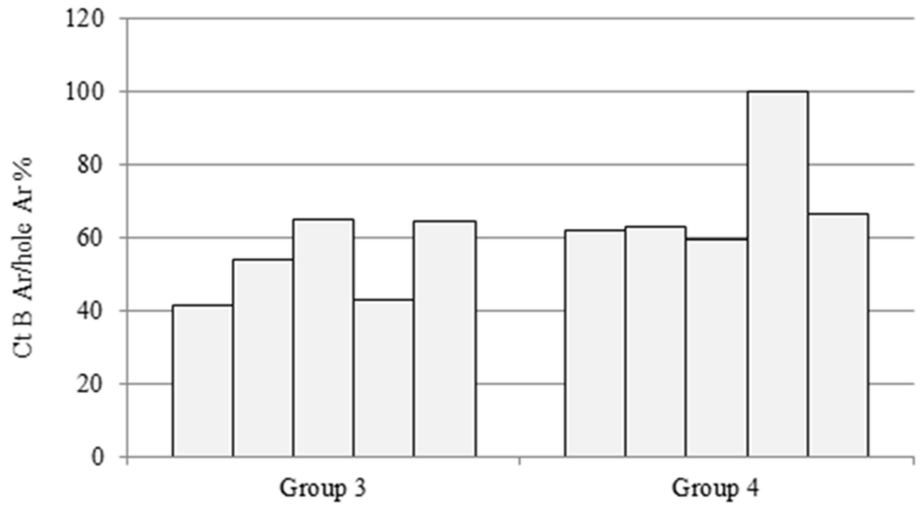

Fig. 5 Histogram reporting the amount of cortical compact bone (Ct B Ar) with respect to hole area (hole Ar), both expressed in percentage values in untreated animals (group 3) and in treated animals (group 4). Ct B Ar is about 70% in treated animals (group 4), about 53% in untreated animals (group 3), though without statistical significance.

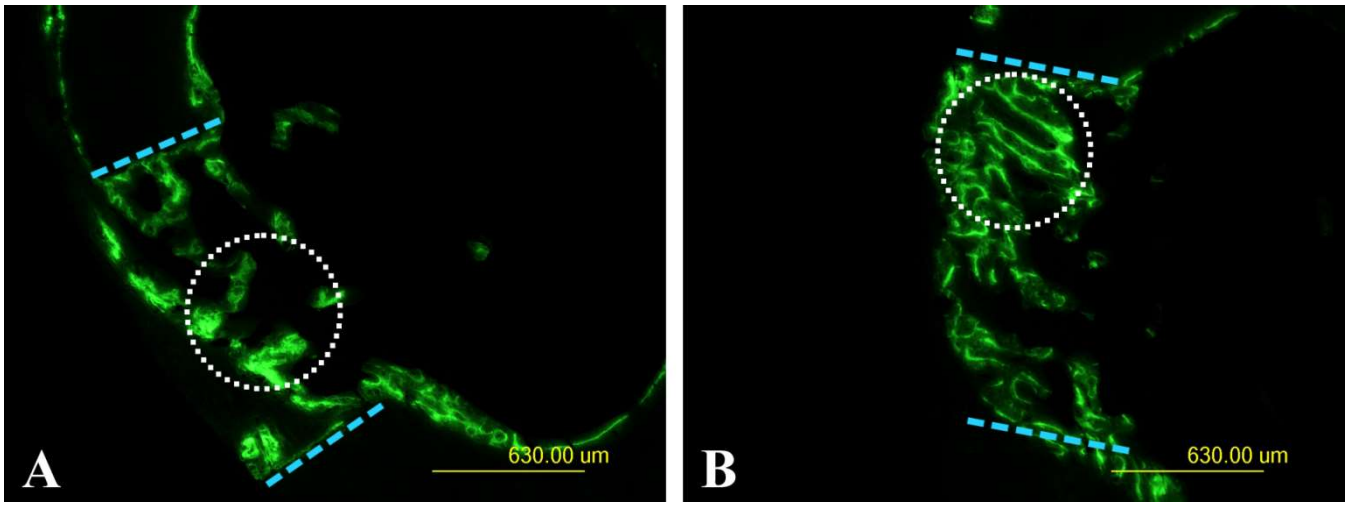

Fig. 6. Fluorescence micrographs of transverse sections of the rat femurs passing through the center of the hole in drilled control (A) and drilled/treated with PTH(1-34) (B) 28 days after drilling. The dotted blue lines indicate the borders of the hole. Green label shows the newly-formed bone at 10 days after drilling. The dotted white circles indicate the different morphology of labeled bone areas in the two animals (irregularly-shaped in A, linear in B). Scale bars: A,B=630µm.

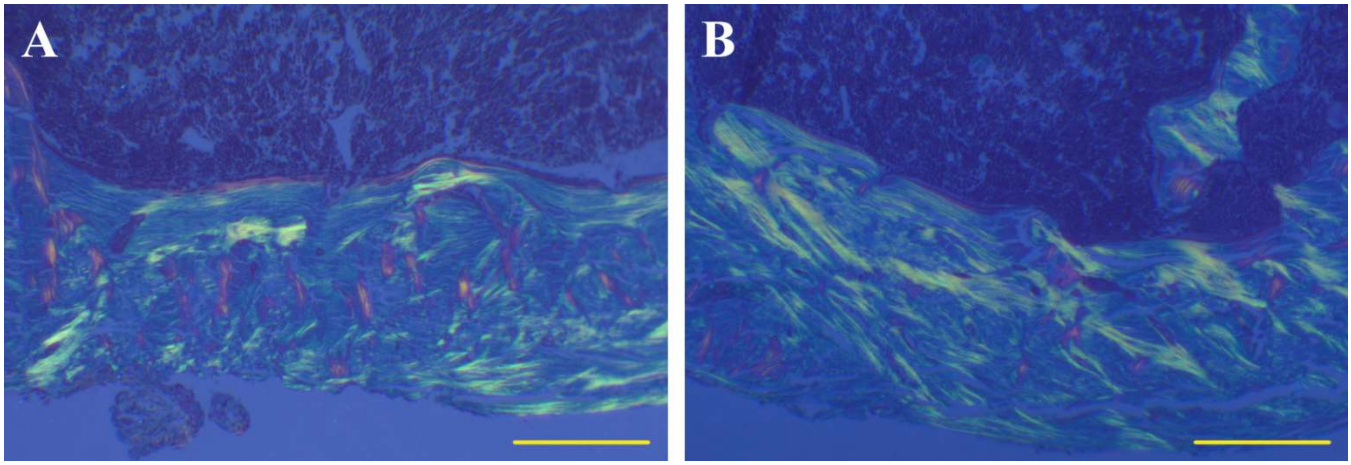

Fig. 7. Sections from drilled control and treated animals, 45 days after drilling, observed under TPL; in the central part of the hole, note in drilled control the abundance of extinguished woven-bone among the primary haversian spaces (A). Note also in drilled/treated with PTH (1-34) the abundance of birefringent lamellar bone outlining the primary haversian osteons (B). Scale bars: A,B=250µm.

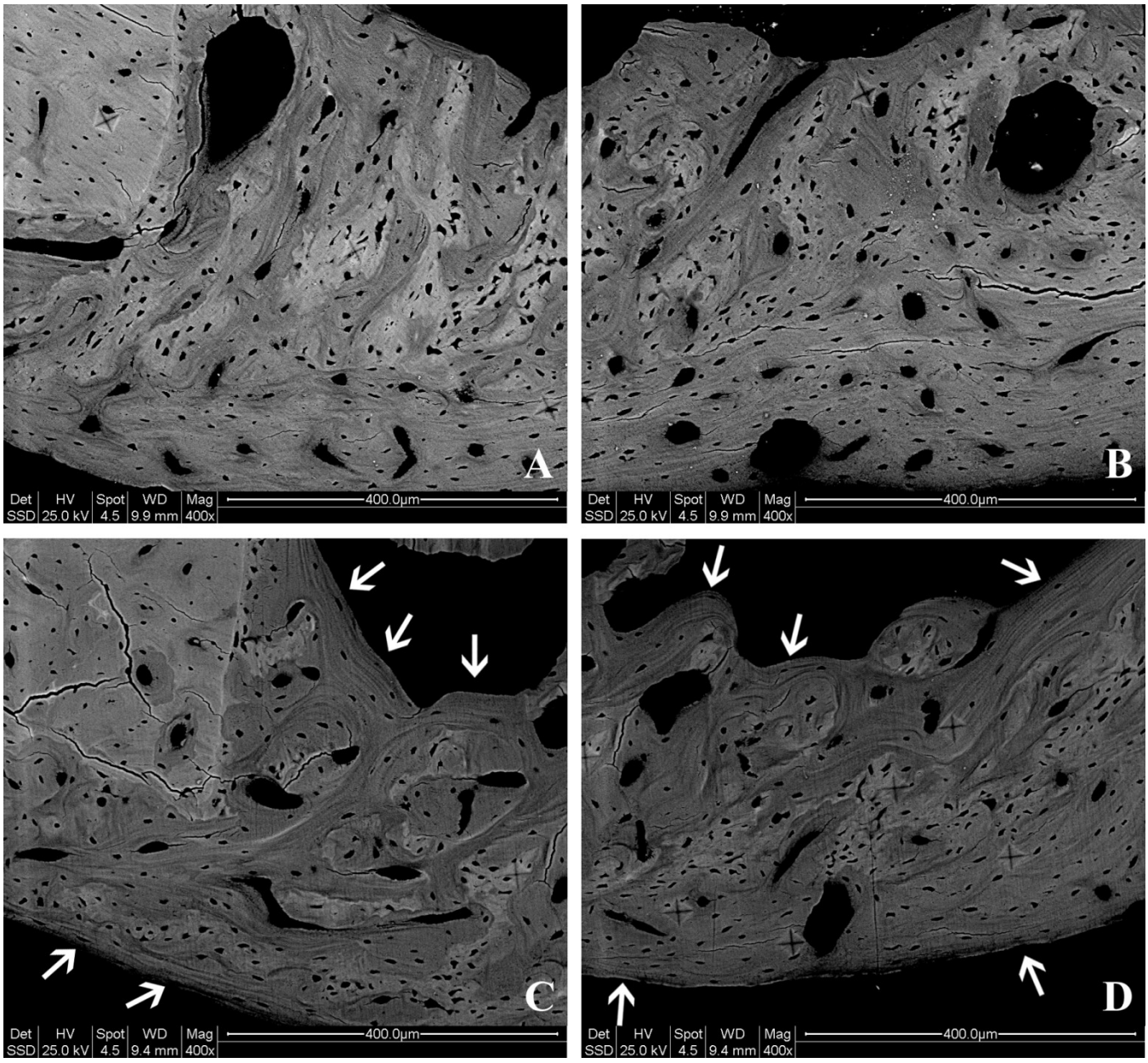

Fig. 8. Sections from drilled control and treated animals, 45 days after drilling, observed under SEM; note in drilled treated animals (C, D) the abundance of lamellar bone (arrows) with respect to the drilled control ones (A, B). Scale bars: A-D=400µm.

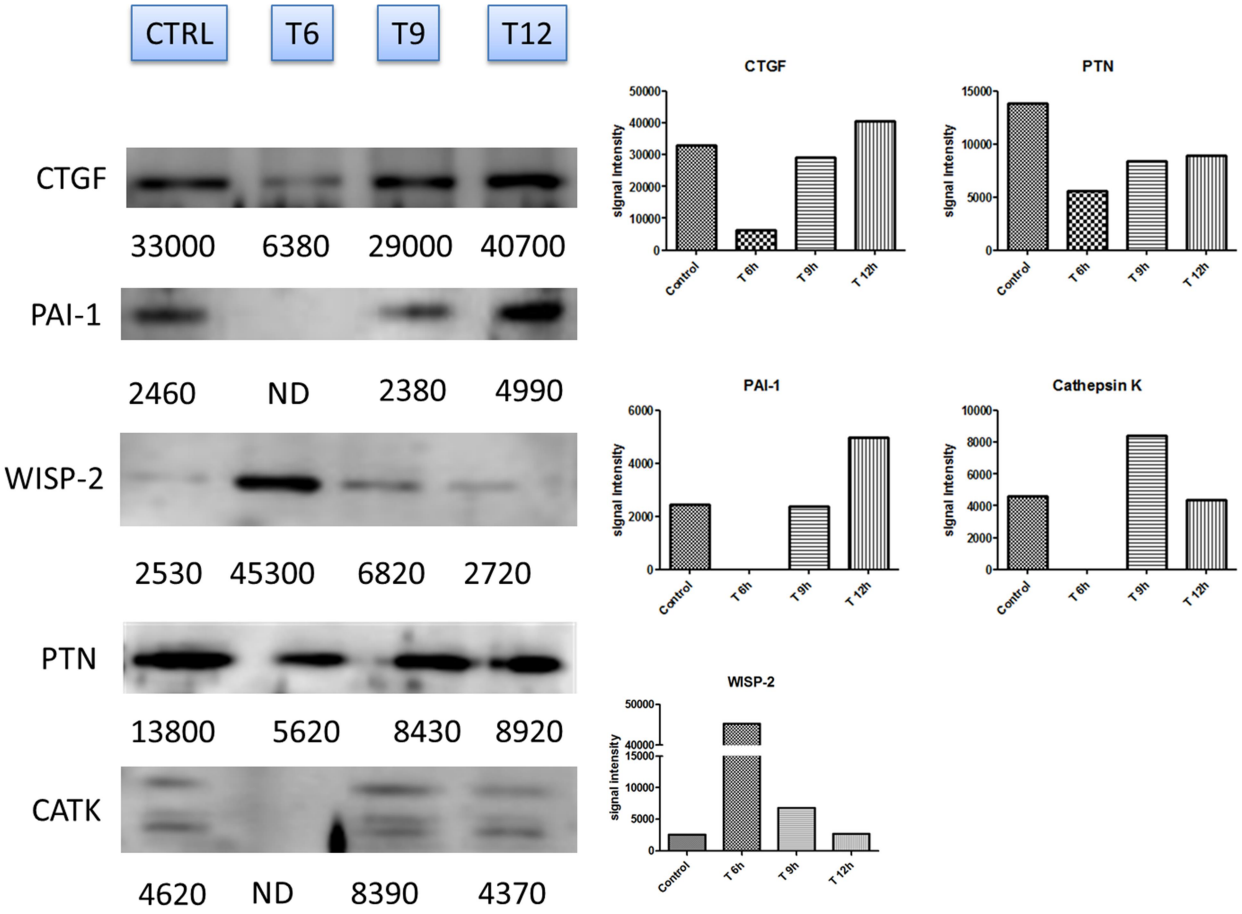

Fig. 9. Western Blots on 5 µg of MLO-Y4 extracted proteins, quantified by Bradford standard method. MLO-Y4 cell line was treated for 6, 9 and 12 hours with Teriparatide and tested for 5 different proteins: CTGF, PAI-1, WISP-2, PTN and CATHEPSIN K. Signal intensity of Western's bands were analyzed with Image Studio Lite 5.0 software (LI-COR Biosciences, NE, USA) and reported in graph.

Table 1. Histomorphometric parameter (BV/TV%) of newly-formed bone in healing holes in sections of femur diaphysis.

Time of sacrifice after drilling	10 days	28 days	45 days
BV/TV% - drilled control	49.9±8.4	68.7±7.4	72.6±5.3
BV/TV% - drilled/treated with PTH(1-34)	46.0±4.8	65.1±9.3	73.0±11.2

All values are expressed as mean±sd. Student's T test, using the Software STATA 11.0 (StataCorp, Texas, USA) was performed to compare means of treated and control groups, according to the time of sacrifice (10, 28, 45 days).

Table 2. Serum parameters (Ca, P, OPG, BALP, CTX, PTH) at the end of the experiments.

Group	Ca mg/dL	P mg/dL	OPG ng/ml	BALP ng/ml	CTX ng/mL	PTH pg/ml
1	10.35±0.29	5.64±0.48	0.84±0.11	7.70±0.62	49.42±8.10	88.17±73.89
2	10.03±0.43	6.74±1.45	0.73±0.04	7.13±0.54	47.64±20.84	51.94±32.34
3	10.16±0.18	6.93±1.29	0.67±0.10	6.54±1.25	56.31±19.20	67.82±36.17
4	9.87±0.34	6.59±1.26	0.73±0.03	6.79±0.37	50.06±21.70	52.29±20.30
5	10.17±0.26	6.84±0.41	0.69±0.05	6.34±1.43	47.59±7.79	77.85±35.81
6	10.25±0.22	6.40±0.42	0.67±0.03	6.72±0.28	64.7±38.48	55.94±23.98

Mean values ±standard deviation of serum parameters at the end of the experiments. Ca=total calcium, P=inorganic phosphorus, OPG=Osteoprotegerin, BALP=specific bone alkaline phosphatase, CTX=Beta CrossLaps; PTH= bioactive-intact PTH(1-84). Group 1: drilled control-10 days; Group 2: drilled/treated with PTH(1-34)-10 days, Group 3: drilled control-28 days; Group 4: drilled/treated with PTH(1-34)-28 days; Group 5: drilled control-45 days; Group 6: drilled/treated with PTH(1-34)-45 days.

Table 3. Gene expression in MLO-Y4, 6 *versus* 9 hours of treatment with Teriparatide.

Gene Symbol	p-value(T 6 vs. T 9)	Fold-Change(T 6 vs. T 9)	Fold-Change(T 6 vs. T 9) Description
Ptn	0,00111009	6,13175	T_6 up vs T_9
Trp53inp1	0,000599073	-5,39765	T_6 down vs T_9
Ctgf	1,65E-06	-8,35315	T_6 down vs T_9
Ptgs2	0,000264633	-5,71823	T_6 down vs T_9
Ddit3	2,95E-06	-5,82632	T_6 down vs T_9
Mlf1	0,000193185	-4,89741	T_6 down vs T_9
Aqp5	0,000110353	4,86473	T_6 up vs T_9
Ms4a4d	2,60E-05	4,26664	T_6 up vs T_9
Wisp2	0,000766724	5,48605	T_6 up vs T_9
Serpine1	0,000639025	-4,5177	T_6 down vs T_9
Slc7a11	0,00028404	-4,49664	T_6 down vs T_9
Car6	0,000427259	-4,45215	T_6 down vs T_9
Lce1g	0,00309292	-7,59097	T_6 down vs T_9
Adam23	0,00259201	4,56705	T_6 up vs T_9
Col6a3	6,14E-08	3,91329	T_6 up vs T_9
Scara3	0,000108654	7,19625	T_6 up vs T_9
Efemp1	4,51E-05	4,56942	T_6 up vs T_9
Eid3	0,000386376	-12,3754	T_6 down vs T_9
Pdlim5	1,17E-05	-4,8986	T_6 down vs T_9
Tchhl1	7,21E-05	-11,8671	T_6 down vs T_9
Plscr4	6,61E-05	4,09527	T_6 up vs T_9
Plcxd2	0,000246052	-4,3352	T_6 down vs T_9
Actr3b	0,000204675	6,78236	T_6 up vs T_9
Pdk1	2,94E-06	4,09838	T_6 up vs T_9
Dusp5	0,0010696	-5,5596	T_6 down vs T_9
Bpifc	7,22E-06	-8,74302	T_6 down vs T_9
Tgm2	9,70E-07	-10,8307	T_6 down vs T_9
Foxq1	1,53E-06	-4,03438	T_6 down vs T_9
Angpt1	0,00328868	4,63389	T_6 up vs T_9
Enpp2	9,81E-06	-4,95172	T_6 down vs T_9
Col6a1	7,92E-06	6,19945	T_6 up vs T_9
Cd68	0,000129271	-4,52759	T_6 down vs T_9
Ctsk	2,00E-08	-4,08632	T_6 down vs T_9
Chac1	7,10E-06	-4,60005	T_6 down vs T_9
Havcr2	8,12E-05	-5,83701	T_6 down vs T_9
Col6a2	2,61E-06	5,15838	T_6 up vs T_9
Tspan17	7,13E-05	4,36627	T_6 up vs T_9

Trib3	5,00E-08	-6,35465	T_6 down vs T_9
Cox6a2	6,61E-05	-5,10223	T_6 down vs T_9
P2rx3	0,000345796	-4,45986	T_6 down vs T_9

Table 4. Gene expression of selected genes with proved bone metabolism functions.

Gene Symbol	p-value(T_6 vs. T_9)	Fold-Change(T_6 vs. T_9)	Fold-Change(T_6 vs. T_9) (Description)
Ptn	0,00111009	6,13175	T_6 up vs T_9
Ctgf	1,65E-06	-8,35315	T_6 down vs T_9
Aqp5	0,000110353	4,86473	T_6 up vs T_9
Wisp2	0,000766724	5,48605	T_6 up vs T_9
Serpine1	0,000639025	-4,5177	T_6 down vs T_9
Scara3	0,000108654	7,19625	T_6 up vs T_9
Efemp1	4,51E-05	4,56942	T_6 up vs T_9
Pdk1	2,94E-06	4,09838	T_6 up vs T_9
Tgm2	9,70E-07	-10,8307	T_6 down vs T_9
Angpt1	0,00328868	4,63389	T_6 up vs T_9
Col6a1	7,92E-06	6,19945	T_6 up vs T_9
Ctsk	2,00E-08	-4,08632	T_6 down vs T_9
Trib3	5,00E-08	-6,35465	T_6 down vs T_9

Table 3 shows the 40 genes with a Fold-change higher than 4, regulated by Teriparatide in MLO-Y4 cell line. 6 hours of treatment were compared with 9 hours of treatment. Table 4 shows the 13 genes (selected among the initial 40) that have been reported in literature to be involved in bone metabolism. Highlighted in blue are the genes selected for further investigation. All the analyses were performed using the analysis of variance (one-way ANOVA) test.

Table 5. Gene names and symbols

Gene Symbol	Gene Title
Ptn	pleiotrophin
Trp53inp1	transformation related protein 53 inducible nuclear protein 1
Ctgf	connective tissue growth factor
Ptgs2	prostaglandin-endoperoxide synthase 2
Ddit3	DNA-damage inducible transcript 3
Mlf1	myeloid leukemia factor 1
Aqp5	aquaporin 5
Ms4a4d	membrane-spanning 4-domains, subfamily A, member 4D
Wisp2	WNT1 inducible signaling pathway protein 2
Serpine1	serine (or cysteine) peptidase inhibitor, clade E, member 1
Slc7a11	solute carrier family 7 (cationic amino acid transporter, y+ system), member 11
Car6	carbonic anhydrase 6
Lce1g	late cornified envelope 1G
Adam23	a disintegrin and metallopeptidase domain 23
Col6a3	collagen, type VI, alpha 3
Scara3	scavenger receptor class A, member 3
Efemp1	epidermal growth factor-containing fibulin-like extracellular matrix protein 1
Eid3	EP300 interacting inhibitor of differentiation 3
Pdlim5	PDZ and LIM domain 5
Tchhl1	trichohyalin-like 1
Plscr4	phospholipid scramblase 4
Plcxd2	phosphatidylinositol-specific phospholipase C, X domain containing 2
Actr3b	ARP3 actin-related protein 3B
Pdk1	pyruvate dehydrogenase kinase, isoenzyme 1
Dusp5	dual specificity phosphatase 5
Bpifc	BPI fold containing family C
Tgm2	transglutaminase 2, C polypeptide
Foxq1	forkhead box Q1
Angpt1	angiopoietin 1
Enpp2	ectonucleotide pyrophosphatase/phosphodiesterase 2
Col6a1	collagen, type VI, alpha 1
Cd68	CD68 antigen
Ctsk	cathepsin K
Chac1	ChaC, cation transport regulator 1
Havcr2	hepatitis A virus cellular receptor 2
Col6a2	collagen, type VI, alpha 2
Tspan17	tetraspanin 17

Trib3	tribbles homolog 3 (Drosophila)
Cox6a2	cytochrome c oxidase subunit VIa polypeptide 2
P2rx3	purinergic receptor P2X, ligand-gated ion channel, 3

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