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The Regulatory Roles of MicroRNAs in Bone Remodeling and Osteoporosis

Dissertation presented by
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Work carried out under the supervision of **Dr. Natalia Garcia Giralt** and **Dr. Adolfo Díez Pérez** in the Musculoskeletal Research Group (Inflammatory and Cardiovascular Disorders Program) at the Institut Hospital del Mar d'Investigacions Mèdiques (IMIM)
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La ciència sense consciència,
no és més que la ruïna de l'ànima

François Rabelais
(1494-1553)

A mi familia,

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– THESIS ABSTRACT –

In bone field, microRNAs (miRNAs) have been described as key factors regulating bone formation, remodeling, and homeostasis. The identification of miRNAs involved in skeletal function will be essential to the development of miRNA-based therapeutic strategies for bone disorders. As with other regulatory molecules, miRNAs are frequently subject to change during the development of human diseases. In this regard, we identified a subset of miRNAs with altered expression in osteoporotic bone and demonstrate the functional involvement of some of those miRNAs in the regulation of bone formation and the pathways regulating the progression of osteoporosis. We also have depicted an overview of miRNAs in the human bone tissue and in primary bone cells. Furthermore, we have identified genetic variants in human osteoblast-related miRNAs associated with bone mineral density and this association was functionally demonstrated in bone and osteoblast samples. This work has provided evidences of the marked complexity behind this regulatory system and opens novel prospect for research and therapy.

- RESUM DE LA TESI -

En l'àmbit de l'estudi de l'òs, els microRNAs (miRNAs) han estat descrits com factors claus en la regulació de la formació, remodelatge i homeòstasis de l' òs. La identificació de miRNAs implicats en la funció esquelètica és imprescindible pel desenvolupament de noves estratègies terapèutiques, basades en miRNAs, dirigides al tractament de malalties òssies. Com en el cas d'altres molècules reguladores, els miRNAs poden patir modificacions durant el desenvolupament de malalties humanes. En aquest sentit, hem identificat un grup de miRNAs amb una expressió alterada en l'òs osteoporòtic i hem demostrat la implicació funcional d'algun d'aquests miRNAs en la regulació de la formació òssia i els mecanismes pels quals es produiria l'osteoporosi. Alhora, també hem ofert una visió general dels miRNAs presents en el teixit ossi humà i en les cèl·lules òssies. També hem identificat variants genètiques dins de les seqüències de miRNAs expressats en osteoblasts, que han estat associades amb la densitat mineral òssia. A més a més, aquesta associació ha estat funcionalment demostrada en òs i osteoblasts. Aquest treball reflexa l'elevada complexitat que hi ha darrera del sistema regulador per miRNAs i obre nous camins per la recerca i la teràpia.

– PREFACE –

Osteoporotic fractures are one of the most common causes of disability and a critical contributor to high medical care cost all over the world. Bone mass and bone microarchitecture are the major contributors to the fracture risk, but the biological mechanisms involved in the osteoporotic phenotype are still yet to be defined. Moreover, even if current anti-osteoporotic drugs have contributed to reduce the risk of fracture, side effects and problems of low adherence derived from those treatments are in the order of the day. Hence, it is necessary to deepen the knowledge about the factors which contribute to the osteoporotic phenotype, the development of a good diagnostic method for patients at fracture risk, and last but not least, the creation of an ideal treatment for patients. Since their discovery around two decades ago, microRNAs have become important regulators in several cellular processes including those related to bone metabolism. Moreover, microRNAs hold great promise due to their dual role as biomarkers and therapeutic targets. This work has performed a deep research on miRNAs involved in the osteoporotic phenotypes using diverse genetic and cellular approaches in order to embrace a wide vision on miRNA functionality in bone context. Our results provide novel insights on the miRNA signature of the bone tissue and how those miRNAs might be helpful to broad our knowledge on bone disorders such as osteoporosis.

– TABLE OF CONTENTS –

– ACRONYMS AND ABBREVIATIONS –

ACTA2	Actin Alpha 2
AGO	Argonaute
ALP	Alkaline Phosphatase
BMD	Bone Mineral Density
BMI	Body Mass Index
BMPs	Bone Morphogenetic Proteins
BSP	Bone Sialoprotein
Cbfa1	Core-Binding Factor α subunit
COLIA1	Collagen Type Iα 1
CT	Computed Tomography
CTX	Cross-linked carboxyterminal-telopeptide
DGCR8	DiGeorge syndrome Critical Region gene
DKK1	Dickkopf-Related Protein 1
DMP-1	Dentin Matrix Protein 1
DXA	Dual energy X-ray densitometry
ECM	Extracellular Matrix
eIFs	Eukaryotic Initiation Factors
ELOSA	Enzyme Linked Oligosorbent Assay
ENCODE	Encyclopedia of DNA Elements
ERα	Estrogen Receptor α
EXP5	Protein Exportin 5
FC	Fold change
FGF2	Fibroblast Growth Factor 2
FGF-23	Fibroblast Growth Factor 23
FN	Femoral Neck
FRAX	Fracture Risk Assessment
GEFOS	Genetic Factors for Osteoporosis
GENOMOS	Genetic Markers for Osteoporosis
GWAS	Genome-Wide Association Studies
hOBs	Primary Human Osteoblasts
hOCs	Primary Human Osteoclasts

HSCs	Hematopoietic Stem Cells
IGF-1	Insulin-like Growth Factor-1
IGF1R	Insulin-like Growth Factor 1 Receptor
ILK	Integrin Linked Kinase
IPA	Ingenuity Pathways Analysis
LRP5	Low Density Lipoprotein Receptor-Related Protein 5
LRP6	Low Density Lipoprotein Receptor-Related Protein 6
LS	Lumbar Spine
LXR	Liver X Receptor
MAPK	Mitogen-Activated Protein Kinase
M-CSF	Macrophage Colony-Stimulating Factor
MEPE	Matrix Extracellular Phosphoglycoprotein
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
MSC	Mesenchymal Stem Cell
nc RNAs	non-coding RNAs
NO	Nitric Oxide
NTX	Cross-linked aminoterminal-telopeptide
OCN	Osteocalcin
OPG	Osteoprotegerin
OPN	Osteopontin
OSX	Osterix
PABP	Poly (A)-Binding Protein
PACT	Protein kinase R-Activating protein
PAZ	PIWI-AGO-ZWILLE
PBMC	Peripheral Blood Mononuclear Cells
PGE2	Prostaglandin E2
PGI2	Prostacyclin
PHEX	Phosphate-regulating gene with Homologies to Endopeptidases on the X chromosome
PICP	Carboxyterminal Propeptide
PINP	Aminoterminal Propeptide
piRNAs	piwi-interacting RNAs
PPARs	Peroxisome Proliferator-Activated Receptors

PTH	Parathyroid Hormone
RANKL	Receptor Activator of NF-κB Ligand
RIN	RNA Integrity Number
RISC	RNA-Induced Silencing Complex
RMA	Robust Multi-chip Average
RQ	Relative Quantification
Runx2	Runt-Related Transcription Factor 2
RXR	Retinoid X Receptor
SD	Standard Deviation
SE	Standard Error
SERMs	Selective Estrogen Receptor Modulators
siRNAs	small interfering RNAs
Snord68	Small nucleolar RNA, C/D box 68
SNPs	Single Nucleotide Polymorphisms
SOCS1	Suppressor of Cytokine Signaling 1
SOST	Sclerostin
STC1	Stanniocalcin 1
TBS	Trabecular Bone Score
TFIIB	Transcription Factor II B
TGF-β	Transforming Growth Factor Beta
TNF-α	Tumor Necrosis Factor Alpha
TRAP	Tartrate-Resistant Acid Phosphatase
TRBP	TAR RNA-Binding Protein
UTR	Untranslated Region
WHO	World Health Organization
XRN1	Exoribonuclease 1

INTRODUCTION

1. Bone tissue

Bone is a mineralized connective tissue that exerts multiple important functions in the body: locomotion, support and protection of vital organs and soft tissues and harboring of bone marrow. It is also the main reservoir of calcium and phosphate in the body, thus contributing to the mineral homeostasis[1]. More recently, bone has been described as an endocrine organ for its whole-body regulatory functions including its role in glucose homeostasis and energy expenditure[2,3], evidencing the complexity and dynamic nature of this tissue.

The bone tissue is made up of cells, vessels and an extracellular matrix. In bone segments, two types of architectures are macroscopically identifiable: cortical and trabecular bone (Figure I-1). Cortical bone, also known as compact bone, represents 80 % of the mature skeleton and provides mechanical strength and protection. It is extremely dense, hard, with low porosity and is mostly calcified. It has a slow turnover rate and constitutes mainly the diaphysis of long bones. Trabecular bone, also known as cancellous bone, is less dense and has a higher turnover rate, which makes trabecular bone more suitable for metabolic functions. It mainly constitutes the epiphysis of long bones as well as flat and isodiametric bones[4].

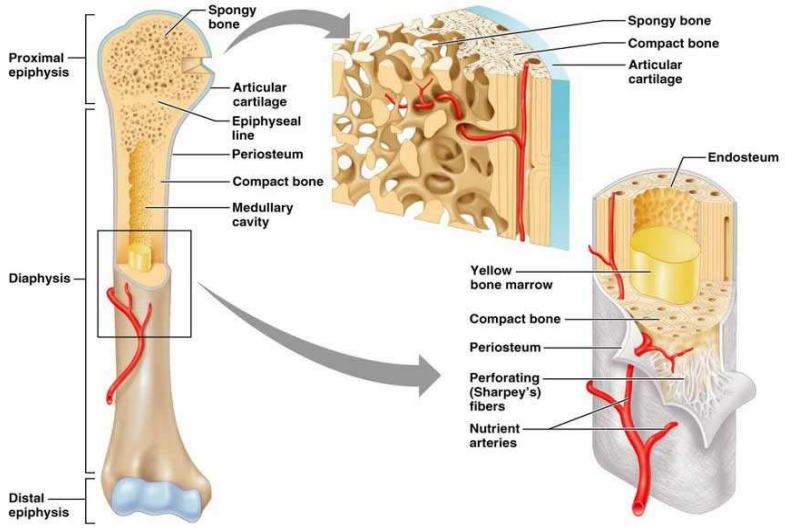


Figure I-1. Schematic structure of long bone: Spongy bone (trabecular) and compact bone (cortical) localization. Extracted from http://classes.midlandstech.edu/carterp/Courses/bio210/chap06/lecture1.html.

1.1 The composition of bone

The bone tissue is formed by mesenchyme-derived cells and an extracellular matrix (ECM) produced by these cells. The extracellular matrix comprises approximately 90% of bone volume while the cells and blood vessels account for the remaining 10%[4].

1.1.1 Extracellular matrix

Bone matrix not only provides a mechanical support for bone cells, but also has a key role in regulating cell behavior which maintains homeostasis, bone remodeling and ability for wound healing[5]. The ECM is mainly synthesized by osteoblasts and is composed of both an organic and inorganic component.

The organic fraction is mainly made up of type I collagen fibers and represents about 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. The organization of collagen fibers is essential for the bone strength and mechanical properties[6-8]. Non-collagenous proteins are also present in the organic matrix, although to a minor extent. They consist in proteoglycans, adhesion molecules such as fibronectin and specialized proteins including bone sialoprotein (BSP), osteocalcin (OCN) and osteopontin (OPN).

The inorganic or mineral fraction represents the major portion of the ECM, and is mainly formed by crystals of hydroxyapatite, which are deposited in the collagen fibers. This inorganic fraction is the main mineral reservoir of the body, containing 99% of the calcium, 85% of phosphates and between 40 to 60 % of the sodium and magnesium[4].

1.1.2. 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 these bone cells.

1.1.2.1 Osteoblasts and Bone Formation

Osteoblasts are well known for their function in bone formation and account for the 4–6% of total resident cells in the bone. Osteoblasts derive from mesenchymal stem cell (MSC) precursors that also have the potential to differentiate into adipocytes, chondrocytes, fibroblasts or myoblasts. Differentiation to the osteochondrogenic lineage depends on the presence of specific sets of cytokines and transcription factors (Figure I-2).

Among factors involved in osteoblast differentiation are Hedgehogs, bone morphogenetic proteins (BMPs), transforming growth factor beta (TGF-β) and parathyroid hormone (PTH)[9,10]. BMPs are expressed in skeletal tissue and are required for adult bone mass maintenance and skeletal development. The role of BMP2 in osteoblast differentiation has been extensively characterized and it has been shown to regulate Runt-related transcription factor 2 (*RUNX2*) expression[11-13]. RUNX2, also known as core-binding factor α subunit (Cbfa1), is the earliest marker of osteogenesis and it plays a crucial role in osteoblast differentiation[10,14]. In fact, Runx2-deficient mice lacked of osteoblasts and exhibited an almost complete absence of mineralized bone tissue[15]. In addition, RUNX2 regulates Osterix (*Osx*), another critical transcription factor involved in bone formation[16]. Moreover, RUNX2 can upregulate other osteoblast-related genes such as Collagen Type Iα 1 (*COLIA1)*, alkaline phosphatase (*ALP*), *BSP* and *OCN*[17].

Wnt signaling pathway is also one of the key pathways involved in osteoblast differentiation and maturation[18]. Wnts are a family of secreted glycoproteins that bind to receptor complexes including Low Density Lipoprotein Receptor-Related Protein 5 (LRP5) and 6 (LRP6), as well as frizzled proteins[19,20]. After this binding, intracellular cascades of events lead to stable β-catenin, which allows the regulation of multiple transcriptions factors[21]. Wnt pathway promotes osteoblast differentiation, is active even in differentiated osteoblasts[22], and it has been shown to enhance bone formation[23]. Indeed, loss or gain of function mutations in LRP5 have been associated with bone diseases characterized by low or high bone mass due to dysregulation in osteoblast activity[24-27]. Additionally, a mutation in LRP6 underlies early onset familial coronary artery disease and osteoporosis[28].

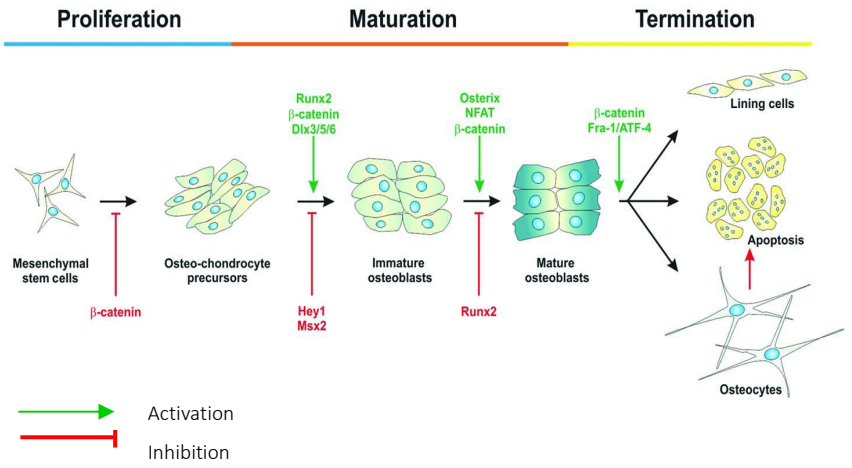


Figure I-2. Mesenchymal stem cell differentiation toward the osteoblastic lineage. Adapted from de Gorter and ten Dijke, 2013[29].

Osteoblasts are responsible of the synthesis of bone matrix, which involves two main steps, deposition of organic matrix and its subsequent mineralization. First, they secrete osteoid, which is the organic component of bone, mainly made of type I collagen and other non-collagen proteins and proteoglycans. Thereafter, matrix vesicles are released from the plasma membrane of osteoblasts into the newly formed bone matrix[30]. These vesicles contain phosphate and calcium ions which nucleate forming the hydroxyapatite crystals. The supersaturation of these ions inside the vesicles leads to the rupture of these matrix vesicles and the hydroxyapatite crystals spread to the surrounding extracellular matrix and deposit between the collagen fibrils[1]. ALP is one of the main proteins required for the deposition of hydroxyapatite-based mineral[31,32]. At this point, osteoblasts can undergo apoptosis or differentiate either to osteocytes or to bone lining cells[33].

Osteoblasts produce crucial factors involved in osteoclast formation, differentiation and survival. They can promote osteoclastogenesis by producing the Receptor Activator of NF-κB Ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) as well as inhibit osteoclastogenesis by the expression of osteoprotegerin (OPG)[34].

A protein often associated with bone metabolism is Leptin, which mostly is known to play a role in weight regulation by adjusting food intake[35]. What is less known about this protein is that 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[36,37].

Osteoblasts also contribute to the endocrine function of bone. Hence, OCN which is mainly produced by osteoblasts and odontoblasts, can affect other organs such as pancreas, testis and adipose tissue[38-40].

1.1.2.2 Osteoclasts and Bone Resorption

Bone resorption is decisive for many skeletal processes including bone growth, tooth eruption, fracture healing, and bone remodeling. Several human diseases of the bone are characterized by an enhanced or decreased bone resorption event, thus emphasizing the key role of this mechanism in the skeleton function.

Osteoclasts, the exclusive bone resorptive cell, approximately represent the 1–2% of total bone resident cells. They are terminally differentiated giant multinucleated cells which derive from the fusion of mononuclear progenitors of the monocyte/macrophage hematopoietic lineage[41].

Osteoclast formation and differentiation is controlled by M-CSF and RANKL, two cytokines expressed by osteoblasts and stromal cells[42] (Figure I-3). Hematopoietic stem cells (HSCs) commit to the myeloid lineage, express on their surface c-Fms and RANK, which are the receptors for M-CSF and RANKL respectively, and differentiate into osteoclasts. M-CSF contributes to the proliferation, differentiation and survival of osteoclasts precursors[43].

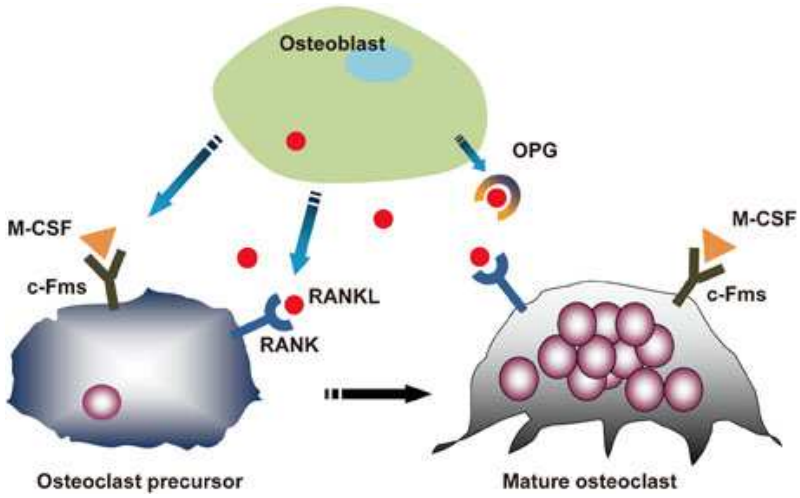


Figure I-3. Osteoclastogenesis regulation mediated by osteoblasts. Osteoblasts promote osteoclast formation by expressing RANKL and M-CSF. In the other hand, osteoblast can inhibit the synthesis of osteoclast by expressing OPG. Extracted from Lee *et al.,* 2010[44].

Other key protein involved in osteoclast formation is OPG, a secreted decoy receptor of RANKL. OPG inhibits osteoclast differentiation by binding to RANKL and preventing its interaction with RANK[34]. Thus, the ratio RANKL/OPG is crucial in the osteoclast formation process and therefore, in the bone resorption regulation[45]. Osteoclastogenesis is also regulated by other factors including vitamin D3, PTH, interleukins such as IL-1β, IL-6, IL-11, IL-17 and tumor necrosis factor alpha (TNF-α)[46-48]. Additionally, it has been

reported that estrogens inhibit osteoclast formation and activity. This phenomenon is mediated by the inhibition of the synthesis of RANKL by osteoblasts and osteocytes[49] and the stimulation of these cells to produce OPG[50] as well as by the reduction of other osteoclastogenic cytokines such as IL-1, IL-6 and TNF-α, among others[51]. Moreover, estrogens can also induce osteoclast apoptosis[52].

Osteoclasts are equipped by efficient and unique machinery made to dissolve mineral and degrade organic bone matrix. The initial event in bone resorption is the attachment of osteoclasts to its target matrix. The psychical interaction between the osteoclast and bone matrix is mediated by integrins such as αvβ3, the main integrin mediating bone resorption[53]. Other event required for the degradation of skeletal tissue is the polarization of osteoclasts which involves the rearrangement of the actin cytoskeleton. This restructuration leads to the formation of the sealing zone and ruffled border in contact with the bone matrix and in the other hand, a basolateral and functional secretory domain which are not in contact with the bone surface[54]. The sealing zone is formed by an actin ring and other proteins involving actin, fibrin and dynamin[1]. Both the ruffled membrane and the actin ring are hallmarks of the degradative capacity of functional osteoclasts.

After migration of osteoclasts to a resorption site, an isolated microenvironment between them and the underlying bone matrix is formed. This space, also referred as the "sealing" zone, is then acidified by a proton pump H^{+-} ATPase located in the ruffled border and a Cl-channel (Figure I-4). Because of this acidification, the mineralized fraction of bone is mobilized and subsequently, the demineralized organic component, mainly type I collagen, is degraded by Cathepsin K[55,56] which has been largely established as an osteoclast marker. Finally, the products released during bone

degradation are removed by vesicular transcytosis[57]. The tartrate-resistant acid phosphatase (TRAP), other well-known osteoclastic marker, participates in the transportation and elimination of the bone matrix degradation products[58].

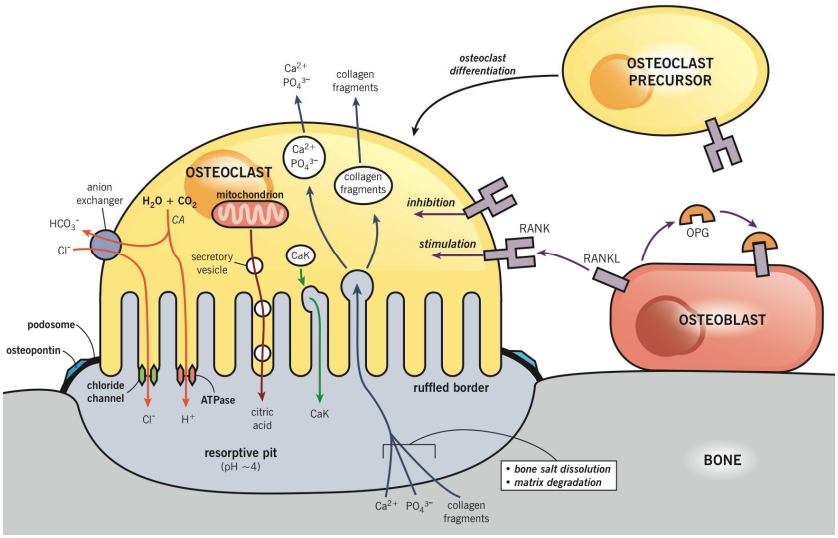


Figure I-4. Osteoclast mediated bone resorption. The osteoclast apical membrane faces the bone and the sealing zone generates an isolated region. Hydrogen ions are also released through the actions of carbonic anhydrase and vacuolar ATPase proton pumps, creating an acidic environment in the resorptive area. Cathepsin K breaks down the collagen bone matrix; the resulting collagen fragments and soluble calcium and phosphate are released to the circulation. Extracted from http://www.pathophys.org/osteoporosis/.

It is important to highlight that other functions, besides bone resorption, are displayed by osteoclasts including the regulation of both osteoblastic cells[45] and the hematopoietic stem cell niche[59].

1.1.2.3 Osteocytes as Bone Orchestrators

Osteocytes, which comprise 90 to 95% of all bone cells in adult bone, are the most abundant and long-lived bone cells, with a lifespan of up to 25 years within their mineralized environment[60]. Osteocytes are terminally differentiated osteoblasts that become surrounded by matrix during the process of bone formation.

Once they become embedded in bone within spaces called *lacunae*, the cell undergoes a transformation from polygonal shape to a dendritic morphology. These cells are connected to each other and to other cells on the bone surface through dendritic processes within small structures called canaliculi[61]. Moreover this communication goes beyond the bone surface up to reaching the bone marrow[62]. This characteristic morphology of osteocytes, stellate shaped cell bodies and dendritic processes, are thought to contribute to their mechanosensory function.

The deeply embedding in which the osteocytes are buried within the bone matrix is ideal for their function as mechanosensors. However, this location make difficult their isolation and study; this is one of the reasons why for years researchers preferred to focus their attention on the more accessible osteoblasts, located on the bone surfaces. Moreover, for a long time osteocytes were considered “passive quiescent cells” without any active role in bone physiology. Thanks to the development of new technologies such as electron microscopy, new animal models, osteocyte-like cell lines, molecular imaging and bioinformatics approaches, osteocytes have become the target of intensive investigation.

During the last decades several studies related to osteocyte differentiation were extensively performed providing new information about osteoblast-to-

osteocyte cell transition. In this regard, Woo *et al*. showed the characterization of a promising cell line called IDG-SW3, which replicated the transition process from late osteoblast to late osteocytes[63].

As the osteoblast transitions to an osteocyte, many of the previously expressed osteoblast markers, including collagen type I and ALP are downregulated or switched off, while osteocyte markers, such as 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 (SOST) and ORP150 are upregulated (Figure I-5).

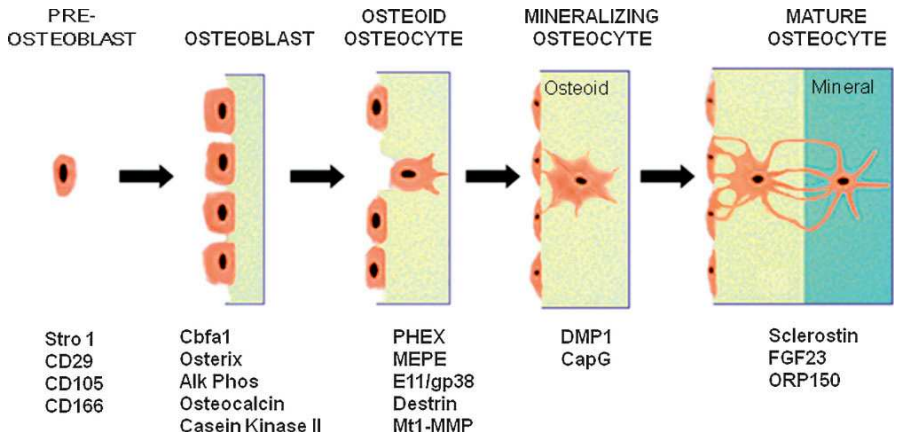


Figure I-5. Expression markers changes during osteoblast to osteocyte differentiation. Extracted from Bonewald *et al*., 2011[60].

Interestingly, 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[64], the so-called osteocytic osteolysis.

Osteocytes play a key regulatory role in the biological, functional and architectural integrity of the skeleton. This contribution is reflected by

several functions displayed by these cells such as modification of its extracellular microenvironment, regulation of bone remodeling by translating the mechanical stimuli into biochemical signals, bone mineralization, calcium and phosphate homeostasis regulation and also their function as an endocrine cells[65-68].

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[67,69,70] (Figure I-6). For instance, mechanical loading stimulates osteocyte to produce factors that exert anabolic action on bone such as prostaglandin E2 (PGE2), prostacyclin (PGI2), nitric oxide (NO), and insulin-like growth factor-1(IGF-1)[1]. In the other hand, mechanical unloading reduces the levels of anabolic factors and stimulates osteocytes to enhance the production of inhibitors of osteoblast activity such as Sclerostin and Dickkopf-related protein 1 (DKK1)[67,71], which are inhibitors of the Wnt//β-catenin signaling. Actually, there is a clear evidence of the involvement of Wnt signaling in osteocytes to sense and transduce the signals of mechanical stimuli to bone cells[72]. For instance, Jahaveri *et al.* demonstrated that deletion of a single allele of β-catenin in osteocytes abolishes the bone anabolic response to loading, suggesting that both alleles are needed for such response[73].

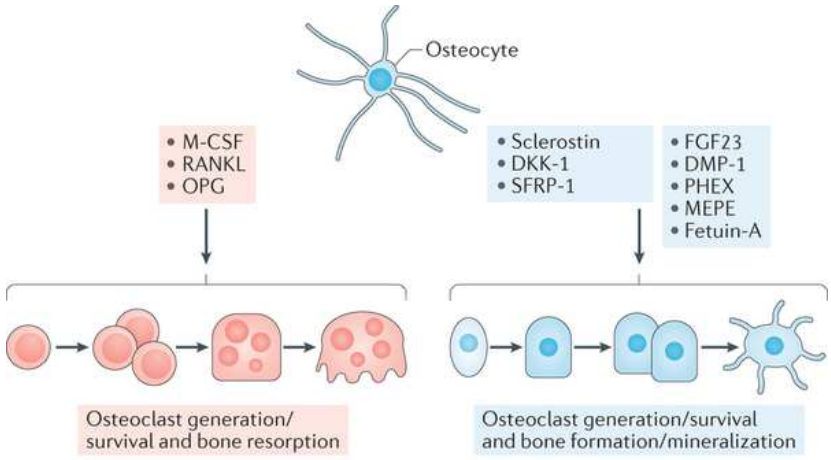


Figure I-6. Osteocytes as master bone orchestrators. Schematic figure of some osteocyte-derived molecules that stimulate or inhibit osteoclast or osteoblast differentiation and function. Extracted from Plotkin *et al*, 2016[74].

Several specific osteocyte proteins have been implied in the regulation of mineral metabolism. Osteocytes regulate phosphate and biomineralization through molecules such as PHEX, DMP-1, MEPE, and FGF-23[75,76]. Moreover FGF-23 can function as a regulator of renal phosphate handling, evidencing the endocrine role of osteocytes in other organs such as kidney[77,78].

Another distinctive feature of osteocytes is their ability to express much higher amount of RANKL than osteoblasts[74]. Mice lacking RANKL specifically in osteocytes developed a severe osteopetrotic phenotype due to a reduced number of osteoclasts, emphasizing the fact that osteocytes are the major source of RANKL in bone remodeling *in vivo*[79]. The expression of this factor leads to the conclusion that osteocytes play a decisive role in orchestrating bone resorption through osteoclast activation[80].

Osteocyte cell death can occur in association with pathologic conditions as osteoporosis and osteoarthritis leading to an increased skeletal fragility

characterized by microfractures[81]. Dying osteocytes are in fact able to recruit osteoclast and to produce both pro-apoptotic and anti-apoptotic molecules[82].

Several factors have been involved in the prevention of osteocyte apoptosis such as bisphosphonates, PGE2, muscle factors, estrogen and the application of fluid flow which, in response to mechanical loading, mimics the flow of the bone fluid through the osteocyte lacuna-canalicular network[72]. 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[83].

1.2 Bone Remodeling

The bone is a dynamic tissue that is constantly being remodeled in response to alterations in physical activity, dietary calcium levels, hormonal changes, bone lesion or injury and local paracrine signals within the bone microenvironment. Bone remodeling occurs at approximately two million microscopic sites in the adult skeleton. 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 throughout life in maintaining the mechanical and mineral homeostasis. This homeostasis is maintained via a balance between bone resorption and bone formation[84].

Bone remodeling implies osteoblast mediated bone formation and osteoclast mediated bone resorption (Figure I-7). It has been shown that also lining cells

play a crucial role in the bone remodeling process. After osteoclast activity, bone-lining cells clean the bone collagen left by osteoclasts in the lacunae, and deposit a thin layer of a collagenous matrix along the lacunae[85]. Alterations of this equilibrium lead to pathological situations, including osteoporosis.

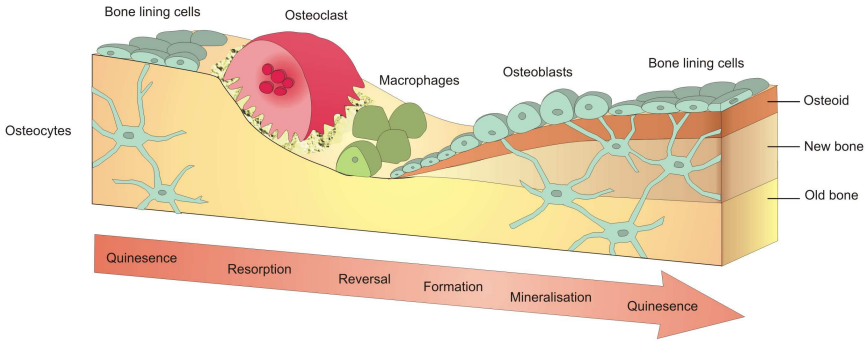


Figure I-7. Phases of bone remodeling. First osteoclasts are activated and resorb the old bone. After resorption and during the reversal phase precursors of osteoblast appear at the resorption site where they proliferate and differentiate. In the formation phase, osteoblasts synthesize the new bone mineralized matrix. At quiescence, osteoblasts become resting cells at the newly formed bone surface. Extracted from http://www.york.ac.uk/res/bonefromblood/background/boneremodelling.html.

This equilibrium is regulated by a set of proteins including the RANK/RANKL/OPG system[34] and other local factors such as cytokines and prostaglandins, or systemic factors such as PTH, calcitonin and estrogens[86-88]. Estrogen is the main sex steroid regulating bone mass in both men and women[89]; the reduction in estrogens levels at perimenopause and during the first years of menopause causes a marked bone loss that leads to osteoporosis[88].

Direct and indirect communications among bone cells occur during bone remodeling, in a process named coupling mechanism, which include soluble coupling factors such as IGFs and BMPs among others, which are stored in the bone matrix and are released during osteoclast bone resorption[90,91]. Recently, semaphorins appeared as a new class of communicators between bone cells during bone remodeling[92,93]. Semaphorin4D is expressed by osteoclasts during bone resorption and it has been shown to inhibit bone formation by binding to its receptor Plexin-B1 located in osteoblasts and inhibiting IGF-1 pathway which is involved in osteoblast differentiation[94].

2. Osteoporosis

Osteoporosis is a systemic skeletal disorder and the most common metabolic bone disease affecting 200 million people worldwide in 2004[95]. It is characterized by low bone mass and micro-architectural deterioration of bone tissue (Figure I-8), which leads to an increased bone fragility and an increased risk of fracture[96]. It is recognized as one of the most common and serious problems facing postmenopausal women and aging persons of both sexes[97]. In America, an estimated 10 million of people aged over 50 years old have osteoporosis and approximately 34 million are at risk[98]. As global population continues to age, the number of people over 50 years old with osteoporosis is expected to increase to 14 million by 2020[99]. Approximately, 12 million women between 50 to 84 years old have osteoporosis in the five largest European countries (Germany, Spain, France, UK and Italy)[100,101]. In Spain, around 35% of women older than 50 years old suffer from postmenopausal osteoporosis[102]. With the increase in aging population, a major number of fragility fractures have been connected with osteoporosis, representing a significant socioeconomic impact and a worldwide public health concern.

According to the World Health Organization (WHO), osteoporosis is defined as a value for bone mineral density (BMD), the T-score, that is more than 2.5 standard deviations (SD) below the young adult mean value, whereas the T-score between −1 and −2.5 have low bone mass or osteopenia[103].

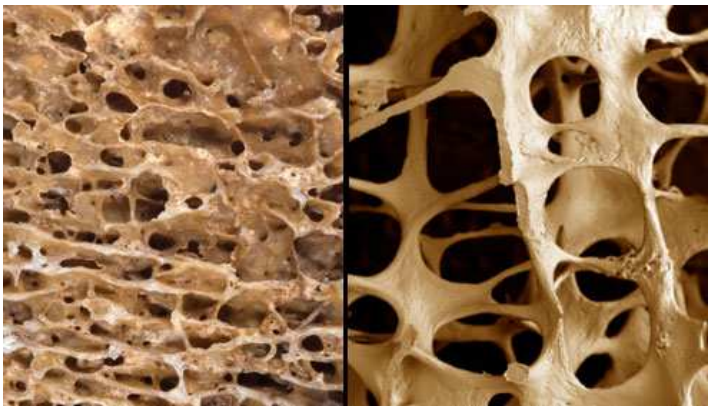


Figure I-8. Bone microarchitecture. In the left side a normal bone is showed meanwhile in the right side an osteoporotic bone with damaged microarchitecture. Extracted from http://www.webmd.com/osteoporosis/guide/what-is-osteoporosis-osteopenia.

Osteoporosis is classified as primary type 1, primary type 2 and secondary. The focus of our research is based on primary type 1, commonly known as postmenopausal osteoporosis. Primary type 2 occurs after the age of 75 years old and it is known as senile osteoporosis, in both men and women. Secondary osteoporosis refers to those conditions that do not start as a skeletal malfunction but the consequence of other factors that may affect BMD such as hyperparathyroidism, treatments with corticosteroids, a not equilibrated diet, vitamin D deficiency and excessive alcohol intake, among others[96,104].

2.1 Bone Mineral Density and Bone Quality

Several elements contribute to the overall skeletal strength in an individual, including bone mass as well as micro and macroarchitecture of the bone. Up to now, great emphasis has been given to bone mass clinically measured as BMD, being the main outcome used to define osteoporosis. It is measured

using dual energy X-ray densitometry (DXA) and the outcome is reported as the mineral weight in grams per cm^2 (g/cm^2) for the tested bone. Currently DXA represents the only non-invasive diagnostic tool for osteoporosis[105].

A healthy person reaches peak bone mass at 30 years old (Figure I-9A). After this time, a gradually loose of bone mass occurred throughout the lifetime. In women, markedly bone loss occurs in the late perimenopause stage[106] and continues to decline rapidly after menopause due to a significant reduction in estrogen levels[96,107] (Figure I-9B).

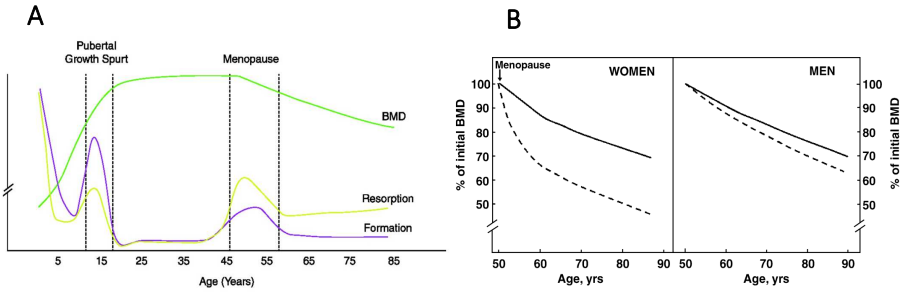


Figure I-9. Schematic representation of bone mass changes throughout life. (A) Scheme of gain and bone loss and the bone remodeling pattern in women during all the age range. **(B)** Bone mass decline after 50 years old in women and men. Solid lines represent cortical bones and dashed lines trabecular bone. Women experienced a more pronounced decrease in terms of bone loss after menopause. Extracted from Kleerekoper, 2013[108] and Drake and Khosla, 2013[109].

BMD can be affected by several factors including age[110], hormone and sex steroids[111], nutrition[112,113] and exercise[114,115], among others. Moreover it is also largely determined by genetic factors[116,117].

Nevertheless, BMD assessment is not sufficient to determine the risk for fractures since it has been shown in some cases in which age increased fracture risk independently of bone density[118]. For this reason, in recent years an especial attention in bone quality has emerged[119,120]. There are

several factors that might influence the resistance to fracture such as biochemical and physical characteristics of bone components (nature of the collagen, degree and type of collagen cross-linking, size and structure of hydroxyapatite crystals and degree of mineralization), the morphology and architecture of the bone and the presence of preexisting microdamage[121]. A special effort has been made in recent years to develop noninvasive imaging techniques capable of assessing bone structure and predicting bone strength as 3-dimensional imaging modalities, including magnetic resonance imaging (MRI), trabecular bone score (TBS) evaluation, and computed tomography (CT). Microindentation appeared as a novel tool to measure the bone tissue mechanical strength in patients[122].

The understanding of all the factors involved in both, bone quantity and bone quality will help to improve the diagnosis of osteoporosis and monitoring of response to treatment.

2.2 Osteoporotic Fractures

Osteoporotic-related fractures have a huge health and economic impact worldwide. In health terms, significant pain, disability, and deformity are some of the clinical manifestations in patients with fractures[123]. The economic burden takes place on patients but also on the whole healthcare system; in North America there are over 1.5 million osteoporotic fractures each year[97]. These osteoporotic fractures are responsible for approximately 500,000 hospitalizations, 800,000 emergency room visits, 2.6 million physician visits, and 180,000 nursing home placements each year; which is translated into around 15 billion of dollars of costs in the US economy[99]. In Europe, osteoporotic fractures account for 2.7 million of cases originating a direct cost of 36 billion of euros[124].

Fragility fractures are defined as fractures that occur from low-energy trauma such as falls from a standing height or less as a result of poor bone quality and low BMD[125]. They can occur in any bone but vertebral body (spine), distal radius (wrist), and hip fracture are the most common[126].

2.2.1 Hip fractures

Although vertebral fractures are the most prevalent[127], hip fractures have been considered as the gold standard of osteoporosis because they are highly related to low BMD values and are related to important complications such as disability, major number of hospitalizations, diminished quality of life, ability to live independent and premature death[100,128].

Hip fractures increases exponentially with age (Figure I-10). As stated above, fractures occurrence will continue to increase as life expectancy continues to rise. In a population demographic study, it was estimated that the number of hip fractures worldwide will increase from I .66 million in 1990 to 6.26 million by 2050[98].

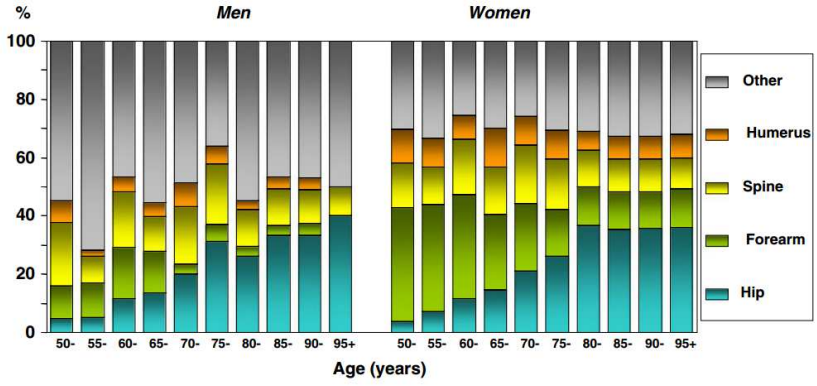


Figure I-10. Pattern of osteoporotic fractures sorted by age worldwide. Pronounced increase in hip fracture cases (in blue) with aging, becoming predominant over 75 years old. Extracted from Strom *et al.*, 2011[101].

The incidence of hip fracture varies among different countries and populations[128] and female are more susceptible to suffer them than men[96](Table I-1). Patients experiencing hip fracture are at considerable risk for subsequent osteoporotic fractures and have increased risk of mortality. In 2010, Haentjens *et al.* performed a meta-analysis to assess the mortality in women or men over the 50 years old after having a hip fracture[129]. They observed that these populations have 5- to 8- fold increased risk for all-cause mortality within the first 3 months after hip fracture.

Table I-1. Probability of fractures (%) in men and women from Sweden population at the age indicated. The risk ratio is referent to the female/male probabilities. Females have a greater risk to suffer any fracture type than men. Extracted from Strom *et al.*, 2011[101].

	At 50 years			At 80 years		
Type of fracture	Men	Women	Risk ratio	Men	Women	Risk ratio
Forearm	4.6	20.8	4.5	1.6	8.9	5.6
Hip	10.7	22.9	2.1	9.1	19.3	2.1
Spine [a]	8.3	15.1	1.8	4.7	8.7	1.9
Proximal humerus	4.1	12.9	3.1	2.5	7.7	3.1
Any of these	22.4	46.4	2.1	15.3	31.7	2.1

[a]Clinical spine fracture

2.2.2 Factors involved in Fractures Risk

Osteoporosis is considered a silent disease because of the absence of symptoms during the bone loss. Therefore, osteoporotic fractures frequently go unrecognized in the clinical setting[130-132]. This notion makes fundamental to have a good diagnostic for fractures risk, in order to detect patients at risk and prevent the fracture.

Fracture risk assessment was largely based on the measurement of BMD, since osteoporosis definition is based on bone mass terms. This relationship has been demonstrated by several studies; the risk of low trauma fracture increases by a factor of 1.4–2.6 for each SD decrease in bone mineral density[133]. Nevertheless, some osteoporotic fractures occur in patients with BMD levels above the ones considered as osteoporotic[125,134]. This phenomenon can be explained by other risk factors partly independent of BMD such as age, previous low-trauma fractures, low body mass index (BMI), parental history, early menopause, etc[135]. In this regard, the Fracture Risk Assessment tool (FRAX) was developed in order to identify the individual risk of osteoporotic fracture on the basis of clinical settings[100,125,136].

Along with the risk factors already described, the contribution of bone turnover markers as fracture risk predictors has also been explored[137]. Bone turnover can be assessed by bone formation and bone resorption markers. The most typical markers for bone formation are carboxyterminal propeptide (PICP) and aminoterminal propeptide (PINP) of type-I collagen, bone-specific ALP and OCN[125]. Products of the collagen degradation performed by osteoclasts during bone resorption are cross-linked aminoterminal-telopeptide (NTX) and cross-linked carboxyterminal-telopeptide (CTX), used as bone resorption markers[125].

Taking into account the multifactorial etiology of fractures, an approach combining all the parameters described, bone mineral density, clinical risk factors for fracture and bone turnover markers, will improve the prediction of fracture risk and the evaluation of patients with osteoporosis.

Even though osteoporotic fractures are associated with substantial healthcare costs and dramatic consequences, less than 40% of patients are

treated within the first year after the fracture occurs and less than 30% of patients who are at risk for fracture are prone to take the recommended medication[138].

2.3 Current Treatments of Osteoporosis

The National Osteoporosis Foundation established the following guidelines to determine which patients should be considered to be treated: (1) patients with a history of hip or vertebral fracture, (2) patients with a T-score of -2.5 or lower at the femoral neck or spine, and (3) patients who have a T-score between -1.0 and -2.5 at the femoral neck or spine and a ten year hip fracture risk of ≥3% or a ten-year risk of a major osteoporosis-related fracture of ≥20% as assessed with FRAX[135].

An imbalance in bone remodeling occurs in postmenopausal osteoporosis and advancing age, and is manifested by high turnover due to the increase in bone resorption. Antiresorptive therapy to decrease high bone turnover has been mainly addressed in these group of patients. In contrast, osteoanabolic agents have been intended to patients with low bone remodeling such as in glucocorticoid osteoporosis[139]. Pharmacological agents belonging to each class of drugs have been shown to enhance BMD levels and reduce the risk of fracture[140-143] (Figure I-11).

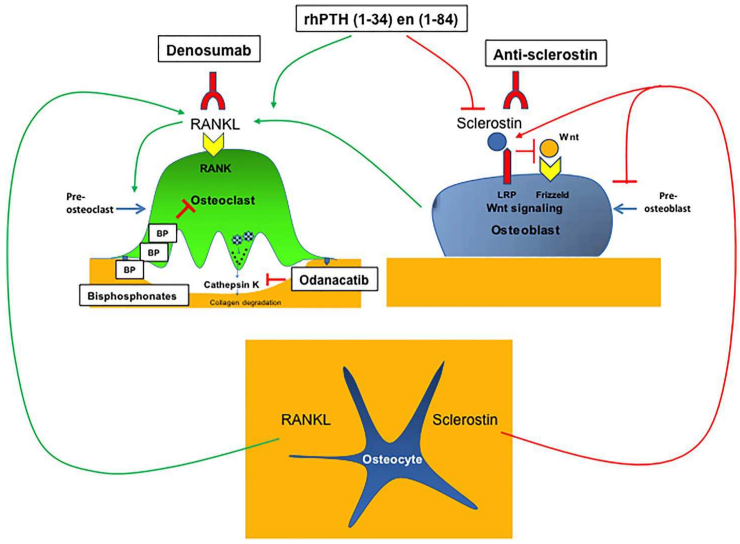


Figure I-11. Representation of the effect on bone cells by the antiresorptive and osteoanabolic drugs. Extracted from Geusens *et al.*, 2015[144].

Among antiresorptive agents there are bisphosphonates, denosumab and selective estrogen receptor modulators (SERMs). Biphosphonates (alendronate, risedronate, ibandronate and zoledronic acid) are a group of compounds analogues to pyrophosphate that have a strong affinity to hydroxyapatite in bone, thus inhibiting osteoclasts mediated bone resorption[145]. Denosumab is a human monoclonal antibody that binds to and consequently inhibits the activity of RANKL, a mediator which promotes osteoclast activity[146,147]. SERMs are ligands for estrogen receptors and can act as estrogen agonist or antagonist depending on the target tissue. Raloxifene inhibits the formation and activity of osteoclasts by mimicking estrogen and it is approved for osteoporosis treatment[100].

Teriparatide is the approved anabolic drug used to treat osteoporosis[148,149]. It is the active fragment (1-34) of the endogenous human PTH[150] and increases bone formation mainly by inhibiting Sclerostin production by osteocytes. At

the same time, it can increase bone resorption through stimulation of RANKL production by osteoblasts and osteocytes but the actions of teriparatide are believed to be mainly anabolic[151].

Emerging therapies for osteoporosis comprise anti-cathepsin K and anti-sclerostin antibodies[152,153]. Osteoclasts express highly amounts of Cathepsin K[154], which degrades type I collagen and other components of the organic bone matrix. Among several Cathepsin K inhibitors that entered into clinical development for metabolic bone disorders, Odanacatib was the most selective and reliable and has reached an advanced stage of development[155,156]. Sclerostin is mainly produced by osteocytes and inhibits osteoblast activity through targeting Wnt signaling pathway. In clinical phase 2 studies it was observed an increase in BMD in the patients treated with the promising anti-sclerostin monoclonal antibody named romosozumab. This drug is at the moment under phase 3 studies in order to evaluate the efficacy of the treatment to reduce fractures, besides tolerability and safety issues[152].

The combination of anabolic and antiresorptive therapies has also been evaluated in an attempt to achieve higher bone mass and strength outcome than the resulted from monotherapy[157-160]. Some studies showed that certain therapy combinations attain greater BMD levels than therapies alone but the BMD outcomes vary depending on skeletal site measurement, assessment tool and type and duration of the pharmacological agents[161]. Furthermore, there is still no data regarding fracture outcome.

Beyond the pharmacological administration of drugs, several life-related measures have been demonstrated to improve and preserve bone health such as adequate dietary intakes of key bone nutrients such as calcium and

vitamin D[162], the avoid of excessive alcohol intake[163] and adequate exercise practice[164].

2.3.1 Barriers to osteoporosis therapy

Even if notable benefits have been undeniably demonstrated with anti-osteoporotic drugs, several studies described side effects derived from these pharmacological treatments. Moreover, low adherence and tolerance have also been associated with these drugs representing a huge problem to osteoporosis, reflected by diminished health benefits and increased healthcare costs.

2.3.1.1 Side effects

Treatment with oral bisphosphonates has been related to cases of renal function impairment, gastrointestinal disorders as dysphagia and esophageal irritation, hypocalcemia, atypical femoral fractures and osteonecrosis of the jaw[165,166]. Raloxifene can increase the risk of venous thromboembolism, including deep vein thrombosis and fatal stroke[142]. In the case of denosumab, as a consequence of the mechanism of action of the drug itself, a reduction in osteoblast mediated bone formation has been observed[167]. For this reason the FDA recommend this medication just in those cases of severe osteoporosis or in patient who did not respond to other therapies. Back pain and skin rashes might also occur after the treatment[141]. Teriparatide is related to few side effects such as leg cramps, mild nausea and transient hypercalcemia[168]. Moreover, an increase in bone resorption has been observed with use of the current anabolic drugs. More detailed adverse effects related to the current pharmacological treatments have been described by many authors[168,169].

2.3.1.2 Adherence

Adherence encompasses two main concepts: persistence and compliance. Persistence refers for how long the medication is taken and it can be expressed as number of days until drop-out. Compliance describes the proximity to the treatment recommendation as given in the official recommendation. A poor adherence to prescribed osteoporosis treatment is widespread in clinical practice leading to treatment discontinuation and unmet benefits expected from medications. A considerable proportion of patients abandon the treatment within the first year after initiation[170-172].

Oral bisphosphonates require dosing regimens and rules that difficult the compliance of the patients. For example, most of them have to be administrated on an empty stomach, first thing at the morning, without any other liquid than water while sitting or standing for 30 minutes from the intake[135]. Those tight restrictions, that cause difficulties to the daily routine of the individuals, added to the side effects previously described, give us an idea of why it is not rare that low adherence is observed to oral bisphosphonates; estimations described that less than 40% of patients are still following the treatment after 1 year[173]. Also some patients reject daily injections required with PTH drugs[152].

Higher efficacy, lower costs and less-frequent dosing regimens are the major preferences required by patients[174]. The knowledge about patients' perceptions and preferences for treatment may be an effective method for improving adherence to current osteoporosis therapies.

Even if remarkable advances in the treatment of osteoporosis have been accomplished over the past two decades, it is evident that the ideal treatment is yet to come. In order to overcome the insufficient adherence,

novel treatments should first solve the problems related to the administration of the drug and then deal with the enhancement in terms of bone strength and fracture prevention, always with a special attention to avoid the previously cited side effects.

New forms of treatment hold the promise of providing a satisfactory and effective treatment of osteoporosis. The main action of microRNA in gene silencing opens the possibility of therapies with pro-osteoblastic activity to stimulate bone formation without resorption enhancement.

2.4 Genetic Study of Osteoporosis

The etiology of osteoporosis is well known to be multifactorial. One of the most important risk factors is a positive family history, which emphasize the crucial role of genetics in the pathogenesis of the disease. The first evidence for the heritability of osteoporosis was showed in twin and family studies. The reported heritability of BMD is between 50% and 85%[175-177]. Besides BMD, other determinants of fracture risk including quantitative ultrasounds properties of bone, femoral neck geometry[178] and bone turnover markers[179] were also found to be heritable. Even though a lack of consensus exists regarding fracture heritability[117], the family history of fracture was catalogued as risk for fracture independently of BMD[180].

After the discovery of the inheritable nature of the osteoporotic phenotypes, several genetic studies were developed with the aim to identify genes, mechanisms or signaling pathways which might broad our current knowledge of the disease. Common diseases like osteoporosis are attributable to the combined effect of many genes, most of which exert subtle effects at the osteoporotic phenotype. Nevertheless, some mutations in bone-related genes were identified with large effects (Figure I-12).

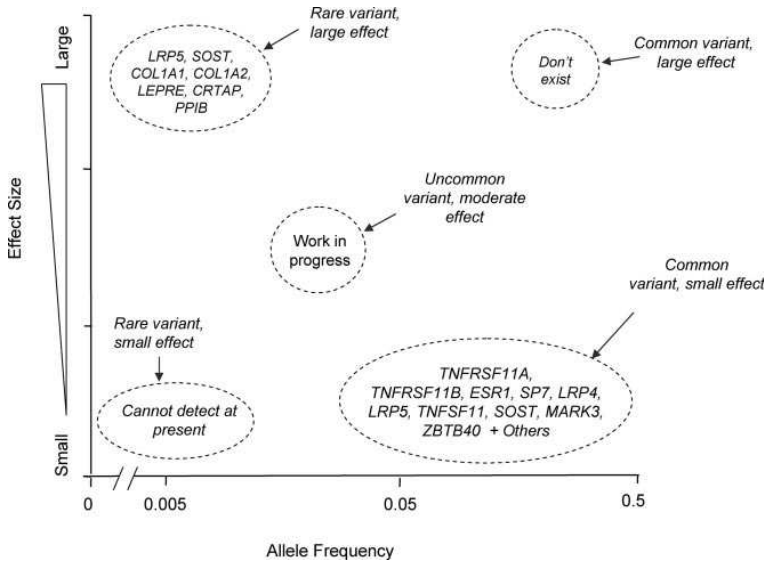


Figure I-12. Scheme of type of variants and their contribution to osteoporosis susceptibility. Variants that have been related to BMD and fracture comprise mainly common variants of small effect (bottom right) or rare variants with large effect (top left). Extracted from Ralston *et al*, 2010[181].

Even though several genes have been associated with osteoporosis outcomes, only a small part of heritability can be explained so far. Epigenetics mechanisms such as microRNAs have been recently appeared as one of the potential mechanisms able to unravel the missing heritability[182].

Diverse approaches have been used with the aim to identify genes involved in the susceptibility to osteoporosis. Initially, the linkage analysis and the candidate gene association studies were the main approaches used in the genetic field. The linkage approach has been largely addressed for the identification of genes responsible for inherited monogenic Mendelian human diseases[181,183]. As they focus their attention on highly penetrant variants (single mutation with strong effect in the disease phenotype), it has very limited success in identifying genes responsible for complex diseases as

osteoporosis. Only few loci related to BMD have been identified applying this approach and the results failed to be replicated among studies[184].

Candidate gene association studies have been widely used in the genetics of complex diseases. This approach analyzes common genetic variants within candidate genes previously known to play a specific role in the disease of interest. Polymorphism is used to describe common genetic variants that are present > 1% in the population, being single nucleotide polymorphisms (SNPs) the most common type within this class of variants.

These association studies are relatively easy to perform and useful to detect small effects from the selected variants. The main limitation of this methodology is the spurious results derived from the use of a small sample size. To address this issue, large scale candidate genes studies have been performed by large consortia. The Genetic Markers for Osteoporosis (GENOMOS) and the Genetic Factors for Osteoporosis (GEFOS) consortiums were created in the osteoporosis field and let the detection of risk alleles with modest effect size.

These studies have identified several genes related to osteoporosis involving *COLIA1*, Estrogen Receptor α (*ERα*), *LRP5, LRP6* and S*OST*[185-187].

Advances in genotyping technologies have offered the possibility to perform association studies on a genome wide basis by assaying up to millions of SNPs in thousands of individuals. In contrast to the candidate gene association studies, genome-wide association studies (GWAS) examine SNPs that are spread across the genome, instead of focusing in specific pre-selected candidate genes. An advantage of GWAS compared to candidate gene studies is that they offer the possibility of ranking the importance of several

association signals across the genome and the identification of novel pathways related to the phenotype studied.

The increase in the amount of data to manage even increases the number of false positive findings, introducing a statistical limitation. Therefore, the number of associations tested across the genome needs an adjustment for multiple testing. In order to reach significant associations withstanding multiple test corrections, large sample sizes are required, leading to the meta-analysis of GWA studies. The meta-analysis studies combine data from several individual GWAS to enhance sample size and obtain more accurate estimations of the effect size of individual genetic variants. These large-scale-meta-analyses require the collaboration and coordination of various research groups.

In the bone field, several GWAS and meta-analyses have been performed with the aim to reveal the contribution of genetics factors to the osteoporotic phenotypes[116,188-197]. The GEFOS consortium published two large-scale GWAS meta-analyses[116,188]. In their first collaborative project, 20 loci associated with BMD were identified, representing a great advance in the bone genetic field. In 2012, they performed the largest meta-analyses of GWAS data on the genetic determinants of BMD performed to date. In this study, 56 loci were identified and among them 14 were also associated with risk of osteoporotic fracture. It is noteworthy to mention the heterogeneity of fractures included in the study and the importance to examine with caution these results before further validations in other cohorts with homogenous fracture types. Another important factor to remark is the reproducibility of these studies. Some significant loci identified in meta-analysis were also found to be statistically significant in individual GWAS, while others were not found to be significant at the genome wide level. At

the same time, significant loci identified in individual GWAS were not replicated in the meta-analysis. These inconsistencies can be attributable to some variables such as the heterogeneity in terms of ethnicities included in different studies[198].

Moreover, SNPs associated with lumbar spine BMD differs from those found to be associated with femoral neck BMD, suggesting a site-specific role of these genes[199]. The most relevant associations between genes and BMD variation at different skeletal sites are represented in Figure I-13.

Most of the published GWAS related to osteoporosis have been focused on BMD. Some of the BMD loci identified in these studies are then selected to test their possible relevance with osteoporotic fracture. Taking into account the partially different genetic determination between BMD and fracture[200], some GWAS assessing fracture as an independent study phenotype have been performed[201,202].

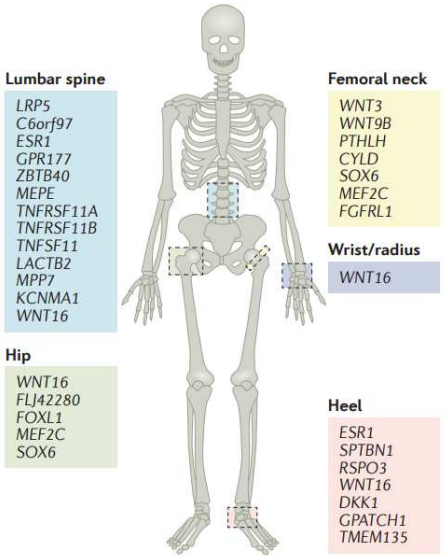


Figure I-13. Important loci associated with BMD variation at different skeletal sites at genome-wide levels. Extracted from Karasik *et al*, 2016[203].

GWAs have proven to be very successful in the identification of several common variants related to common human diseases. Nevertheless, the identification of rare variants with larger effects may be missed by this type of approach[204]. However, recent advances in next-generation sequencing technologies have improved the detection of functional rare variants[205]. By sequencing the whole genome or a locus of interest, it is possible to generate a complete catalog of all variants present within a given DNA sequence[206] and therefore to identify uncommon variants that had been previously dismissed.

The identification of variants and genes involved in BMD regulation and fracture offers the prospect to identify novel molecules that might be used as targets for new drug design for the prevention and treatment of osteoporosis.

3. MicroRNAs

One of the major discoveries that has arisen in the last decade is the discovery of pervasive transcription in the human genome[207], which refers to the idea that the vast majority of the mammalian genome is transcribed. Though the initial studies were based on tiling arrays, this has been further corroborated by different researchers suggesting a completely new class of transcripts arising from regions of the genome, which have not been previously characterized as coding. Even more surprising was the fact that many of these newly discovered transcripts have no apparent potential to code for proteins. These transcripts have been categorized as noncoding RNAs (ncRNAs)[208].This discovery changed the way to understand how our genome works; what at first was considered as junk DNA turned into a crucial element in gene regulation. Interestingly, the Encyclopedia of DNA elements (ENCODE) project assigned biochemical functions for approximately 80% of the genome, particularly outside of the well-studied protein-coding regions; once again highlighting the importance of this novel non-coding transcripts[209].

Lee *et al*.[210] identified the first two microRNAs encoded by the lin-4 gene (control the timing for *C. elegans* early larval development events). The lin-4 RNAs were found to be complimentary to a repeated sequence in the three prime untranslated region (3'UTR) of lin-14 and to regulate lin-14 translation by an antisense mechanism. Afterwards, miRNA let-7 was discovered to direct the later stages of *C. elegans* development in a similar way to lin-4[211].

Lin-4 and lin-7 were found to be evolutionary conserved from flies to humans, implicating an extensive role of these genes in animals[212].

MicroRNAs (miRNAs) are single strand non-coding RNAs about 20-25 nucleotides in length that post-transcriptionally regulate gene expression by binding at the 3'UTR of their targets messenger RNAs (mRNA). This binding is mediated through the bond of the seed region of the mature miRNA (located around the 2 to 7 or 8 nucleotide positions in the 5'end) with the complementary bases of the 3'-UTR of the target mRNAs[213]. Some miRNAs have also been found to target the 5'-UTRs of mRNAs and to induce target translation[214].

One miRNA can regulate hundreds of genes[215] and one mRNA can be targeted by several miRNAs[216]. The latest release of the most comprehensive miRNA database (miRBase) has identified 2,588 mature miRNAs in humans. In mammals, more than 60% of protein coding genes are predicted to be regulated by miRNAs[217]. In addition to miRNAs, there are other major types of small silencing RNAs in animals: siRNAs (small interfering RNAs) and piRNAs (piwi-interacting RNAs), which present different processing enzymes involved in their genesis and have distinctive functions[218].

3.1 miRNAs Biogenesis

MiRNAs can evolve from several distinct genomic sources. Most of the miRNAs are encoded in the genome as independent transcriptional units or reside in introns of protein-coding genes[219].

MiRNA genes are transcribed by either RNA polymerase II or RNA polymerase III into primary miRNA transcripts (pri-miRNA)[218,220]. Both RNA polymerases

are differently regulated and recognize specific promoter and terminator elements, offering a wide variety of regulatory options.

MiRNA maturation is a multi-step process involving various RNase enzymes. They are first transcribed as long pri-miRNAs, which contain characteristic hairpin loop structures. Pri-miRNAs are over 1kb in length and are typically capped and polyadenylated structures. The pri-miRNAs hairpin helps the microprocessor complex containing the RNAse II endonuclease Drosha and some cofactors, including the double-strand RNA binding-protein DGCR8 (diGeorge syndrome critical region gene) to recognize them from similar structures present in the nucleus[221,222]. Neither Drosha nor DGCR8 alone is active in pri-miRNA processing, but the combination of both restore this activity, therefore underlying the importance of those two proteins in pri-miRNA processing[223].

Drosha and Dicer are two major enzymes involved in the miRNAs biogenesis process. It has been reported that germline deficiency of either Drosha or Dicer causes lethality at embryonic stage in mice, reflecting the decisive role of miRNAs in development[218].

As Drosha cleavage defines the terminus of a miRNA and therefore defines its specificity, it is important that Microprocessor precisely recognizes and cleaves a pri-miRNA**.** Drosha cleaves the hairpin at approximately 11 bp away from the basal junction between single stranded RNA and dsRNA, and approximately 22 bp away from the apical junction linked to the terminal loop, generating a double-strand miRNA precursor (pre-miRNA) of approximately 60 to 80 nucleotide in length. Following Drosha processing, pre-miRNA is exported into the cytoplasm, where maturation can be completed. The protein exportin 5 (EXP5) forms a transport complex with the

GTP-binding nuclear protein RAN-GTP and a pre-miRNA. After translocation trough the nuclear pore complex, GTP is hydrolyzed, resulting in the disassembly of the complex and the release of the pre-miRNA into the cytoplasm[218].

Once pre-miRNA is located in the cytoplasm, pre-miRNA is cleaved near the terminal loop by a second endonuclease called Dicer, generating a small RNA duplex around 21 to 24 nucleotides. Dicer is an RNase III-type endonuclease that forms a C-terminal intramolecular dimer to create a catalytic center. The N-terminal helicase domain facilitates pre-miRNA recognition while the PAZ (PIWI-AGO-ZWILLE) domain binds to the termini of pre-miRNA. Dicer binds to pre-miRNA with a preference for a two-nucleotide-long 3' overhang, previously generated by Drosha.

The small RNA duplex generated after Dicer processing is then loaded onto an Argonaute (AGO) protein to generate a complex called RNA-induced silencing complex (RISC). All human AGO proteins (AGO1–4) are capable of inducing translational repression and decay of target mRNAs but only AGO2 can slice perfectly matched target mRNAs. In humans the four AGO proteins are associated with almost indistinguishable sets of miRNAs[218,224].

To constitute the active RISC responsible for the gene silencing, the double-stranded RNA needs to be first separated into the functional guide strand which is complementary to the mRNA target, and the passenger strand which is degraded (Figure I-14). The guide strand is determined during the AGO loading step, mainly based on the relative thermodynamic stability of the two ends of the small RNA duplex. The strand with a relatively unstable terminus at the 5' side is typically selected as the guide strand[218]. An additional determinant for strand choice is the first nucleotide sequence: AGO proteins

select for guide strands those with a U at first nucleotide position[225]. Moreover, the cell or tissue specific differences in the relative amount of the AGOs proteins suggest that the efficacy of mRNA repression mediated by miRNAs might be different between different cell types[226].

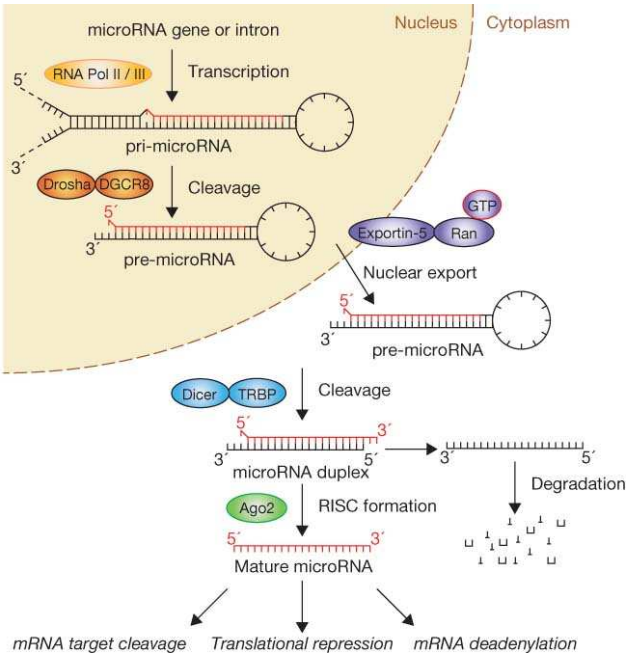


Figure I-14. Schematic representation of miRNA's biogenesis. MiRNAs are transcribed into pri-miRNAs in the nucleus and recognized then by Drosha and DGCR8 to generate a pre-miRNA that is exported to the cytoplasm by Exportin 5. A microRNA duplex is formed after the cleavage performed by Dicer. RNA-induced silencing complex (RISC) recruits the selected miRNA strand and drives the passenger strand to degradation. Depending on the miRNA-mRNA complementarity, the target mRNA is inhibited or degraded by the miRNA. Extracted from Winter *et al.*, 2009[227].

Even if the vast majority of identified miRNAs are produced by the mentioned canonical pathway, there are alternative pathways for miRNA biogenesis. Mirtrons, microRNAs located in the introns of the mRNA encoding host genes, represent a widespread class of intron-derived miRNAs in animals

that are generated by a non-canonical biogenesis pathway which bypasses the pri-miRNA processing step by Drosha[228,229].

3.2 MiRNA function

The domain at the 5' end of miRNAs that spans from nucleotide position 2 to 7 is crucial for target recognition and has been termed the "miRNA seed". Nevertheless, the downstream nucleotides of miRNA sequences can also be mediators of miRNA-mRNA interactions[230]. The seed region is responsible for the identification of the complementary bases of the 3'-UTR of the target mRNAs and to trigger their cleavage and degradation[231]. However, there is usually an imperfect base pairing between the seed region and the target mRNA; resulting in protein expression decrease due to translational suppression.

3.2.1 MiRNA mediated mRNA repression

Translation process is divided into three steps: initiation, elongation and termination. Any of these phases can be interrupted by miRNAs with a final effect on mRNA repression, being initiation the most common target for translational control.

Eukaryotic transcribed mRNAs have an m^7GpppN group (N is any nucleotide) at the 5' cap that facilitates ribosome recruitment to the mRNA. Most of the mRNAs are translated via cap-dependent mechanism, even if some cellular and viral mRNAs are translated using alternative cap-independent mechanisms.

In a cap-dependent translation, the small ribosomal subunit 40S in a complex with several eukaryotic initiation factors (eIFs) binds the mRNA near the

5'cap and goes through the mRNA sequence in a 5 →3 direction until it finds a starting codon (AUG). The eIF4F complex binds to the 5'cap and the poly (A)-binding protein (PABP) binds to the 3 poly (A) tail, facilitating the recruitment of ribosomes to the mRNA[232].

Many mechanisms have been described to explain the repression of mRNA translation initiation. Nevertheless, inconsistences between theories are present. One model proposes that miRISC competes with eIF4E for binding to the mRNA 5'cap structure or that miRISC interferes with elf4E function. A second model suggests that miRISC stimulates deadenylation of the mRNA tail and therefore translation is repressed because of the inability of 5'cap and the PABP-free tail to circularize. The third model proposes that miRISC blocks the association between the 60S ribosomal subunit and the 40S preinitiation complex by the recruitment of eIF6[232] (Figure I-15).

There are also some studies trying to understand how miRNA repression works at post initiation steps. Among the mechanisms there is a possible inhibition of ribosome elongation, induction of ribosome drop-off or facilitation of proteolysis of nascent polypeptides.

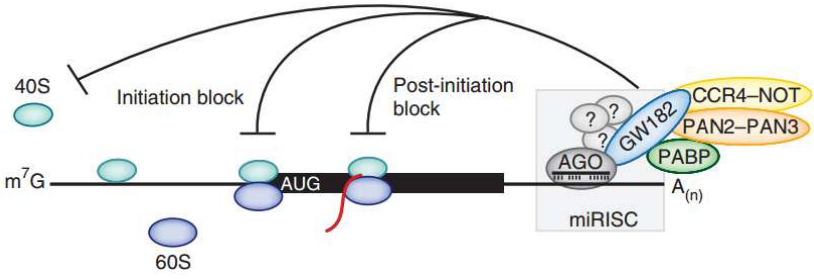


Figure I-15. Diagram of miRNA-mediated translational repression. Repression can either be performed at initiation or post-initiation stage of translation. miRISC can interfere with eIF4F-cap recognition and 40S small ribosomal subunit recruitment or by antagonizing 60S subunit joining and preventing 80S ribosomal complex formation. Translational repression can also be performed after initiation stage, for instance by inhibition of ribosome elongation. Extracted from Fabian *et al*., 2012[233].

3.2.2 MiRNA mediated mRNA degradation

In addition to mRNA repression, miRNAs also trigger mRNA degradation. AGO2 is the only argonaute protein able to induce the guide strand-mediated cleavage of target mRNA by the catalytically competent RISC. Nevertheless, miRNA targeted mRNAs significantly reduce their abundance due to an increase in mRNA degradation that is not mediated by the mentioned Ago-catalyzed mRNA cleavage but for another mechanisms such as deadenylation, deccaping and exonucleolytic digestion of the mRNA[232] (Figure I-16).

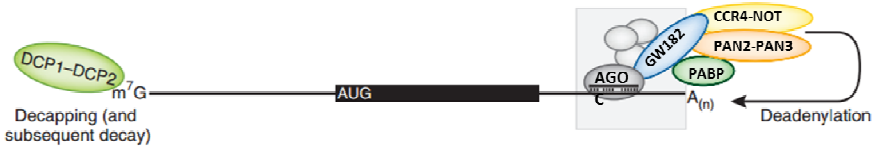


Figure I-16. Representation of the miRNA-mediated mRNA decline. Deadenylation requires the direct interaction of the GW182 protein with the poly(A)-binding protein (PABP) After deadenylation, the 5' cap (m7G) is removed by the decapping DCP1-DCP2 complex. Adapted from Fabian *et al.*, 2012[233].

In this mechanism, mRNAs are first deadenylated by the consecutive and partially redundant action of the deadenylase complexes such as CCR4–NOT. Deadenylated mRNAs are then decapped by the decapping DCP1-DCP2 complex, which requires additional cofactors for full activity. Finally, deadenylated and decapped mRNAs are degraded by the exoribonuclease 1 (XRN1)[234]. Consecutive steps in the mRNA decay are coupled through a network of direct interactions between subunits of the catalytic complexes involved.

AGO and GW182, components of the miRISC, are required for miRNA-mediated gene silencing. AGO proteins seems to be involved in the

recruitment of GW182 to the mRNA and in turns, GW182 recruits the mRNA decay machinery to miRNA targeted mRNAs by promoting the removal of the poly(A) tail. In addition to their interaction with AGO proteins and deadenylase complexes, GW182 proteins bind to PABP, which also plays an important role in gene silencing[234].

3.3 Regulation of miRNA Expression

The creation of a mature and functional miRNA is a complex procedure characterized by an intricate multistep biogenesis process followed by a sophisticated regulation process. Any of the molecules involved in the synthesis pathway is susceptible to changes and modifications by other factors. The sum of all changes that might occur has to be taken as an overall instead of independent facts in order to have an accurate comprehension of the whole mechanism. MiRNA expression is very variable and depends on cell-cycle status (differentiation, proliferation) as well as environment (hormones, cytokines and other signaling factors).

3.3.1 Transcriptional control of miRNA biogenesis

One of the major regulatory steps in the miRNA biosynthesis is transcription. It has been shown that several characteristics in miRNA gene promoters are similar to the ones of the promoters of protein coding genes, such as the presence of CpG islands, TATA box and transcription factor II B (TFIIB) recognition[235]. There are also some DNA-binding factors that regulate miRNA transcription which are shared with those that control protein-coding genes, such as c-myc and p53[236,237]. The proto-oncogene c-myc encodes a transcription factor able to regulate 10 to 15 % of human genes[238]. Interestingly, c-myc was found to decrease the expression of several tumor suppressor miRNA genes such as miR-29, miR-34 and let-7 families[239].

Some mechanisms of epigenetic control that occurs in protein coding genes regulation are also present in miRNA genes for instance, DNA methylation and histones modifications has been already described[240,241].

3.3.2 Control of miRNA processing

Various strategies are applied by the cell to interfere with or to facilitate each step involved in miRNA maturation. The total levels of Drosha and DGCR8 in the cell are tightly controlled and need to be closely coupled for a satisfactory pri-miRNA processing. There is a regulatory loop between these two proteins; Drosha maintains a highly regulated level of DGCR8 through the Drosha microprocessor mediated cleavage of DGCR8 mRNA. Furthermore, DGCR8 stabilizes Drosha protein levels and ensures the tight coupling of the core microprocessor proteins[242]. Any alteration in this regulatory loop between both proteins, or in the expression or activity of any of the components of the microprocessor complex can modify the correct functioning of the system and therefore in the pre-miRNA generation. For instance, the RNA helicases p68 and p72 (components of the Drosha microprocessor complex) interfere in the processing mediated by Drosha of some miRNAs[243]. Other proteins, including Smads, p53 and ERα can also interact with p68 helicase and interfere in the pri-miRNA processing[242].

DICER plays a key role in the second processing step from pre-miRNA to a final mature miRNA. Several Dicer-associated proteins have been identified, including TAR RNA-binding protein (TRBP) and protein kinase R-activating protein (PACT). The association of both proteins with Dicer enhances Dicer stability and processing activity[244]. The expression or activity of Dicer can also be modulated by cellular signaling pathways. For example, Mitogen-Activated Protein Kinase (MAPK/ERK) signaling was found to promote the

phosphorylation of TRBP. Phosphorylated TRBP enhances miRNA production by increasing the stability of Dicer. Remarkably, increased abundance of Dicer is correlated with the increase in growth-promoting miRNAs and decrease of let-7 which has a tumor suppressor activity[242].

Total levels of the Ago proteins also contribute to global miRNA regulation and biogenesis. Ectopic expression of Ago proteins results in a dramatic increase in mature miRNAs[242].

Besides the regulatory mechanisms already described, there are other steps that might be regulated. For instance, the export of the pre-miRNA to the cytoplasm might be differently regulated under specific physiological conditions or the regulation of the loop in the pri-miRNA processing[242]. The regulation can also occur through miRNA tailing. Many authors reported that uridylation or adenylation modifies the pre-miRNA and mature miRNA and affect their processing and stability[245-247]. Regarding miRNA activity, alternative polyadenylation in mRNAs can produce different mRNA transcript isoforms with diverse 3'UTRs, thus creating or eliminating miRNA binding sites[228].

Despite extensive analysis of the multiple processes involved in the generation of miRNAs, relatively little is known regarding the miRNAs half time and stability. It has been proposed that several nucleases can cleave and degrade miRNAs, but the precise mechanism is still to be defined. One of the first reports on this mechanism was performed in *Arabidopsis thaliana*, in which a group of 3'-5' exoribonucleases known as small-RNA-degrading nucleases, degrades miRNAs[248]. Moreover, it has been already published that the target mRNA can modulate the stability of the miRNA itself in both directions, either stabilizing them or promoting their degradation[218,245].

Recently a new class of non-coding RNAs named circular RNAs has been discovered (circRNAs). circRNAs were found to contain up to multiple specific miRNAs binding sites and indeed were found to antagonize miRNA activity by a sponge-like mechanism[249,250], adding a new step of complexity in the miRNA regulation.

3.3.3 Genetic variants affecting miRNA biogenesis and functionality

The coding sequences of miRNAs genes like any other genes are subject to genetic variations. Genetic variants found in miRNA genes (miR-SNPs) can alter their biogenesis and/or affect their target specificity[251] (Figure I-17). Furthermore, variants in miRNA promoters[252] and other regulatory regions might alter the transcription rate. Variants within miRNA transcripts can also change the binding affinity of biogenesis enzymes like Drosha and Dicer to the miRNA hairpin altering the processing accuracy or modifying strand loading preference into RISC[251].

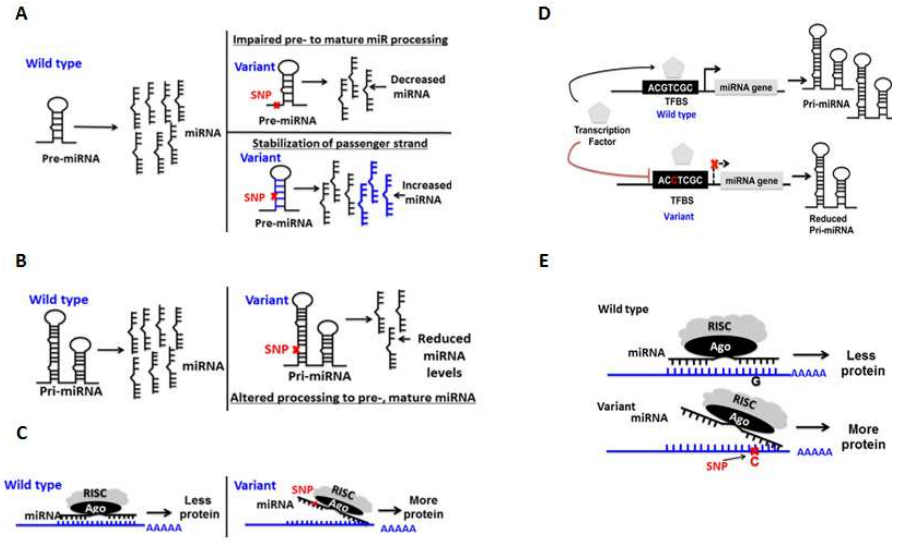


Figure I-17. Schematic representation of how SNPs are able to modulate miRNA transcription, processing or function depending on their location. (A) SNPs in the pri-miRNA sequence can alter miRNA biogenesis. **(B)** As in the previous case SNPs in the pre-miRNA sequence can affect miRNA processing. **(C)** SNPs in mature miRNA itself can affect their functional activity. **(D)** SNPs within miRNA promoter regions can alter transcription factor binding sites. **(E)** SNPs in miRNA target site can create new or delete existing miRNA target sites, or even reduce or increase the affinity of some miRNAs. Adapted from Dole *et al.*, 2016[253]

Genetic variants either in the mature miRNA itself or within the 3'UTR region of target genes (miR-TS-SNP) may affect the binding affinity leading in a loss of function of this regulatory system[254,255].

These variants had been related to many human diseases phenotypes[256]. Taking into account that a single miRNA could target hundreds of genes and therefore regulate multiple pathways, a variant in a miRNA gene resulting in functional impairment could impact several regulatory pathways.

There are an increasing number of studies associating polymorphisms in miRNA binding sites with some human diseases. In the light of bone

metabolism, variants within bone-related miRNAs or in the miRNA binding sites have been described and associated with skeletal phenotypes. For instance, miR-146a plays a crucial role in regulating bone remodeling for his involvement in osteoblast formation[257] and contributing to the suppression of osteoclast differentiation[258]. The SNP rs57095329 located in the promoter region of this miRNA was first identified as strongly associated with risk of systemic lupus erythematous. The risk-associated allele was linked to a reduced activity of the miR-146a promoter, likely by reducing the binding affinity of Ets1 to the miR-146a promoter and therefore leading to low miR-146 levels[253]. Another example can be found in the miR-125 members, where increasing levels of miR-125a were found during human and mouse RANKL-stimulated osteoclastogenesis *in vitro*[253]. The SNP rs12975333 found in the pri-miR125a was shown to modulate miRNA expression and activity. The minor allele decreases the binding of Drosha to the pri-miRNA, thus affecting the formation of the pre-miRNA and resulting in low mature miRNA levels. This variant was also found in the mature miRNA affecting the targeting. More SNPs have been found in the pri-miRNA sequence even if their possible functionality had not been defined yet. Thus, impairment in miR-125a levels due to miR-SNPs could modulate osteoclastogenesis and disrupt the balance in bone remodeling[253].

As previously mentioned, SNPs affecting miRNA-mRNA regulation can also be found at the targeted miRNA binding sites (miR-TS-SNP). In a study published by Qin *et al.*, osteoporosis associated GWAS SNPs located at bone-related genes were found to affect the binding of several miRNAs[259]. Three polymorphisms in the 3'UTR of fibroblast growth factor 2 gene (FGF2) which are located in potentially binding sites for some miRNAs including miR-146a were found to be significantly associated with femoral neck BMD[260]. The

rs1054204 located at the 3'UTR of Osteonectin was found to be associated with low bone mass in a men cohort with idiopathic osteoporosis. *Osteonectin* is one of the main non-collagenous extracellular matrix components of bone and it has been largely involved in osteoblast differentiation[261]. The minor allele variant caused the generation of a new target site for miR-433, a miRNA who has been related to osteoblasts regulation[262], triggering lower amounts of Osteonectin in the bone tissue.

Interestingly, large-scale *in silico* analyses of SNPs in human miRNA genes have demonstrated lower SNP densities in the miRNA sequences compared to their flanking regions or the human genome. Moreover, conserved miRNAs tend to have lower SNP densities than non-conserved families[263]. Hence, these data reinforce the relevant functions of miRNAs and the deleterious effect of mutations in the miRNA genes.

3.4 Identification of miRNAs involved in diseases

There are three main strategies to study the involvement of a miRNA in the pathogenesis of a disease: miRNA profiling based methods, genetic approach and functional assays. To elucidate the specific role of a miRNA, any of these strategies should not be considered as independent, to the contrary, as an indissociably set, considering that every one of them contribute to an specific miRNA characteristics that cannot be determined by the other.

In the miRNA profiling approach, samples from a group of patients are compared with the control group (generally the same tissue in a healthy individuals or a non-affected tissue in the same group of patients). The profiling comparison allows determining miRNAs differently expressed, providing an idea of the set of miRNAs that might be involved in the

mechanism undergoing the pathophysiology of the disease. For this purpose, there are different technologies available as microarray, Real-Time PCR (RT-PCR) and RNA sequencing. There are several limitations to this approach; for example, these miRNA expression signatures in pathological samples do not provide evidence of causality because of the altered pattern may be a consequence of the disease or even unrelated to the pathogenesis. Other limitations are tissue accessibility and the variability of miRNAs half-lives[251].

In the genetic approach, a linkage or association analysis between one variant within a candidate miRNA or in a miRNA target site and one disease related outcome is performed. In this case, the associated variant is likely involved in the pathophysiology or confers susceptibility to develop the disease. However it is possible that the variant detected is in linkage disequilibrium with the true functional variant. Actually, possible changes in RNA secondary structure or effects in target binding caused by the variants might be assessed *in silico* with the use of different prediction tools[251].

Nevertheless, in order to elucidate the role of one miRNA or one genetic variant in the pathophysiology of the disease, a functional validation in cell-based assays is crucial. There is a large repertoire of functional assays that can be performed, depending on the location of the variant or depending on the role that the miRNA might have in the tissue of interest.

3.5 miRNAs related to Osteoporosis

Identifying the specific functions of miRNAs in bone may give insights into the treatment of skeleton disorders. Several miRNAs have been reported to regulate the differentiation and activity of osteoblasts (Figure I-18) and osteoclasts (Figure I-19) by targeting genes with a key role in bone turnover. Although the important role of osteocytes in bone, very few studies have

been done in the miRNA field related to osteocytes, likely due to the difficulty to work with them.

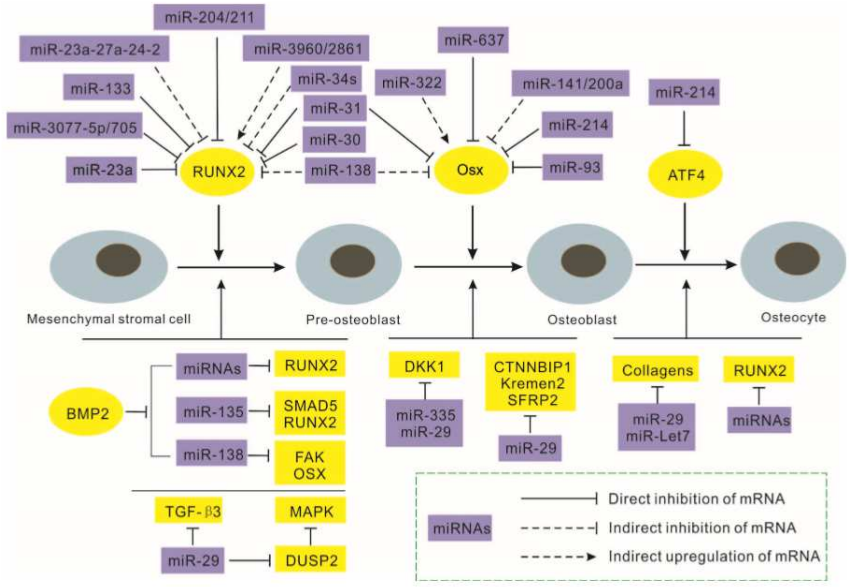


Figure I-18. Schematic representation of miRNAs involved in osteoblast differentiation. The regulation mediated by miRNAs occurs through the entire differentiation process from mesenchymal stem cells to osteoblasts. Selected miRNAs are in purple and their targets in yellow. Extracted from Jing *et al.*, 2015[213].

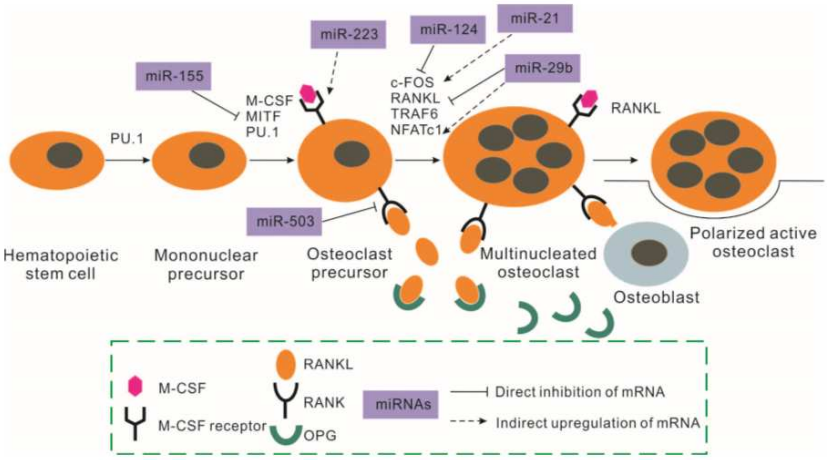


Figure I-19. Schematic representation of miRNAs involved in osteoclast differentiation. MiRNAs target different factors involved in osteoclast differentiation, such as RANK or M-CSF receptor, among others. M-CSF, macrophage colony-stimulating factor; OPG, osteoprotegerin; MITF, microphthalmia associated transcription factor; RANK, receptor activator for nuclear factor kB; RANKL, RANK ligand; NFATc1, nuclear factor of activated T-cell calcineurin-dependent 1. Extracted from Jing *et al.*, 2015[213].

Numerous signaling communications that affect both osteoblast and osteoclast lineage have also been targeted by some miRNAs which exhibit a dimorphic role by regulating both pathways. One example of this dual role is the regulation of Suppressor of Cytokine Signaling 1 (SOCS1) by miR-155[213]. SOCS1 is a negative regulator of TNF-α. TNF-α can either promotes or inhibit osteoblastogenesis[264,265] and is also able to stimulate osteoclast bone resorption[266]. Thus, a dysregulation of SOCS1 mediated by miR-155 can affect TNF-α expression resulting in an altered bone formation and bone resorption at the same time.

In this regard, the relation of miRNAs with bone loss disorders as osteoporosis has been investigated by several groups. Li *et al.*[267] described a mutation in pre-miR-2861 that blocked expression of miR-2861 and caused primary osteoporosis in two related adolescents. In parallel with our work,

Seeliger *et al*.[268] and Garmilla-Ezquerra *et al*.[269] performed a microRNA analysis from total bone tissue comparing osteoporotic versus non-osteoporotic bone. Seeliger *et al*.[268] have identified 6 miRNAs upregulated in bone tissue of osteoporotic fracture patients: miR-21, miR23a, miR-24, miR-25, miR-100 and miR-125b meanwhile Garmilla-Ezquerra *et al*.[269] detected miR-187 and miR518f as differentially expressed between sample groups. Weilner *et al*.[270] published that miR-22-3p, miR-328-3p and let-7g-5p exhibited significantly different serum levels in response to osteoporotic hip fracture and Panach *et al*.[271] found that the circulating microRNAs: miR-122-5p, miR-125b-5p and miR-21-5p were significantly upregulated in the patients with osteoporotic hip fractures compared to those without fracture.

3.6 miRNAs as novel and prominent therapeutics targets

The use of biomaterials for the repair of bone defects and the restoration of bone function has expanded with the onset of miRNAs[272], which have become promising therapeutic target for new drug research[213,273]. In order to miRNAs become successful therapeutic agents, many challenges remain to be overcome, including their efficient delivery, biological stability, long-term presentation and toxicity.

Numerous *in vivo* and *in vitro* studies have shown a prospect on the clinical application of miRNAs for the treatment of osteoporosis. MiR-29b combined with a synthetic cell-penetrating peptide was efficiently delivered into human mesenchymal stem cells and was able to promote osteogenesis by directly down-regulating osteogenic inhibitors[274]. In mice, miR-34a, inhibits osteoclast function and stimulates osteoblast function. Osteoclastic miR-34a-overexpression in transgenic mice exhibits lower bone resorption and higher bone mass. Conversely, miR-34a knockout and heterozygous mice exhibit

elevated bone resorption and reduced bone mass[275]. In this study they found that ovariectomy-induced osteoporosis as well as bone metastasis were reduced in osteoclastic miR-34a deficient transgenic mice and effectively attenuated by miR-34a chitosan nanoparticle treatment[275]. In other study published by Deng *et al*., critical canine medial orbital bone defects were repaired by scaffolds seeded with miR-31 bone marrow mesenchymal stem cells[276]. In a recent study, the 3D two-stage miR-26a delivery enhanced multiple osteogenic genes, *in vitro* and *in vivo*, resulting in repair of the critical-sized calvarial bone defects in osteoporotic mice[277]. Really recently, it has been observed that local implantation of scaffolds loaded with siRNA-*Semaphorin4d* near fracture sites in an osteoporotic rat model have improved new bone formation[278].

It is now clear that miRNAs constitute a complex regulatory network in the human body. Alterations in the expression levels of miRNAs associated with bone metabolism can lead to osteoporosis disease. Therefore, prolonged aberrant expression of miRNAs may contribute to the onset and progression of osteoporosis outcomes. In the recent years, significant progress has been made to identify altered miRNA profiling in osteoporosis. However, the understanding of mechanisms responsible for these specific miRNA signatures in the pathologic bone is still unknown. So far, miRNAs could become novel diagnostic biomarkers for the disease. Furthermore, these findings along with the development of bone compatible biomaterials have led miRNAs to be used as drug target for treating bone loss and enhancing fracture healing in osteoporotic patients.

OBJECTIVES

Notorious advances have been arisen in the understanding of the mechanisms underlying osteoporosis physiopathology. Nevertheless, molecular factors responsible for osteoporotic fractures have not been completely defined. Epigenetics mechanisms such as microRNAs have been recently appeared as one of the potential mechanisms able to broaden our knowledge in this bone disease.

The **general objective** of this thesis is to investigate the role of microRNAs in the osteoporosis disease. To this aim we focused on:

1. Study of miRNAs related to the osteoporotic fracture.

    1.1 Identification of miRNAs altered in the osteoporotic bone using a microRNA expression array, comparing fractured bone tissue vs non-osteoporotic bone.

    1.2 Mapping of microRNA expression patterns in bone cells using microRNA expression arrays in primary osteoblasts and osteoclasts.

    1.3 *In vitro* functional assays of osteoporotic-related miRNAs in order to reveal their role in the pathological process of osteoporosis.

2. Identification of miRNAs involved in the differentiation process from osteoblast to osteocyte.

3. To identify putative functional SNPs within candidate bone-related miRNAs (miR-SNPs) associated with the osteoporotic phenotype.

3.1 Association study of SNPs in the pri-miRNA sequences with BMD in the OSTEOMED2 cohort.

3.2 To validate the association results in bone tissue and in primary osteoblasts.

3.3 *In vitro* functional experiments of miRNAs harboring the associated variants, in order to reveal their role in bone metabolism.

METHODS AND RESULTS

CHAPTER 1

MicroRNAs Involvement in the Osteoporosis Physiopathology

MicroRNAs Involvement in the Osteoporosis Physiopathology

- **MANUSCRIPT 1**

MiRNA profiling of whole trabecular bone: identification of osteoporosis-related changes in miRNAs in human hip bones

De-Ugarte L, Yoskovitz G, Balcells S, Güerri-Fernández R, Martinez-Diaz S, Mellibovsky L, et al. MiRNA profiling of whole trabecular bone: identification of osteoporosis-related changes in MiRNAs in human hip bones. BMC Med Genomics. 2016 Jan 10;8(1):75. DOI: 10.1186/ s12920-015-0149-2

- **MANUSCRIPT 2**

Estudio del patrón de expresión de microRNAs en el hueso osteoporótico

García-Giralt N, De-Ugarte L, Yoskovitz G, Güerri R, Grinberg D, Nogués X, Mellibovsky L, Balcells S, Díez-Pérez S. Estudio del patrón de expresión de microRNAs en el hueso osteoporótico. Rev Osteoporos Metab Miner. 2016; 8 (1): 5-14.

CHAPTER 2

Exploring the functional role of miR-320a in osteoblasts

Exploring the functional role of miR-320a in osteoblasts

SUMMARY

Bone-related miRNAs involved in the osteoporotic fracture have been previously identified. MiR-320a was expressed in primary human osteoblasts (hOBs), with a putative regulatory role of genes required for bone metabolism. In this regard, functional assays have been performed with the aim to elucidate the action mechanism of miR-320a in osteoblastic cells. For this purpose miR-320a was either overexpressed or inhibited in hOB and gene expression changes were evaluated trough microarray analysis and the Ingenuity Pathways Analysis (IPA) software. In addition, osteoblast functionality was evaluated by assessing matrix mineralization and alkaline phosphatase activity. The effect of miR-320a on cell proliferation and viability of hOB and osteosarcoma cell line was also evaluated.

Array analysis identified 92 genes that were differentially expressed after miR-320a transfection of which 57 were mapped by IPA. Overall regulated genes were found to be involved in processes such as cellular movement, cellular growth and proliferation and skeletal and muscular system development. PTGS2 and BMP2 were the top upregulated genes while ACTA2 and miR-296 were the top downregulated genes.

A significant reduction in osteoblast matrix mineralization was observed after miR-320a overexpression. Nevertheless, any effect was observed in osteoblast differentiation after miR-320a transfection. Inhibition of miR-320a into U2OS significantly increased cell proliferation, while no effect was detected in hOB cells.

CHAPTER 2
Methods

1. Cell culture

1.1. Primary osteoblast culture

Human primary osteoblasts (hOBs) were obtained from trabecular bone of postmenopausal women who underwent hip and knee replacement due to osteoarthritis. Bony tissue was cut up into small pieces, washed in phosphate buffered solution (PBS, Gibco by Life Technologies; Paisley, UK) to remove non-adherent cells, and placed on a 140 mm culture plate. Samples were incubated in hOB medium: Dulbecco's Modified Eagle Medium (DMEM; Gibco; Invitrogen, Paisley, UK), supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; St. Louis, USA), 100 U/ml penicillin/streptomycin (Sigma-Aldrich; St. Louis, USA), 0.4% fungizone (Gibco by Life Technologies; Paisley, UK), and 100 µg/ml ascorbic acid (Sigma-Aldrich; Steinheim, Germany). This allowed osteoblastic cells to migrate from the fragments and proliferate. All experiments were performed at maximum passage 2.

1.2. Cell lines cultures

U2OS human osteosarcoma cells were grown in the same conditions as described for the culture of human osteoblasts without the addition of fungizone.

2. Cell transfection

Cells were seeded at the following conditions:

- 6-well plate at 180.000 cells/well for the gene array
- 12-well plate at 90.000 cells/well for miRNA transfection evaluation and gene target validation by qPCR

- 96-well plates at 12.000 cells/well for MTS experiments and ALP activity assays
- 24-well plate at 45.000 cells/well for Alizarin Red assays

Once cells reached 60-70% of confluence, transient transfections were performed using *mir*Vana mimics or inhibitors of hsa-miR-320a. *Mir*Vana™ miRNA Mimic Negative Control #1 and *mir*Vana™ miRNA Inhibitor Negative Control #1 were used as controls. All products were purchased from Ambion® Life Technologies. Mimics and controls mimics were used at 100 nM and inhibitors and control inhibitors at 400 nM. In order to monitor transfection efficiency, miRIDIAN microRNA Mimic Transfection Control with Dy547 (Dharmacon) was transfected at the same conditions. Transfection of miRNAs was carried out using Lipofectamine RNAiMAX (Invitrogen; Carlsbad, USA) according to the manufacturer's instruction.

3. RNA extraction

Total RNA was extracted using miRNeasy mini kit (Qiagen) or High Pure RNA Isolation kit (Roche Diagnostics, Indianapolis, USA) according to manufacturer's instructions.

4. Gene expression microarray analysis

RNA quantity and purity was determined on the ND-2000 Spectrophotometer (NanoDrop Technologies) and RNA integrity was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies). All samples met the quality standards (RNA integrity number (RIN) >7) and were subsequently used in microarray experiments.

We measured changes in gene expression levels 48 hours after transfection of hOBs (n=2) with miR-320a mimic or inhibitor by microarrays. Amplification, labeling and hybridizations were performed according to protocol GeneChip WT PLUS Reagent kit (Affymetrix) and then hybridized to GeneChip Human Gene 2.0 ST Array (Affymetrix) in a GeneChip Hybridization Oven 640. Washing and scanning were performed using the Expression Wash and Stain and the GeneChip System of Affymetrix (GeneChip Fluidics Station 450 and GeneChip Scanner 3000 7G). After quality control of raw data, data were background corrected, quantile-normalized and summarized to a $\log_2$ gene-level using the robust multi-chip average (RMA)[279] obtaining a total of 48144 transcript clusters, excluding controls, which roughly correspond to genes or other mRNAs as miRNAs or lincRNAs.

Gene expression levels were analyzed for detecting differences due to miRNA transfection following these criteria:

- Transfection with mimic or inhibitor was performed independently. S1 and S2 correspond to the two hOB samples in which experiments were performed.
- The $\log_2$ Fold Change ($\log_2$FC) was calculated for each sample as following:

  S1.320.ΔM = S1.320.M - S1.320.CM

  S2.320.ΔM = S2.320.M – S2.320.CM

  S1.320.ΔI = S1.320.CI - S1.320.I

  S2.320.ΔI = S2.320.CI – S2.320.I

  - M= Mimic; CM= Control Mimic; I= Inhibitor; CI= Control Inhibitor
  - The order of the factors in the subtractions is inverted given that the expected effect of the Mimic and the Inhibitor are reverse.

- Therefore we have 4 groups:

  S1: S1.320.ΔM; S1.320.ΔI; S2: S2.320.ΔM; S2.320.ΔI

- Each group is sorted by $\log_2$FC. The genes selected were those with a) the same sign in the two samples (S1 and S2), b) the same sign for mimic and inhibitor transfection and c) having at least one $|\log_2 FC| \geq 1$ in one of the columns:

S1_320.ΔM	S1_320.ΔI	S2_320.ΔM	S2_320.ΔI	Selection
+	+	+	+	**YES**
-	-	-	-	**YES**

- Genes differentially expressed (upregulated and downregulated) underwent a further functional analysis using Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com). For functional analysis, the maximum value among samples (S1 and S2) and treatments (mimic or inhibitor) was considered for each gene. At this point, the software does not distinguish if the effect is caused by the miRNA mimic or inhibitor in the gene network interactions.

Data analyses were performed in R (v 3.1.1) with aroma.affymetrix package.

5. Real Time PCR (qRT-PCR)

a) In order to evaluate the miR-320a levels after transfection of hOBs, total RNA extraction was performed 48 hours after transfection. Then, 1 µg of total RNA was reverse-transcribed in 20 µl reactions using the miScript II RT kit (Qiagen). cDNA was diluted 1/8 and 2 µl

were assayed in 10 µl PCR reactions in 384-well plates using MiScript SYBR Green PCR kit according to the protocol. The sequence of the mature miR-320a, according to the mirBase web site, was used as a forward primer and the Universal primer as a reverse. U6 snRNA was used as the reference gene for normalization. Preliminary experiments were conducted to optimize PCR conditions.

Forward primer sequences:
MiR-320a: 5'-AAAAGCTGGGTTGAGAGGGCGA-3'
U6: 5'-CGCAAGGATGACACGCAAATTC-3'

b) In order to assess the mRNA levels of miR-320a gene targets after transfection, cDNA synthesis was performed using 500 ng of the total extracted RNA from 3 independent hOB samples. The product was diluted by half with RNAse-free pure water, and 2 µl were assayed in 10 µl PCR reactions in 384-well plates using commercially available TaqMan Gene Expression assays (Thermo Fisher Scientific). Gene expression levels were calculated against beta-actin expression and then normalized to an internal sample (relative quantification) using arbitrary units.

All qPCR reactions for each sample were performed in triplicate. Amplification was performed in a QuantStudio 12K Flex Real-Time PCR (Applied Biosystems), and the ExpressionSuite software v.1.0.3 (Life Technologies) was used both for determination of relative quantification (RQ) (by 2-ΔΔCt method) and for melting curve analysis.

6. Cell proliferation assay (MTS)

Viable cells were determined 48 hours post-transfection in hOBs (n=3) using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega; WI, USA) according to manufacturer's instructions. To measure the amount of soluble formazan produced by cellular reduction of MTS, the absorbance at 490 nm was measured using a multi-well plate reader Infinite M200 (Tecan; Grödig, Austria).

7. Alizarin red quantification

hOBs (n=4) were cultured during 28 days with hOB medium supplemented with 5mM β-glicerophosphate (Sigma-Aldrich, St Louis, MO, USA). Cells were transfected with both mimic and inhibitor of miR-320a and the corresponding controls at day 1 and day 14 after seeding. During the cell culture time period, the medium was changed every 3 days.

At day 28, cells were stained with alizarin red to quantify mineralized nodules. At this time point, media was removed from the cell monolayer and gently washed 3 times with PBS. The cells were then fixed in 10% buffered formalin for 10 minutes at room temperature. Fixative was removed and cultures washed in PBS. The cell layer was stained with 2 % Alizarin-S (Sigma-Aldrich, St Louis, MO, USA) at ~ pH 4.2 for 20 minutes. Cell preparations were washed with PBS to eliminate nonspecific staining. To quantify calcium deposition, the dye was leached from the monolayer by the addition of 10% cetylpyridinium chloride until all of dye had been drawn from the monolayer. 100µl of the solution (in duplicate) was transferred to a clean 96 well plate. Optical density was then quantified by spectrophotometry at 550 nm, using 10% cetylpyridinium chloride as a blank reference.

8. ALP activity assay

ALP activity was measured in hOBs 48 hours after miR-320 transfection (n=4) using the Alkaline Phosphatase Assay Kit (Colorimetric) (Abcam; Cambridge, UK) according to the manufacturer's instructions.

9. Statistical analysis

Mann-Whitney U test in the SPSS v.12.0 for Windows was performed to stablish comparisons between cells transfected with miRNAs and their respective controls in the Alizarin Red quantification, alkaline phosphatase activity, cell proliferation assay and detection of miRNA and mRNA levels by qPCR. . All analyses were two-tailed, and p-values<0.05 were considered significant. .

CHAPTER 2
Results

1. Exploring the functional role of miRNA-320a in osteoblasts

In order to evaluate the role of miR-320a in bone metabolism, we performed a gene microarray analysis after either overexpressing or inhibiting the miR-320a on hOBs. As a first step, we monitored the efficiency of miRNA transfection using miRIDIAN microRNA Mimic Transfection Control with Dy547 (Figure R-1A) and by determining miR-320a levels after mimic or inhibitor transfection (Figure R-1B). Results demonstrated a high transfection efficiency by increasing the miR-320a levels more than 400-fold after mimic transfection (p=0.002) and a markedly downregulation by the inhibitor transfection (p=0.002)

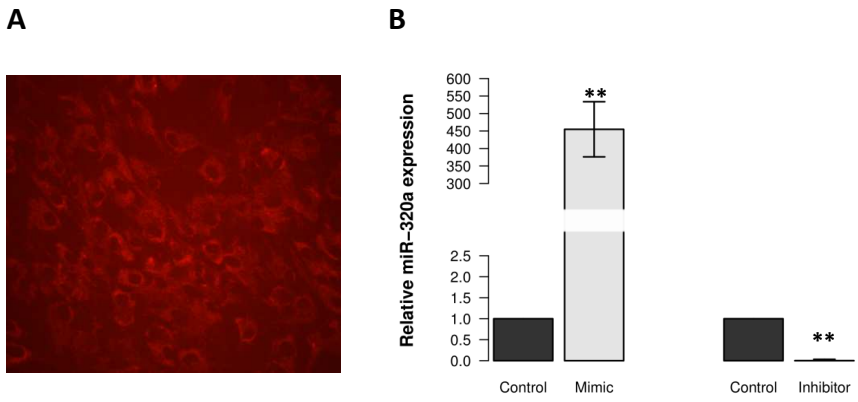


Figure R-1. Transfection efficiency of miRNA transfection in hOBs. (A) miRIDIAN microRNA Mimic Transfection Control with Dy547 at 100nM was efficiently delivered into hOBs (Magnification 20x). **(B)** hOBs were transfected with mimic (100 nM) and inhibitor (400 nM) of miR-320a and the respective miRNA controls. MiRNA levels were measured 48 hours post-transfection by qRT-PCR. Data represent the mean ± SD (n=2). **p<0.01

1.1 Gene microarray analysis of miR-320a transfection

mRNA expression levels were assessed 48 hours after miR-320a transfection on hOB samples (n=2). A total of 92 genes were found differently expressed after miR-320a overexpression or inhibition. Out of the 92 genes, only 57

genes had Affymetrix annotations and were finally used in the functional analysis performed by IPA (Table R-1). Using hierarchical clustering heat map, the 57 differently expressed genes were visually illustrated (Figure R-2).

Table R-1. Differentially expressed genes selected for functional analysis by IPA. The log_2 Fold Change (log_2FC) is calculated for each sample subtracting the log_2 normalized gene expression of the mimic with his own control or the opposite in the case of inhibitor as described in methods section. In bold, values of $|log_2FC| \geq 1$.

Affymetrix Symbol	S1.320.ΔM	S2.320.ΔM	S1.320.ΔI	S2.320.ΔI
PPIAL4B	-0,37	-0,35	**-1,08**	**-1,06**
PTGS2	**2,03**	**1,53**	0,16	0,31
MIR1537	-0,53	**-1,09**	-0,96	-0,53
RP11-464F9.21	**1,01**	0,59	0,23	0,38
TUBB8	0,10	0,08	**1,02**	0,07
ACTA2	-0,03	**-1,22**	-0,29	-0,03
OR9I1	0,51	0,49	0,13	**1,05**
OR10P1	-0,10	-0,67	**-1,04**	-0,19
SNORA2A	**-1,27**	-0,22	-0,58	-0,98
RNY3P10	0,23	**1,03**	0,18	0,35
RNA5SP25	-0,94	-0,62	-0,56	**-1,06**
RNY3P3	-0,67	-0,07	-0,33	**-1,24**
RP11-404P21.5	**1,42**	0,30	0,10	0,07
RP11-638I2.4	**-1,10**	-0,49	-0,42	-0,70
LOC642131	-0,29	**-1,31**	-0,24	-0,64
SNORD115-45	0,03	0,27	**1,34**	0,96
CTD-3092A11.1	0,27	0,50	0,38	**1,05**
SCARNA15	-0,42	-0,41	-0,73	**-1,02**
RNU7-24P	0,72	**1,08**	0,24	0,35
LINC00854	0,47	0,33	0,68	**1,02**
SLC16A6	**1,01**	**1,12**	0,13	0,34
RNA5SP92	0,30	0,55	0,12	1,32
RNU6-951P	-0,26	-0,51	**-1,02**	-0,15
CCL20	**1,19**	0,27	0,25	0,53
NR4A2	**1,08**	**1,16**	0,03	0,21

Affymetrix Symbol	S1.320.ΔM	S2.320.ΔM	S1.320.ΔI	S2.320.ΔI
ANKRD44-IT1	**1,03**	0,53	0,24	0,12
BMP2	**1,65**	**1,60**	0,27	0,08
RP3-461P17.9	-0,90	**-1,87**	-0,24	-0,32
RNA5SP479	0,20	1,09	0,04	0,59
MIR296	-0,24	-0,07	**-1,27**	-0,36
AP001434.2	-0,94	**-1,06**	-0,49	-0,26
DGCR11	-0,47	-0,30	-0,04	**-1,08**
LIF	**1,28**	**1,10**	0,08	0,34
RNU7-110P	**-1,08**	-0,42	-0,51	-0,89
AREG	**1,11**	0,68	0,18	0,52
AREG	**1,27**	0,78	0,13	0,73
SLC7A11	**1,16**	**1,20**	0,20	0,80
NPY6R	-0,03	-0,16	-0,64	**-1,00**
MIR1271	0,18	**1,25**	0,56	0,45
RNU6-522P	**1,46**	0,85	0,24	0,07
HIST1H3H	-0,51	**-1,07**	0,00	-0,14
FKBP1C	0,15	0,45	**1,22**	0,16
SNORD101	-0,07	**-1,09**	-0,72	-0,42
LPA	0,24	0,76	0,08	**1,00**
SNORD93	-0,01	-0,41	-0,39	**-1,15**
IGFBP1	0,91	**1,38**	0,03	0,20
STC1	**1,42**	**1,02**	0,11	0,44
RNU7-181P	0,61	0,60	0,09	**1,01**
IL33	**1,41**	0,77	0,12	0,13
RN7SKP120	0,05	0,00	0,15	**1,06**
PGM5-AS1	**-1,06**	-0,72	-0,64	-0,06
RP11-60I3.4	-0,27	-0,26	-0,53	**-1,09**
C5	-0,12	**-1,05**	-0,28	-0,10
LOC101927635	0,38	0,22	0,22	**1,10**
CDY2A	0,04	0,83	**1,06**	**1,06**
LOC728763	**-1,07**	-0,17	-0,21	-0,08
AC003092.1	**1,20**	1,39	0,03	0,48

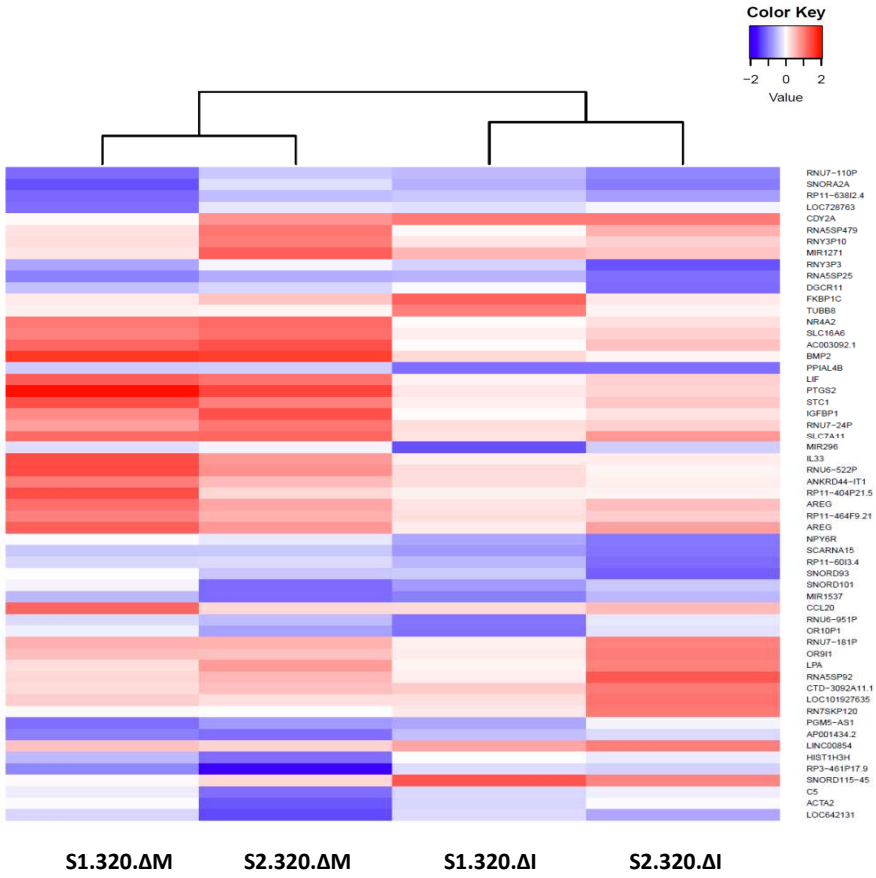


Figure R-2. Heatmaps of differently expressed mRNA expression after miR-320a transfection. Out of the 92 differently expressed mRNAs detected between treatment and control, a heatmap was generated for the 57 mRNAs with annotated symbol in Affymetrix which were further used in the functional analysis. Experiments were performed in two hOB samples, S1 and S2. The first two columns represent a hierachial cluster analysis of the differently expressed mRNAs after overexpression of miR-320a compared to the control (Mimic-Control), while the third and fourth column show the differently expressed mRNAs after inhibition of miR-320a compared to the control (Control-Inhibitor). Each row represents an mRNA and each column, a sample. The mRNA clustering tree is shown at the top of the panel. The color scale illustrates the relative level of the corresponding mRNA expression: red, higher than the reference and blue below the reference.

1.2 *In-silico* functional analysis of canonical pathways and cellular functions related to miR-320a

Among the 57 genes that were differentially expressed after miR-320a transfection, 33 were up-regulated and 24 were down-regulated. A systemic analysis of our microarray data in a biological context was performed with IPA. The significant canonical pathways identified are represented in Figure R-3. Top statistically significant canonical pathways included LXR/RXR activation, agranulocyte adhesion and diapedesis, and ILK signaling. Interestingly, we found the role of osteoblasts, osteoclasts and chondrocytes as one of the most significant pathways (Figure R-3; *in bold*). Statistical significance was based on the ratio of differently expressed genes in our data set to all genes involved in the pathway. We then investigated these genes in terms of cellular function (Figure R-4). Important and statistically significant functions included cellular movement, cellular development and cell growth and proliferation. Remarkably, genes involved in skeletal system have been affected by miR-320a transfection.

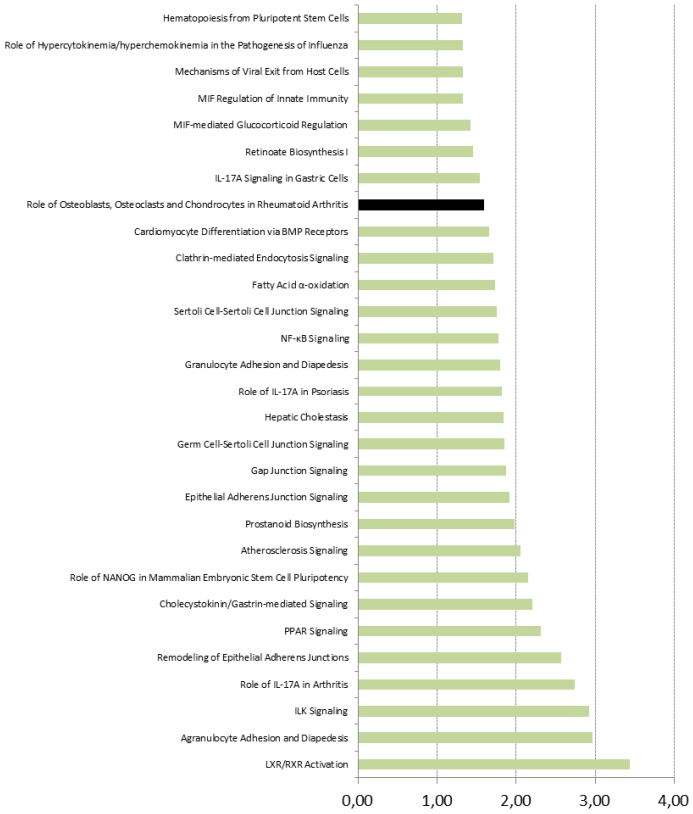


Figure R-3. Significant pathways generated by Ingenuity Pathway Analysis (IPA) from the differentially expressed (upregulated and downregulated) mRNAs after miR-320a transfection. Significant pathways are sorted by score –log (p-value) from the Fisher's exact test across all observations.

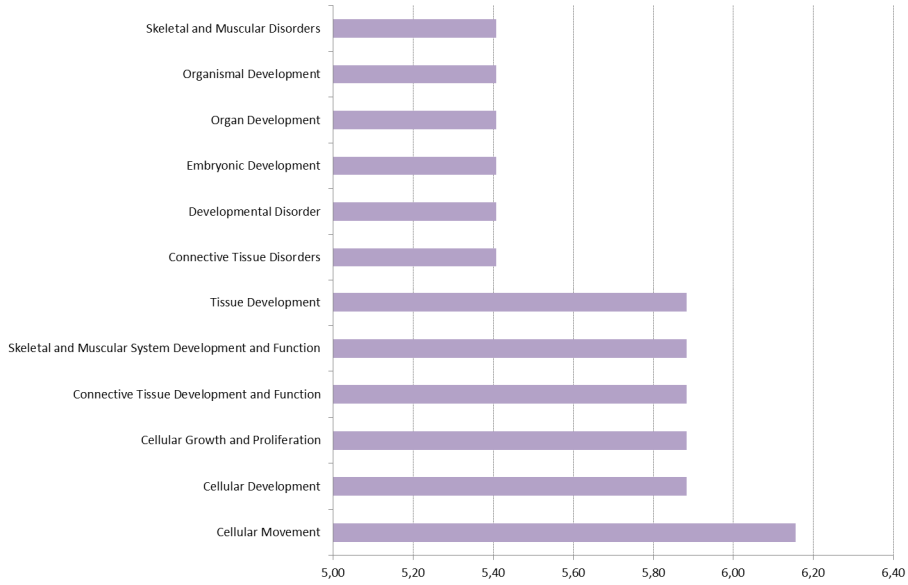
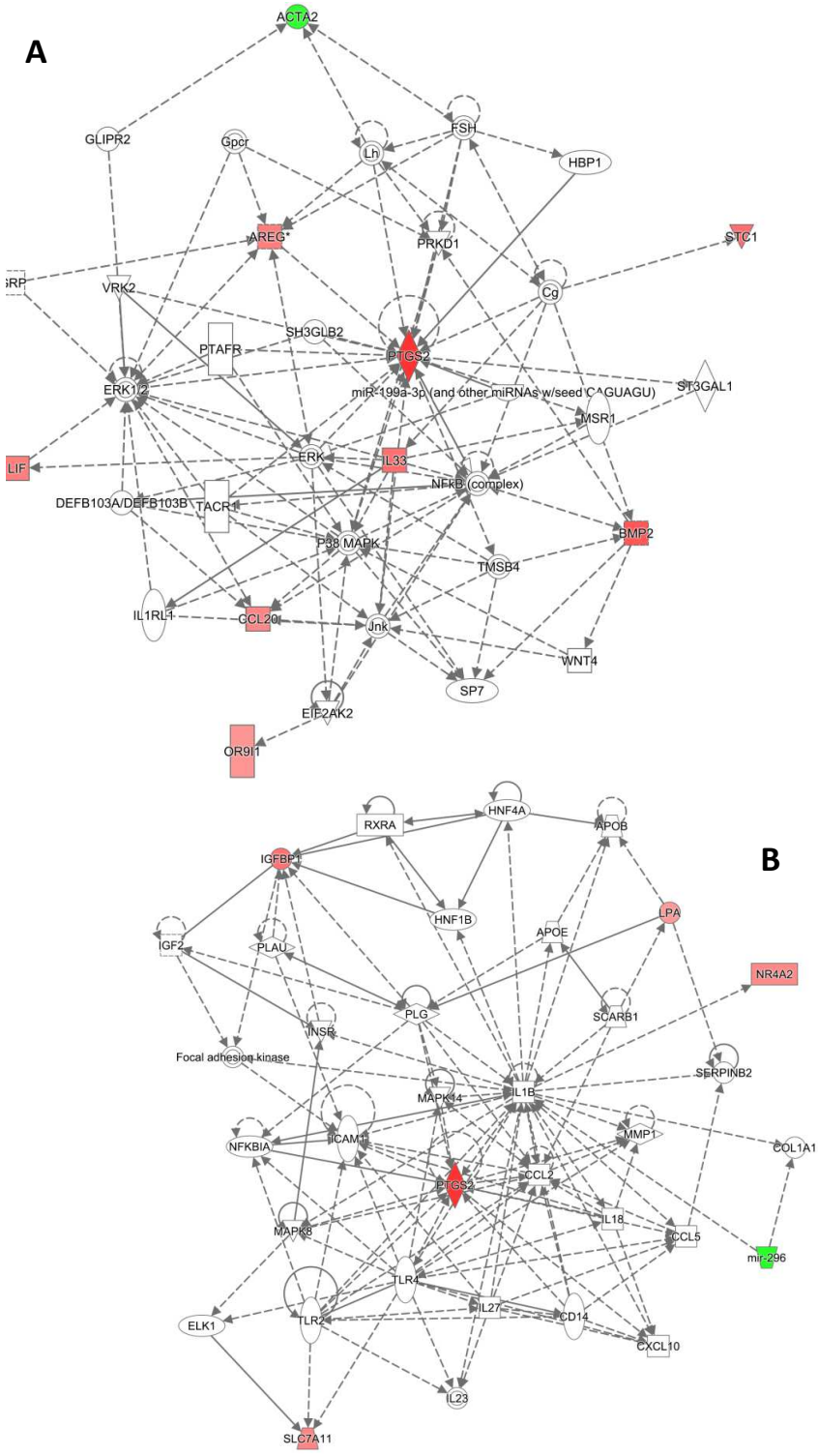


Figure R-4. Diseases and functions enriched for differentially expressed transcripts in miR-320a transfected hOBs. Enrichment was determined using IPA. The P-value was calculated using the Fisher's exact test. The 12 most significant categories, among a total of 75, are represented. Significant categories are sorted by score –log (p-value).

The differently expressed genes were then overlaid onto a global molecular network developed from information contained in the IPA knowledge base. The top two networks identified are represented in Figure R-5. The interactions between the genes involved in both networks were mainly indirect. Interestingly, important genes related to bone metabolism such as SP7 (Osx)[16] and Wnt4[280] have been found to be indirectly regulated by BMP2 (Figure R-5A), and Col1A1 regulated by miR-296 (Figure R-5B). These networks identified a considerable number of cytokines, such as IL33, IL1B, IL18, IL27 and IL23, and MAPK related to miR-320a targets.


A
ACTA2
GLIPR2
Gpcr
FSH
Lh
HBP1
AREG*
PRKD1
STC1
VRK2
Cg
SH3GLB2
PTAFR
PTGS2
ERK1/2
miR-199a-3p (and other miRNAs w/seed CAGUAGU)
ST3GAL1
MSR1
ERK
IL33
LIF
NFkB (complex)
DEFB103A/DEFB103B
TACR1
BMP2
P38 MAPK
TMSB4
IL1RL1
CCL20
Jnk
WNT4
SP7
EIF2AK2
OR9I1
B
HNF4A
RXRA
APOB
IGFBP1
HNF1B
LPA
IGF2
PLAU
APOE
NR4A2
PLG
SCARB1
INSR
Focal adhesion kinase
SERPINB2
MAPK14
IL1B
MMP1
NFKBIA
ICAM1
COL1A1
CCL2
PTGS2
IL18
MAPK8
CCL5
miR-296
TLR4
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CD14
ELK1
TLR2
CXCL10
IL23
SLC7A11

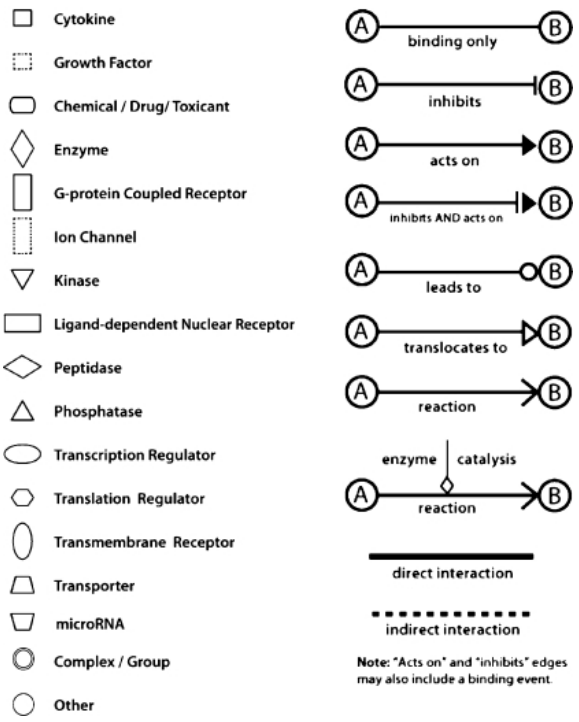

Figure R-5. Most significant two gene networks of differently expressed genes after miR-320a transfection in hOBs. The intensity of the node color-(red) indicated the degree of up-regulation. The node color-(green) indicated gene down-regulation. Genes in uncolored notes were not identified as differentially expressed in our experiment and were integrated into the computationally generated networks on the basis of the evidence stored in the IPA knowledge memory indicating a relevance to this network.

1.3 Top miR-320a regulated genes underwent validation by qRT-PCR

Among the genes differently expressed after miR-320a transfection, we selected 6 of the top regulated genes which showed highest fold change after miRNA transfection compared to the control and/or have been linked with bone regulation. Genes selected were the upregulated genes *PTGS2, BMP2, STC1* and *IGFBP1* and the downregulated genes *ACTA2* and *miR-296*.

In fact, PTGS2[281,282], BMP2[283-286], STC1[287-289] and IGFBP1[290-293], have been related to biological/molecular functions on bone metabolism.

Gene expression values were analyzed following the same criteria applied in the microarray data analysis. Hence, each transfection condition (mimic or inhibitor) was treated individually. Genes maintaining the same sign between conditions and the same sign found in the array were considered as validated. Only miR-296 was validated in the new set of samples (ΔM= -0.62; ΔI= -0.17). BMP2 (ΔM=1.3), STC1 (ΔM=1) and ACTA2 (ΔM=-0.6) were only validated for transfection with the mimic. IGFBP1 was differently regulated after transfecting with mimic or inhibitor but showed an opposite trend of regulation than the observed in the microarray analysis.

1.4 The role of miR-320a in osteoblast function

1.4.1 Matrix mineralization is negatively regulated by miR-320a

The role of miR-320a on osteoblast function was *in vitro* investigated. Primary hOBs (n=3) were transfected with miR-320a mimic or inhibitor and the respective controls. A significant reduction in matrix mineralization was observed after overexpressing miR-320a in hOB at 28-day culture (p=0.002) (Figure R-6). Moreover, an increase in matrix mineralization was observed after the inhibition of miR-320a although data does not reach to a significant level (p=0.065) (Figure R-6).

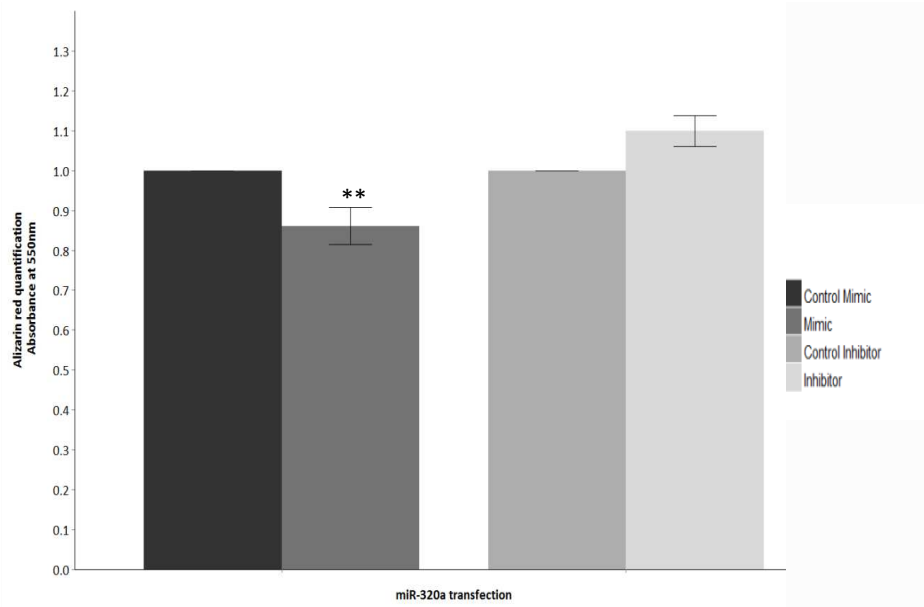

Figure R-6. Effect of miR-320a transfection in osteoblast matrix mineralization. Alizarin Red quantification was assayed in hOB transfected with either mimic or inhibitor of miR-320a and the respective controls at 28 day culture. Data represent the mean ± SEM (n=3). **p<0.01

On the other hand, no significant differences in ALP activity were observed 48 hours after miR-320a transfection.

1.3.2 Inhibition of miR-320a increases osteosarcoma cell proliferation

Functional data generated by IPA showed that genes regulated by miR-320a were significantly associated with cell proliferation. In this context, mimic and inhibitor of miR-320a and its respective controls were transfected into primary hOB and U2OS cell line. The overexpression or inhibition of miR-320a did not cause any significant change in hOB proliferation at any of the conditions tested (Figure R-7). In contrast, a reduction in cell viability was found when miR-320a was overexpressed at 400 nM in U2OS cell line, although this reduction did not reached significance (Figure R-7). In the other

hand, the inhibition of miR-320a significantly increased cell proliferation in U2OS cells (p=0.029) (Figure R-7).

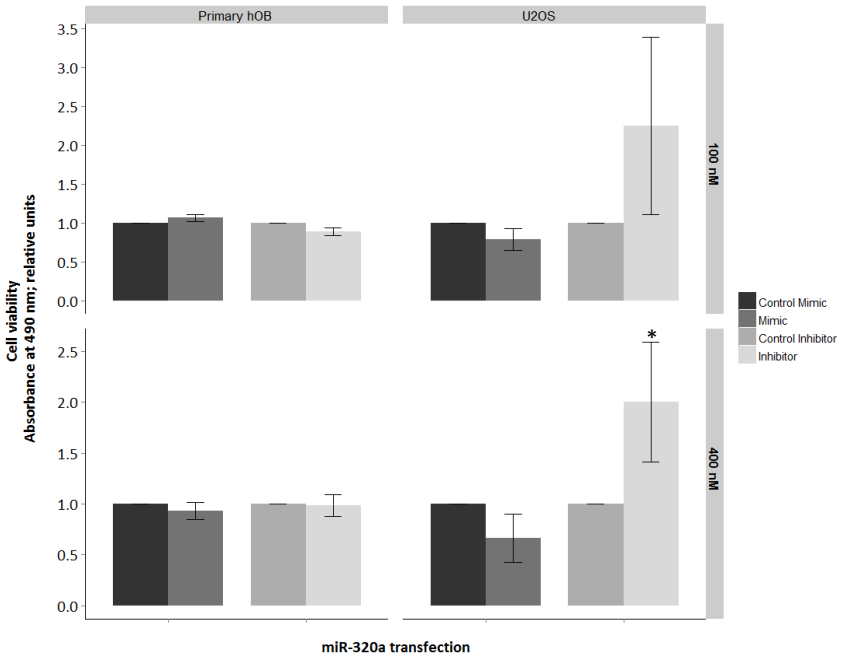

Figure R-7. Effect of miR-320a in osteoblast proliferation. Cell viability was determined in primary hOBs (n=3) and U2OS cells (n=4) transfected with mimic and inhibitor of miR-320a and its respective controls 48 hours post-transfection. Data represent the mean ± SEM (n=3). *p<0.05

CHAPTER 3

Characterization of miRNAs expressed in bone tissue

Characterization of miRNAs expressed in bone tissue

SUMMARY

Bone tissue is a complex network of several cell types with very different functions, which interact among them and with the extracellular matrix. Each specific cell type expresses theirs own miRNAs that are needed to perform the cell function at this time. The set of total miRNAs expressed for all cell types will configure the specific signature of the bone tissue at this physiological situation.

The first aim of this project was to obtain a miRNA expression profile of whole bone tissue from postmenopausal women. Additionally, we needed to perform a miRNA expression profiling of human primary osteoblasts and osteoclasts in order to assess their origin.

Whole bone tissue was obtained from trabecular bone after hip replacement and was analyzed in fresh conditions (n=6). Primary osteoblasts were obtained from pieces of this trabecular bone after culturing them during 1 month (n=4). Human osteoclasts were obtained from monocytes precursors after 21 to 24 days of *in vitro* RANKL and M-CSF stimulation (n= 5).

A microRNA expression profiling was obtained for each sample by means of a microarray. Thereafter, a global miRNAs analysis was performed combining the data acquired in the three miRNA arrays.

CHAPTER 3
Methods

1. Whole trabecular bone tissue and hOBs were obtained according to the protocol previously explained in methods of chapter 1.

2. Obtaining of human Osteoclast from Peripheral Blood Mononuclear cells

Blood samples were obtained from postmenopausal women (n=5) in accordance with the approved ethical procedures of Clinical Research Ethics Committee of Parc de Salut MAR (Table M-1). Monocytes, the osteoclast precursors, were isolated from peripheral blood mononuclear cells (PBMC) using Human Monocyte Enrichment Cocktail RosetteSep™ (Stemcell technologies, Vancouver, BC, Canada) following the manufacturer's instructions. After isolating the monocyte fraction, the CD14 marker, strongly expressed on monocytes, was determined by flow cytometry, usually obtaining a culture purity of 90% CD14+ cells. Monocytes were seeded at 5 x 10^5 cells on 10mm glass coverslips (Marienfeld, Lauda-Königshofen, Germany) and were incubated for 2 hours at 37°C in 5% CO_2 in completed medium, consisting in: alpha-MEM (Gibco) supplemented with 10% FBS (Gibco), 2mM Glutamax (Gibco) and 1% penicillin/streptomycin (Gibco). Non-adherent cells were washed out and adherent cells were used for the osteoclast differentiation. The adherent cells were cultured in completed medium supplemented with 25ng/ml M-CSF (Peprotech) and 50ng/ml RANKL (Peprotech). All the cultures were maintained until the generation of multinucleated giant cells, which occurred around 21-24 days of culture. Culture medium was changed once every 3 days and M-CSF and RANKL were added at each medium change.

Table M-1. Baseline characteristics of the postmenopsaual women

Patient characteristics	Mean±SD (n=5)
Age (years)	65.4 ± 5.08
BMI (kg/m^2)	22.85 ± 2.56
LS BMD (g/cm^2)	0.80 ± 0.13
FN BMD (g/cm^2)	0.65 ± 0.08

Abbreviations: BMI=body mass index; BMD=bone mineral density; LS=lumbar spine; FN=femoral neck

2.1 Osteoclast characterization

a) Phalloidin and DAPI staining

Osteoclasts have a ring structure composed of actin. This actin ring is a hallmark of the degradative capacity of functional osteoclasts, without this actin ring, osteoclasts cannot resorb bone efficiently[56].The obtained osteoclasts were washed with PBS and fixed with 3.7% (W/V) paraformaldehyde (PFA) for 10 min. Then cells were permeabilized with 0.1% Triton-X-100 in PBS for 3-5 min. The cytoskeletal actin was stained with TRITC-Phalloidin (Sigma-Aldrich; Steinheim, Germany) and cell nuclei with 4', 6-Diamidino-2-phenylindole (DAPI) (Sigma-Aldrich; Steinheim, Germany). The cytoskeleton actin and DAPI-stained nuclei were visualized by Leica Confocal TCS SP5 Upright microscopy.

b) Tartrate-resistant acid phosphatase (TRAP) staining

TRAP staining was performed using the Leukocyte Acid Phosphatase Assay Kit (Sigma-Aldrich, St. Louis, Missouri, USA) according to the manufacturer's instructions.

3. RNA extraction of osteoclasts

Total RNA was extracted using miRNeasy mini kit (Qiagen) according to manufacturer's instructions.

4. MiRNA microarray analysis of osteoclasts

RNA quantity and purity was determined on the ND-2000 Spectrophotometer (NanoDrop Technologies) and RNA integrity was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies).

For microarray analysis, samples were poly (A)-tailed and biotin-ligated using the Genisphere FlahTag™ Biotin HSR RNA Labelling Kit. After sample processing and before hybridization, biotin labelling was confirmed with the Enzyme Linked Oligosorbent Assay (ELOSA). Samples were then hybridized into the GeneChip® miRNA 4.0 arrays during 16 hours at 48 °C and 60 rpm in a GeneChip® Hybridization Oven 640. Following hybridization, the arrays were washed and stained in the GeneChip® Fluidics Station 450, using the GeneChip® Hybridization, Wash and Stain kit. The stained arrays were scanned with the GeneChip® Scanner 3000 7G, generating CEL files for each array. Data quality control was assessed using Affymetrix Expression Console software. All arrays met the quality control criteria.

Normalization was performed with R (version 3.2.3) using the Robust Multi-array Average algorithm included in the aroma.affymetrix package. MiRNAs with expression values higher than three times the 25th percentile of the overall intensity values per array in at least two of the samples were selected.

5. Analysis of array results from osteoblasts, osteoclasts and whole bone tissue

Previously, a miRNA array analysis was performed in total bone tissue and human primary osteoblasts at Exiqon Services. The methodology used was

previously described in chapter 1[294]. In the case of Exiqon arrays, miRNAs with expression values higher than 1.2 times the 25th percentile of the overall intensity values per array in at least two osteoblasts samples or in three samples of total bone tissue were selected.

The intersections of the expressed miRNAs in osteoclast, osteoblast and fresh bone were made using R programming with the package Vennerable.

CHAPTER 3
Results

1. Characterization of microRNAs expressed in bone tissue

1.1 Characterization of primary osteoblasts

ALP was used as marker for determining the osteoblast differentiation. Results were previously explained in chapter 1.

1.2 Formation and characterization of primary osteoclasts

Like osteoclasts are very difficult to isolate from bone because they are very adherent to the bone matrix and present at very low frequency in the mature skeleton, human osteoclasts (hOCs) were differentiated from monocytic progenitors. Specific characteristics were tested, including TRAP and osteoclasts actin ring staining, to confirm the osteoclasts differentiation in culture conditions.

Mature TRAP-positive giant multinucleated osteoclasts were detected at 21 to 24 days of culture in the presence of M-CSF and RANKL (Figure R-8A). Functional osteoclasts were confirmed microscopically by the presence of three or more nuclei, the presence of podosomes, and the formation of actin ring (Figure R-8B)

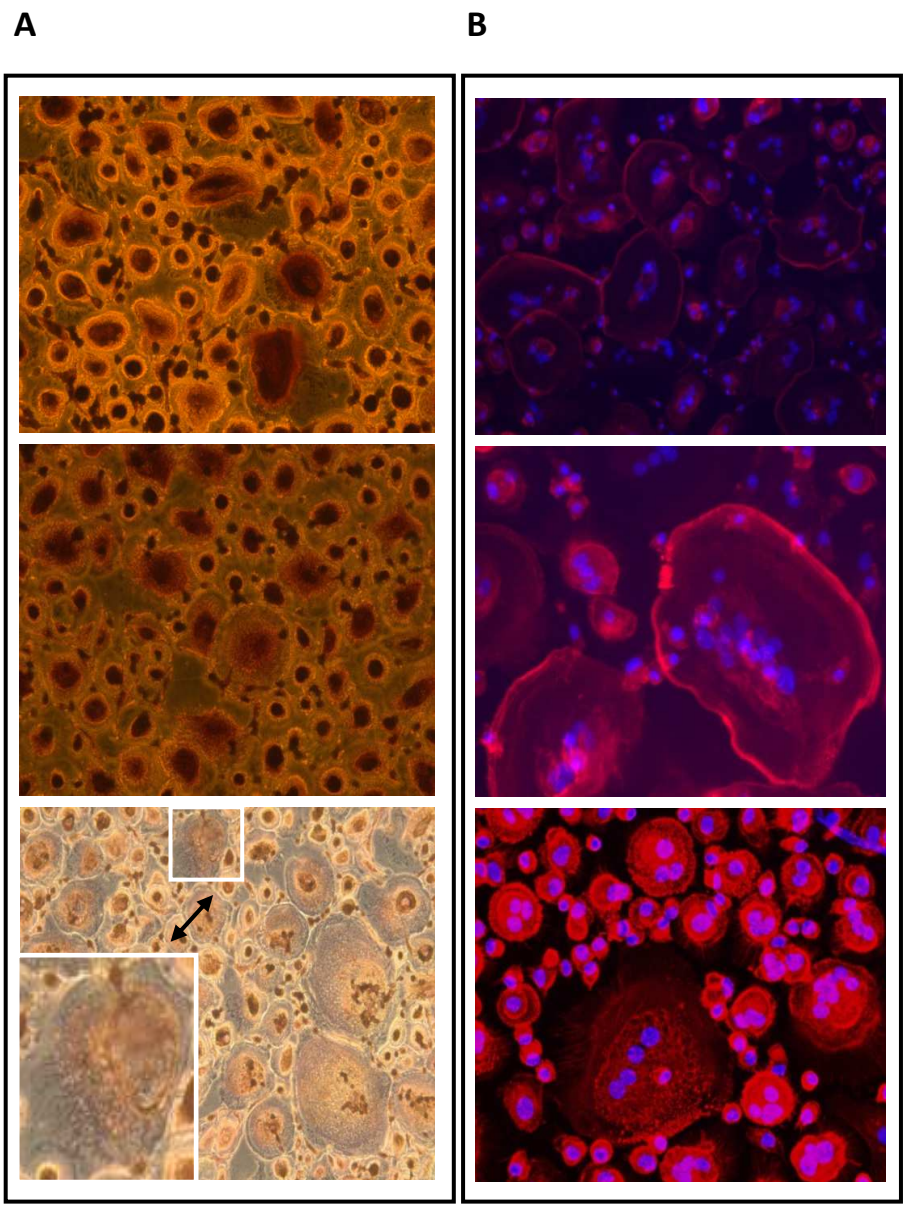

Figure R-8. Characterization of human primary osteoclasts. A confluent layer of multinucleated cells was observed in all cultures at 21 to 24 days. **(A)** More than 90% of multinucleated cells were TRAP positive after 21 days of culture **(B)** The multinucleated cells form actin rings observed by confocal microscopy after phalloidine labeling of actin (red); nuclei were stained with DAPI (blue). **(A & B)** The presence of podosomes was observed by both light **(A insert)** and confocal microscope **(B, bottom)**. Magnification 20X (B2: 40X)

1.3 Global bone miRNA analysis

Three microRNA arrays were performed: from total fresh bone, from hOBs and from *in vitro* differentiated hOCs. In total, 6 healthy bone samples, 4 hOB samples and 5 hOC samples were used for the miRNA global analysis. Bone and osteoblast arrays were performed in Exiqon services while osteoclasts array was done in Affymetrix platform. Only shared miRNAs (n=2057) between platforms were included in the study. MiRNAs expressed at least in 2/4 of the hOB, 2/5 hOC and 3/6 bone samples were included for the miRNA intersection analysis, resulting in 801 miRNAs (Figure R-9).

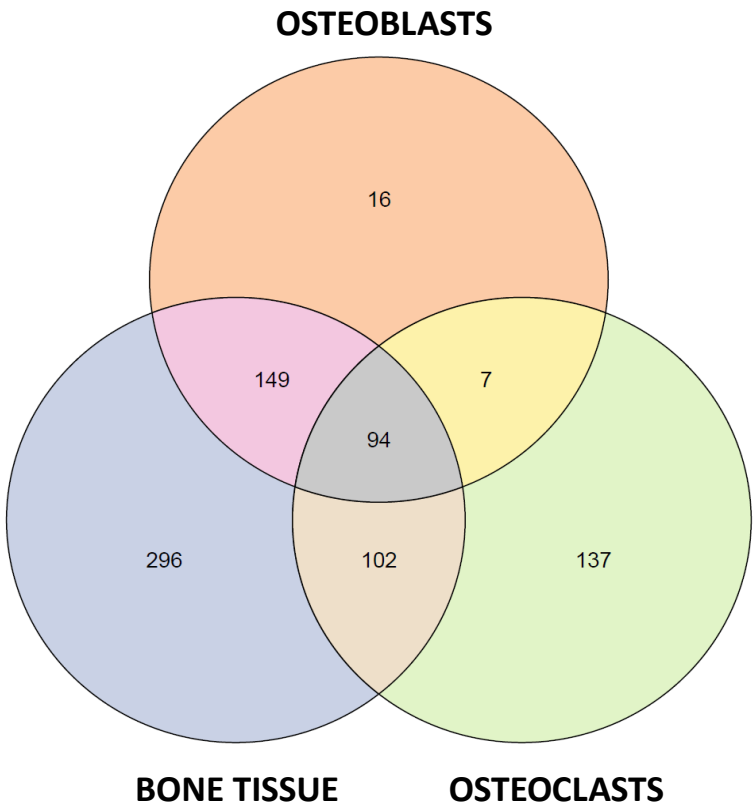

Figure R-9. Global expression analysis of bone related miRNAs. Venn diagram representing data obtained from the microarray analysis of miRNAs detected in fresh bone samples, osteoblasts and osteoclasts.

From the 641 miRNAs detected in bone samples, a 54% (n=345) was found to be present in osteoblasts and/or osteoclasts. The other 46 % of miRNAs detected in bone (n=296) were not identified in any of the bone cells analyzed. This group of miRNAs might be specific of other bone cells such as osteocytes which are the most prevalent cells of bone (90 to 95%), while osteoblasts are estimated to constitute 4 to 6% and osteoclasts around 1 to 2%. Other cell sources may be lining cells, MSC or even adipocytes.

MiRNAs from each array were then sorted in terms of expression levels. The 65 % (n=13) of the top 20 most expressed bone-related miRNAs were found in osteoblasts and/or osteoclasts. In Table R-2 are shown the top ten most expressed miRNAs in bone and their heterogenic localization.

Table R-2. Top ten most expressed miRNAs in the bone tissue array and their localization.

miRNA	Localization
miR-26b-5p	OB
miR-20a-5p	OC
miR-16-5p	OB & OC
miR-126-3p	OC
miR-4284	OB & OC
let-7a-5p	OB & OC
miR-223-3p	ONLY BONE
miR-4791	OB
miR-27a-3p	OC
let-7g-5p	OB & OC

A total of 266 miRNAs were detected in osteoblasts, of which 243 (91 %) were also present in total bone samples, representing a 38 % of all 641 bone miRNAs. Of them, 94 were found to be also expressed in osteoclasts. Thereafter, miRNAs present in all osteoblast samples were sorted in terms of expression levels. The 75% of the top 20 most expressed miRNAs were also found in bone tissue whereas the other 25% were only detected in the osteoblast array. These last miRNAs might be expressed in osteoblasts due to the *in vitro* conditions of cultured cells that create an artificial environment leading to an altered miRNA expression.

In osteoclasts, 340 miRNAs were detected, of which 196 (58 %) were also present in the bone tissue array, representing a 31 % of all miRNAs detected in total bone tissue. In this case, an important proportion of osteoclast-related miRNAs was not found in the bone tissue array. One feasible reason to explain this fact might be the low percentage of osteoclasts in bone, making it difficult to detect osteoclast-specific miRNAs when total bone is analyzed. Hence, only high expressed miRNAs in osteoclasts can be caught in the bone tissue array. Other explanation may be the methodology used to obtain osteoclasts cells as they were *in vitro* differentiated from blood mononuclear cells instead of isolating them *in-situ* from bone tissue. Thereafter, miRNAs present in all osteoclast samples were sorted in terms of expression levels. Remarkably, all top 20 most expressed osteoclast-related miRNAs were found in bone tissue corroborating the first hypothesis.

Seven miRNAs detected in both cell types were not detected in bone tissue samples. These miRNAs might be attributable to some aspect related to the *in vitro* culture conditions.

CHAPTER 4

MicroRNAs involved in Genetics of Osteoporosis

- **MANUSCRIPT 3**

Association between SNP Regulation of miRNA Expression and Osteoporosis

This manuscript has been submitted to Scientific Reports

Association between SNP Regulation of miRNA Expression and Osteoporosis

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Abstract

Biogenesis and function of microRNAs can be influenced by genetic variants in the pri-miRNA sequences leading to phenotypic variability. This study aims to identify single nucleotide polymorphisms (SNPs) affecting the expression levels of bone-related mature microRNAs and thus, triggering an osteoporotic phenotype.

An association analysis of SNPs located in pri-miRNA sequences with bone mineral density (BMD) was performed in the OSTEOMED2 cohort (n=2183). Functional studies were performed for assessing the role of BMD-associated miRNAs in bone cells.

Two SNPs, rs6430498 in the miR-3679 and rs12512664 in the miR-4274, were significantly associated with femoral neck BMD. Further, we measured these BMD-associated microRNAs in trabecular bone from osteoporotic hip fractures comparing to non-osteoporotic bone by qPCR. Both microRNAs were found overexpressed in fractured bone. Increased matrix mineralization was observed after miR-3679-3p inhibition in human osteoblastic cells.

Finally, genotypes of rs6430498 and rs12512664 were correlated with expression levels of miR-3679 and miR-4274, respectively, in osteoblasts. In both cases, the allele that generated higher microRNA expression levels was associated with lower BMD values.

In conclusion, two osteoblast-expressed microRNAs, miR-3679 and miR-4274, were associated with BMD; their overexpression could contribute to the osteoporotic phenotype. These findings open new areas for the study of bone disorders.

Introduction

MicroRNAs (miRNAs) have opened a new field of research for complex diseases with a genetic basis. These small non-coding RNAs inhibit the expression of target mRNAs by binding to their 3'-untranslated regions (3'UTRs). These molecules have added a new step of complexity in gene regulation, but may also help to increase our understanding of many multifactorial diseases that have been a mystery up to now.

miRNAs have been extensively studied in bone research, particularly their relationship to osteoporosis [1] [2] [3]. These studies clearly showed altered miRNAs profiling in serum from patients with osteoporosis, as well as in bone tissue after osteoporotic (OP) fracture. However, these miRNA expression signatures observed in patients with osteoporosis do not provide evidence of causality because the altered pattern could be a consequence of the disease or even unrelated to the pathogenesis.

Another approach in miRNAs studies is the association analysis between one SNP within a candidate miRNA (miR-SNP) or in a miRNA target site and one disease related-outcome. In this case, the associated variant is likely involved in the pathophysiology or confers susceptibility to develop the disease. Moreover, many evidences suggest that the genetics of complex traits are attributable to genetic variations that modulate gene expression, rather than the variations resulting in protein structure changes [4]. However, functional assays are needed in order to elucidate the role of the associated variants in the pathophysiology of the disease since the SNP could be in linkage disequilibrium with the true functional variant.

The aim of this study was to identify SNPs within candidate miRNAs in order to perform an association study between those SNPs and bone mineral density (BMD), the main outcome used to define osteoporosis. First, we searched for miR-SNPs in the primary miRNA transcript (pri-miRNA), which has a hairpin structure with a terminal loop and two single-stranded flanking regions. The pri-miRNAs are recognized and cleaved by the Drosha and DCGR8 complex, resulting in a shorter structure called pre-miRNA. In this step, the sequences at the unpaired flanking arms and within the hairpin double-stranded stem structure are crucial to correct binding and cleavage by the Drosha-DCGR8 complex. Thus, the existence of genetic variants within the pri-miRNA sequences could lead to an alteration of the hairpin structure, affecting molecular processing and the underlying miRNA maturation [5]. Changes in miRNA maturation would trigger changes in miRNA abundancy, and consequently a deregulation of the expression levels of target genes. Supporting this idea, large-scale *in silico* analyses of SNPs in human miRNA genes have demonstrated lower SNP densities in the miRNA sequence than their flanking regions or the human genome [6]. Hence, our study was based on the detection and subsequent genetic association analysis of putative functional miR-SNPs. Furthermore, associated miR-SNPs were explored in bone cells in order to validate the association with the OP phenotype.

Results

Association analysis with BMD

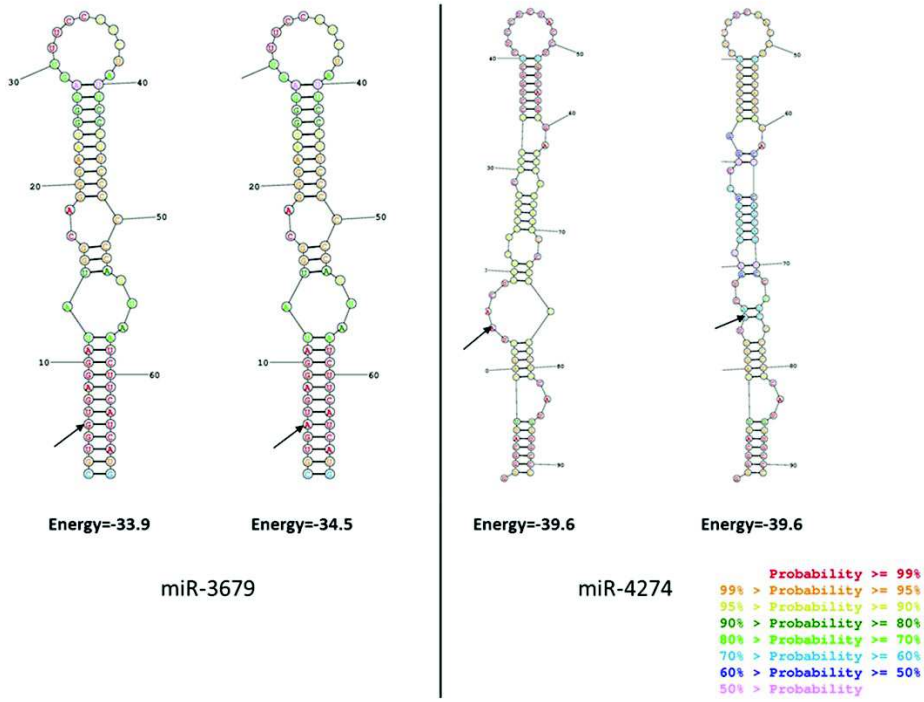
Validated SNPs with a MAF>0.01 (n=5) were genotyped in the OSTEOMED2 cohort to assess their association with LS BMD and FN BMD (Table 1A and B). SNP rs6430498 in the miR-3679 and rs12512664 in the miR-4274 were significantly associated with FN BMD in a recessive model (*p* value= 0.021, Beta coefficient [95% CI]= -0.017 [-0.032 to -0.003]; *p* value= 0.01, Beta coefficient [95% CI]= 0.015 [0.004 to 0.027], respectively). For both SNPs, the genotyping efficiency was approximately 98%. The A alleles for rs6430498 (minority allele) and rs12512664 (majority allele) were found to be associated with lower BMD values.

Quantification of miRNA expression levels in total FN bone samples

The anthropometric features of the OP and Control groups were shown in Table 2. Using the Mann–Whitney U test, no statistical differences in any features were observed between both groups.

MiR-3679 and miR-4274, which harbored BMD-associated SNPs in their pre-miRNA sequence (Fig. 1), were quantified by qPCR in order to compare the expression levels between OP and non-OP bone samples.

Figure 1: Predicted changes in secondary structure provoked by the BMD-associated SNPs according to RNAstructure web server.



Black arrows tag the corresponding allele for rs6430498 in the miR-3679 and rs12512664 in the miR-4274

MiR-3679-5p was not detected in total bone samples or in cultured human osteoblast cells, but miR-3679-3p was present in both sample types. Therefore, only mature miR-3679-3p was assayed in

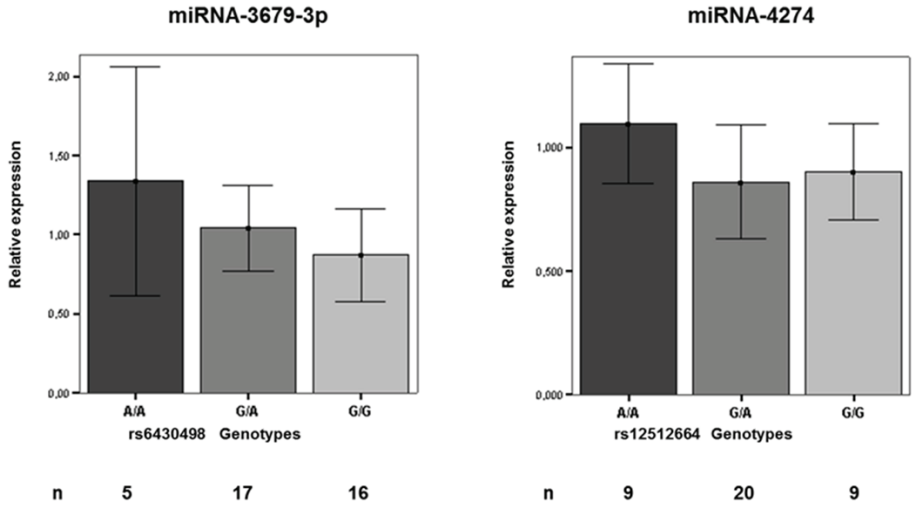
the qPCR. Both miRNAs miR-3679-3p and miR-4274 were significantly overexpressed in the OP samples (Table 3).

Other bone miRNAs, miR-631 and miR-574-5p [1], were also tested in order to preclude a non-specific phenomenon related to a general upregulation of gene expression. No differences were found between osteoporotic and non-osteoporotic bone samples (p=0.626 and p= 0.183 respectively).

Association analysis of miRNA expression levels with genotypes of BMD-associated SNPs

Human primary osteoblasts (n=38) were cultured for DNA and RNA extraction and sorted by genotype for both rs6430498 and rs12512664. Association between expression levels of mature miRNA miR-3679-3p and miR-4274 with its corresponding own genotype was analyzed. A significant correlation was observed between miRNA levels and the genetic variant (Fig. 2). The A allele for both SNPs was associated with higher expression of each corresponding miRNA (miR-3674; log-additive model; p-value= 0.015, and miR-4274; dominant model; p-value=0.013).

Figure 2: Correlation between miRNA expression levels and genotypes for miR-3679 and miR-4274.



MiRNA expression levels are represented as a mean±SD of the relative expression in Real-Time PCR. U6 was used for normalization. Samples of 38 human primary osteoblasts were used for experiments. (n) is the number of samples for each genotype group.

Additionally, in order to corroborate that the differences among expression levels are due to genotypes "per se" and not for other cellular circumstances, another bone-related miRNAs [1,7] were checked in these cells. Expression levels of miR-320a, and miR-22-3p were measured and no differences were found irrespective of genotypes. Moreover, as a sensitivity test, we analyzed the miR-3679-3p and miR-4274 expression levels separated according to reverse genotypes corresponding to the other miRNA (rs12512664 and rs6430498 respectively). Once again, no differences were found in both miRNA analyzed (miR-3679-3p; rs12512664: p-value=0.713 and miR-4274; rs6430498: p-value=0.645).

Bioinformatic analysis

A subtle effect of the BMD-associated SNPs on secondary pre-miRNA structure was detected. The genetic variants rs6430498 and rs12512664 did not provoke evident changes in the loop formation on the RNAstructure web server (Fig. 1).

Predicted targets for miR-3679-3p are *WHSC1, SMAD2, LRP6, SOX4, WNT7A, and MGAT5B*. The miR-3679 is encoded within MGAT5 gene. The protein encoded by this gene, the mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase, is one of the most important enzymes involved in the regulation of the biosynthesis of glycoprotein oligosaccharides. The most significant pathway regulated by this miRNA is the MAPK signaling pathway (p-value=0.002).

Predicted targets for miR-4274 are *BTF3, SOCS5, RAB10*, and *ESRRG* genes. ERBB (p-value=$3.2x10^{-5}$) and mTOR (p-value= $1x10^{-4}$) signaling pathways are the top pathways involving gene targets for this miRNA.

In-vitro assessment of osteoblast activity and matrix mineralization

hOBs were treated with miR-3679-3p and miR-4274 mimics or inhibitors and their respective controls for investigating their role in osteoblast function.

Inhibition of miR-3679-3p significantly enhanced matrix mineralization levels at 28-day culture (p value = 0.004). No differences in ALP activity and cell proliferation were observed after miR-3679-3p transfection.

For miR-4274 transfections, no changes in any parameter evaluated were found.

Discussion

Two osteoblast-related microRNAs, miR-3679 and miR-4274, were found to be associated with femoral neck BMD and overexpressed in OP fractured bone. These miRNAs harbor genetic

variants in their pre-miRNA sequences that were correlated with the miRNA expression levels in human osteoblasts.

The first approach used in our study was to identify functional variants within microRNAs involved in bone metabolism. The MAF of many of the miR-SNPs found in databases has not been assessed in European population; therefore, a validation step was necessary. We tested the identified variants in the BARCOS subcohort. Of the 53 miR-SNPs tested, 14 were validated in this cohort. However, only 5 SNPs had a MAF >0.01 and were finally genotyped in the OSTEOMED2 cohort.

The lower density of genetic variants in miRNA genes compared to other non-codificant genomic regions suggests that SNPs can have a remarkable biological role in pri-miRNAs by differential regulation of their target genes [8]. Hence, we chose genetic variants within the pri-miRNA sequences for their putative functionality. Moreover, the BMD-associated miR-SNPs in our study were located in the pre-miRNA molecules, which exhibit high evolutionary conservation.

SNPs within the pri-miRNA sequence can affect the miRNA maturation in multiple aspects, modifying the hairpin structure or changing the binding affinity of biogenesis enzymes to the miRNA hairpin. Moreover, variants can affect alternative cleavage sites of biogenesis enzymes, producing novel isomiRs or changing their frequency. These changes in maturation can result in altered expression of the functional miRNA, resulting in deregulation of target genes. Hence, several studies have suggested that functional SNPs in pri-miRNAs affect the processing and expression levels of mature miRNAs [9].

Two mir-SNPs, rs6430498 and rs12512664, within the pre-miRNA sequence of miR-3679 and miR-4234, respectively, were associated with FN BMD in the OSTEOMED2 cohort. Bioinformatic analysis of the hairpin structure of these BMD-associated miRNAs showed no striking effect on its secondary structure caused by allelic changes but we cannot rule out that the expression was altered by affecting the affinity binding of the processing enzymes or their accessory proteins. In this regard, other studies have demonstrated that miR-SNPs in pre-miRNA sequences lead to altered levels of mature miRNA *in vivo* even though changes in its secondary structure are not predicted by the RNAstructure web [10], [11].

In addition, in our study osteoblastic cells carrying the AA genotype both for rs6430498 and rs12512664 showed higher expression levels of miR-3679-3p and miR-4274, respectively. Moreover, both miR-SNPs fall into a low linkage disequilibrium region according to hapmap information, suggesting a possible functionality of these variants. Further supporting these results, the miRNAs miR-3679-3p and miR-4274 were overexpressed in bone OP samples, which is consistent with the association results. Therefore, the A allele for both SNPs was associated with higher expression levels of respective miRNAs and with lower BMD levels in FN, which would explain why these miRNAs were overexpressed in OP bone samples. Consistently with miR-3679-

3p overexpression in OP fractured bone, transfection results suggest that miR-3679-3p negatively regulates matrix mineralization by osteoblasts. Altogether, these findings suggest that these miRNAs can play a role in bone metabolism, that their overexpression could be detrimental, and that it could lead to an OP phenotype. Since information about these two miRNAs is very scarce, this study could be considered as a preliminary approach presenting these variants within the complex network of bone regulation.

One limitation is the use of osteoarthritic samples as control group. Due to obvious ethical reasons the collection of bone from healthy individuals is not allowed. However, in an attempt to minimize this potential limitation, we obtained the bone samples from a location distant from the interface between bone and cartilage and, therefore, as far away as possible from the osteoarthritic lesion. Other limitation of the study is the need to replicate the association results in other cohorts because these miR-SNPs lacked linkage information with other tag-SNPs related to bone phenotypes. However, this is the first time that two genetic variants in human osteoblast-related miRNAs have been associated with FN BMD in an extensive cohort of postmenopausal women, and this association was functionally demonstrated in bone samples. In conclusion, two putative functional SNPs in the pre-miRNA sequence of miR-3679 and miR-4274 were associated with femoral neck BMD. In both cases, the allele associated with lower BMD was correlated with higher expression levels of mature miRNA in human osteoblastic cells, leading to an increased risk for OP fracture. Our results open new exploratory avenues for future studies in the bone field.

Methods

Study subjects

Genetic association analysis was performed in an observational, clinical cohort study (OSTEOMED2) of patients recruited from 14 medical centers in several regions of Spain. All patients were consecutive, unselected, postmenopausal women attended in an outpatient clinic. Patients were prospectively recruited regardless of BMD value (Table 4). Exclusion criteria were any history of metabolic or endocrine disease, chronic renal failure, chronic liver disease, malignancy (except superficial skin cancer), Paget's disease of bone, malabsorption syndrome, and any anti-OP or bone-affecting treatment. In addition, women with early menopause (before the age of 40) were excluded from analysis.

BMD (g/cm^2) was measured at the lumbar spine (LS) L2-L4 and at the non-dominant femoral neck (FN) using the dual-energy X-ray densitometers available in each participating center (Supplemental table 1).

Whole bone samples for qPCR miRNA quantification were obtained from the transcervical region of the femoral neck of postmenopausal women undergoing hip replacement due to either OP fracture (n=10) or osteoarthritis in the absence of osteoporosis (n=10) (T-score measurements [mean±SD]: 0.616 ± 0.523) (Table 2). Bone was taken from a location distant from the interface between bone and cartilage and, therefore, as far away as possible from the osteoarthritic lesion. Again, none of the participants had a history of metabolic or endocrine disease, chronic renal failure, chronic liver disease, malignancy, Paget's disease of bone, malabsorption syndrome, hormone replacement therapy, anti-resorptive or anabolic agents, oral corticosteroids, anti-epileptic drugs, or treatment with lithium, heparin, or warfarin.

The study protocols for obtaining DNA from blood samples and primary osteoblasts were approved by the CEIC-Parc de Salut MAR, the coordinating center (Registry number 2010/3882/I).

SNPs selection in pri-miRNA sequences

In the first step, 9 candidate genes were selected for their well-known function in bone regulation: *ESR1*, *TGFB2*, *PTH1R*, *RUNX2*, *IL6R*, *LRP5*, *IL6*, *VDR*, and *CYP24A1* [12 13 14 15 16 17] (Table 1A). Then, miRNAs that bind to the mRNA 3'UTR of these genes were identified using the microRNA.org website. The algorithm used is based on sequence complementarity, binding energy of the miRNA–target duplex, evolutionary conservation of the target site sequence and target position in aligned UTRs of homologous genes. Some listed miRNAs target more than one bone-related gene, as for example, miR-130b, miR106b, miR148b and miR-93 that target both *ESR1* and *TGFB2* genes. These miRNAs were located in *ESR1* target miRNAs in order to simplify the table 1A. Next, miRNAs with the highest expression levels in primary human osteoblasts (hOB) were selected (Table 1B) according to results obtained in a previous miRNA array performed in our group (GEGSE74211; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE74211) [1]. Finally, SNPs in the pri-miRNA sequences were searched using the Ensembl (www.ensembl.org), UCSC (http://genome.ucsc.edu/), and HapMap (www.hapmap.org) databases.

Only those SNPs with a minor allele frequency (MAF) in Utah residents with ancestry from northern and western Europe (CEU) > 0.01 were included in the study; SNPs lacking published MAF were validated in participants from the BARCOS cohort [18] (included in OSTEOMED2) by means of PCR-RFLP, genotyping or Sanger sequencing.

Polymorphism genotyping

DNA was obtained from peripheral blood collected in EDTA tubes and genotyping was performed in LGC Genomics platform using KASPar v4.0 genotyping systems. To ensure genotyping quality, a

random sample (5% of the total number of samples) was also genotyped in a separate control plate. There was 100% concordance between these results.

Human osteoblasts culture

Human primary osteoblasts (hOB) were obtained from trabecular bone of patients who underwent knee or hip replacement due to osteoarthritis. Bony tissue was cut up into small pieces, washed in phosphate buffered solution (PBS, Gibco by Life Technologies; Paisley, UK) to remove non-adherent cells, and placed on a 140 mm culture plate. Samples were incubated in hOB medium: Dulbecco's Modified Eagle Medium (DMEM; Gibco; Invitrogen, Paisley, UK), supplemented with 10% fetal calf serum (FBS; Sigma-Aldrich; St. Louis, USA), 100 U/ml penicillin/streptomycin (Sigma-Aldrich; St. Louis, USA), 0.4% fungizone (Gibco by Life Technologies; Paisley, UK), and 100 ug/ml ascorbic acid (Sigma; Steinheim, Germany). Alkaline Phosphatase (ALP) activity and osteocalcin gene expression were measured in order to confirm the osteoblastic phenotype (data not shown). All experiments were performed at maximum passage 2.

Thirty eight samples were grown in parallel: one for DNA extraction and the other for RNA extraction. Additionally, 11 hOB samples were used for Alizarin Red, ALP and MTS assays.

Human osteoblast DNA extraction and sequencing

DNA was extracted from cultured hOBs using the Wizard® Genomic DNA Purification Kit (Promega) according to manufacturer's instructions. Genotypes for rs6430498 and rs12512664 were assessed by Sanger sequencing using the BigDye® Terminator v3.1 (Applied Biosystems) in the genomic services of Universitat Pompeu Fabra. Primers (Sigma-Aldrich) were designed using the Primer3 imput (v. 0.4.0) and the UCSC website: for rs6430498 in miR-3679, F: 5'-CGGTGAGGAGTTTTCTGAATG-3' and R: 5'- CACCAAGCATAATAGCTAAAAATCAA-3' (fragment size: 400 bp) and for rs12512664 in miR-4274, F: 5'- CATCCACTTTGGGGAGAAGT-3' and R: 5'-CCAAGGTACCACTGCCTCAT-3' (fragment size: 392 bp).

RNA extraction of osteoblasts and total bone

miRNA-enriched fraction from hOB cultures was extracted using MiRNeasy mini kit and RNeasy MinElute Cleanup (Qiagen) according to manufacturer instructions.

For RNA extraction of total bone, a piece of tissue was cut out and added to QIAzol Lysis Reagent (Qiagen), then homogenized for 5 min using the TissueLyser system. Chloroform was added to each sample, followed by centrifugation for 15 min (12000 g). The upper water phase was collected and the extraction continued according to manufacturer's instructions. The quality of the total RNA

was verified by an Agilent 2100 Bioanalyzer profile. The concentration of the purified RNA was analyzed on a spectrophotometer (Nanodrop, Thermo Fisher Scientific Inc).

miRNA quantification by qPCR

Using the miScript II RT kit (Qiagen), 1 µg of total RNA was reverse-transcribed in 20 µl reactions. cDNA from total bone was diluted x8 and cDNA from hOBs was diluted x16; in both cases, 2 µl were assayed in 10 µl PCR reactions in 384-well plates using MiScript Syber Green PCR kit according to the protocol. The sequence of the mature miRNAs selected, according to the mirBase web site, was used as a forward primer and the Universal primer as a reverse. U6 snRNA was used as the reference gene for normalization. Amplification was performed in a QuantStudio 12K Flex Real-Time PCR (Applied Biosystems), and the ExpressionSuite software was used both for determination of relative quantification (RQ) (by $2^{-\Delta\Delta Ct}$ method) and for melting curve analysis.

Bioinformatics analyses of BMD-associated miRNAs

Gene target prediction was assessed using the following six programs: PicTar (http://pictar.mdc-berlin.de), TargetScan Human (http://www.targetscan.org), miRDB (http://mirdb.org), MiRanda (http://www.microrna.org), DIANA-TarBase (http://diana.imis.athena-innovation.gr), and miRTarBase (http://mirtarbase.mbc.nctu.edu.tw). The DIANA-mirPath web-based computational tool [19] was used to identify molecular pathways potentially altered by the intersection of miRNAs differentially expressed in fractured bone.

miRNASNP database [8], RNAstructure (http://rna.urmc.rochester.edu/RNAstructureWeb) and RNAfold software (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) were used to predict the effect of the variants on the miRNA secondary structure. These predictions are based on the assumption that variants can destabilize the hairpin and therefore reduce the mature miRNA levels.

Cell transfection

Primary hOB cells were seeded at the following conditions: 96-well plate at 12.000 cells/well for MTS and ALP activity assays and 24-well plate at 45.000 cells/well for Alizarin Red quantification.

Once cells reached 60-70% of confluence, transient transfections were performed using *mir*Vana mimics and inhibitors of both hsa-miR-3679-3p and hsa-miR-4274. *Mir*Vana™ miRNA Mimic Negative Control #1 and *mir*Vana™ miRNA Inhibitor Negative Control #1 were used as controls. All products were purchased from Ambion® Life Technologies. Mimics and controls mimics were used at 100 nM and inhibitors and control inhibitors at 400 nM. In order to monitor transfection efficiency, miRIDIAN microRNA Mimic Transfection Control with Dy547 (Dharmacon) was transfected into the cells at the same conditions as the miRNAs tested. Transfection of miRNAs was carried out using Lipofectamine RNAiMAX (Invitrogen; Carlsbad, USA) according to the manufacturer's instruction.

Alizarin Red quantification

To induce osteoblastic mineralization, hOBs (n=4) were cultured during 28 days with hOB medium supplemented with 5mM β-glicerophosphate (Sigma-Aldrich, St Louis, MO, USA). Cells were transfected with both mimics and inhibitors of miR-3679-3p and miR-4274, and their corresponding controls at day 1, and day 14 after seeding. During the cell culture time period, the medium was changed once every 3 days.

At day 28, cells were stained with alizarin red to quantify mineralized nodules. At this time point, media was removed from the cell monolayer and gently washed 3 times with PBS. The cells were then fixed in 10% buffered formalin for 10 minutes at room temperature. Fixative was removed and cultures were washed in PBS. The cell layer was stained with 2% Alizarin-S (Sigma-Aldrich, St Louis, MO, USA) at ≃ pH 4.2 for 20 minutes. Cell preparations were washed with PBS to eliminate nonspecific staining. To quantify calcium deposition, the dye was leached by the addition of 10% cetylpyridinium chloride until all of dye had been drawn. Optical density was then quantified by spectrophotometry at 550 nm, using 10% cetylpyridinium chloride as a blank reference.

ALP activity assay

ALP activity was measured 48 hours after miRNA transfection of hOBs (n=4) using the Alkaline Phosphatase Assay Kit (Colorimetric) (Abcam; Cambridge, UK) according to the manufacturer's instructions.

Cell proliferation assay (MTS)

Viable cells were determined 48 hours post-transfection of hOBs (n=3) using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega; WI, USA), according to the manufacturer's instructions.

Statistical methods

Hardy-Weinberg equilibrium (HWE) was calculated using the chi-square test. Multivariate linear regression models were fitted to assess the association between genotyped SNPs and BMD. Potential confounders considered for adjustment were densitometer devices, body mass index (BMI) and age. Association analyses were performed using R software version 2.13.2 with the *SNPassoc, foreign, gdata* and *multtest* packages. Mann-Whitney U test was performed for OP and non-OP group comparisons in the SPSS v.12.0 for Windows. This test was also used for treatment comparisons between miRNA mimics or inhibitors and their respective controls.

Linear regression was used to analyze the association between miRNA expression levels and sample genotypes. Correlation analyses were performed using R software version 2.13.2 with the *SNPassoc* and *foreign* packages.

All analyses were two-tailed, and p-values<0.05 were considered significant.

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Author Contributions

LDU, NGG and ADP conceived the concept for this paper. MAGP, JMO, MSH, RPC, CGA, LDL JMA, JABC, JGM, JPM, MMT, MDC, JM, AC, JLPC, XN performed patient and data collection. JABC, ADP and NGG designed the OSTEOMED2 network. MR managed the patient database and performed statistical analyses. LDU, ECM and NGG set up the experiments and recorded data. All authors participate in the analyses of data, and discussed the results and context of the manuscript. All authors read and approved the final manuscript.

Additional Information

Competing financial interests: The authors declare no competing financial interests.

Figure Legends

Fig. 1 Predicted changes in secondary structure provoked by the BMD-associated SNPs according to RNAstructure web server. Black arrows tag the corresponding allele for rs6430498 in the miR-3679 and rs12512664 in the miR-4274

Fig. 2 Correlation between miRNA expression levels and genotypes for miR-3679 and miR-4274. MiRNA expression levels are represented as a mean±SD of the relative expression in Real-Time PCR. U6 was used for normalization. Samples of 38 human primary osteoblasts were used for experiments. (n) is the number of samples for each genotype group

Tables

Table 1A. Validation of miR-SNPs for the BMD association analysis. MiRNAs that target bone-related genes

TARGET GENE	miRNA	SNP	MAFB
ESR1	miR-106b	rs72631827	Monomorphic
	miR-130b	rs72631822	Monomorphic
	miR-148b	rs74878365	Monomorphic
	miR-18a	rs41275866	Monomorphic
	miR-222	rs72631825	Monomorphic
	miR-373	rs80338016	Monomorphic
	miR-520c	rs7255628	Monomorphic
	miR-93	rs72631824	Monomorphic
	miR-96	rs41274239	0.0033
		rs73159662	0.0058
TGFB2	miR-141	rs34385807	Monomorphic
	miR-149	rs71428439	Monomorphic
	miR-182	rs77586312	Monomorphic
		rs75953509	Monomorphic
		rs80041074	0.0033
	miR-199b	rs72631835	Monomorphic
	miR-193a	rs60406007	Monomorphic
	miR-200b	rs72563729	Monomorphic
	miR-33a	rs77809319	Monomorphic
	miR-431	rs76090066	0.00083
		rs128840'05	Monomorphic
	miR-590	rs6971711	Monomorphic
	miR-7-1	rs76662330	Monomorphic
	miR-7-2	rs41276930	0.005
		rs75737367	Monomorphic
PTH1R	miR-339	rs13232101	Monomorphic
		rs72631820	Monomorphic
		rs72631831	Monomorphic
RUNX2	miR-122	rs41292412	0.0033
	miR-154	rs41286570	0.0004
IL6R	miR-124-2	rs72631829	Monomorphic
	miR-124-3	rs34059726	Monomorphic
	miR-125a	rs12975333	Monomorphic
	miR-140	rs7205289	Monomorphic
	miR-320d-1	rs74826059	Monomorphic
	miR-499	**rs3746444**	**0.21**
		rs7267163	0.0025
LRP5	**miR-27a**	**rs11671784**	**0.0162**
IL6	**miR-146a**	**rs2910164**	**0.26**
	miR-146b	rs76149940	Monomorphic
	miR-202	rs12355840	Monomorphic
	miR-365-2	rs35143473	Monomorphic
VDR	miR-10a	rs72631828	Monomorphic
	miR-223	rs34952329	Monomorphic
CYP24A1	miR-30b	rs111424617	Monomorphic
	miR-30e	rs112439044	Monomorphic
	miR-183	rs72631833	Monomorphic
		rs41281222	Monomorphic
	miR-101-2	rs78851134	0.0004

Table 1B. Validation of miR-SNPs for the BMD association analysis. MiRNAs with the highest expression levels in primary human osteoblasts

Highly expressed in HObs	miR-1282	rs11269	Monomorphic
	miR-3679	**rs6430498**	**0.35**
		rs10175383	Monomorphic
	miR-4274	**rs12512664**	**0.47**

MAFB; Minor allele frequency in BARCOS cohort

In bold; Validated SNPs for genotyping in total OSTEOMED2 cohort

Table 2. Patient characteristics for osteoporotic fracture and non-osteoporotic groups.

Biological groups	n	Age (Mean ± SD)	BMI (kg/m^2) (Mean ± SD)	BMD (g/cm^2) (Mean ± SD)
Osteoporotic	10	75.6 ± 6.38	27.11 ± 2.94	Fragility fracture
Non-osteoporotic	10	71.7 ± 7.36	27.42 ± 3.15	0.882 ± 0.137

Abbreviations: SD: Standard Deviation; BMI: Body Mass Index; BMD: Bone Mineral Density

Table 3. miRNA expression levels, comparison between osteoporotic and non-osteoporotic bone samples

miRNA	Biological Group	RQ (Median)	IQR	*P* value
miR-3679	Osteoporotic	89.601	220.636	0.001
	Control	1.423	0.964	
miR-4274	Osteoporotic	144.268	318.409	0.001
	Control	1.197	2.154	

Abbreviations: RQ= Relative quantification; IQR= Interquartile Range

Table 4. Baseline characteristics of the OSTEOMED2 cohort

Patient characteristic	Mean ± SD	
	LS BMD n=2183	**FN BMD** n=2015
Age (years)	57.61 ± 9.26	58.80 ± 8.99
Age of menopause (years)	48.7 ± 3.94	48.7 ± 3.92
BMI (kg/m^2)	26.56 ± 4.18	26.48 ± 4.13
BMD (g/cm^2)	0.870 ± 0.16	0.707 ± 0.14

Abbreviations: BMI=body mass index; BMD=bone mineral density; LS=lumbar spine; FN=femoral neck

CHAPTER 5

Identification of miRNAs involved in Osteoblast to Osteocyte Differentiation

Identification of miRNAs involved in Osteoblast to Osteocyte Differentiation

SUMMARY

It is now appreciated that miRNAs play an important role in bone formation and osteoblast differentiation. Many key signaling pathways essential for the onset of osteogenesis and for maintaining bone mass are dependent on miRNAs. However little is known about the role of miRNAs in the regulation of osteoblast to osteocyte differentiation.

The aim of this study is to identify the miRNAs involved in the differentiation process from osteoblasts to early osteocytes and from early osteocytes to late osteocytes as well as to discern the role of those miRNAs in determining osteocyte phenotype and function.

Although osteocytes are the most abundant cells in bone, they are the most challenging bone cell to study because they are embedded in a mineralized matrix and are difficult to isolate with increasing skeletal age accompanied by increasing mineralization. In order to address this issue, the IDG-SW3 cell line, which replicates osteoblast to early osteocyte and early osteocyte to late osteocyte differentiation in culture[63] was been used as a model of this transition process between osteoblasts and osteocytes.

To begin to understand the role of miRNAs in this process, we performed miRNA array analyses of the different stages of IDG-SW3 cells; from late osteoblast to early osteocyte and to mature osteocyte. Total RNA was extracted at 0, 3, 7, 14, 21 and 28 days of culture to perform miRNA qPCR based global expression profiling arrays (Fluidigim). From 133 detected miRNAs, 56 miRNAs were significantly up- or downregulated during the

differentiation process with ≥2 fold change. Among the highest significant miRNAs, 6 (miR-146b, miR-195a, miR-497, miR-224, miR-322 and miR-351) were selected for validation by SYBR Green qRT-PCR in IDG-SW3 cells. Expression of all of them correlated with and validated the results from profiling arrays.

In addition, bioinformatics analysis was performed on the results from the profiling arrays in order to identify pathways regulated by the miRNAs which showed the highest significant changes during IDG-SW3 differentiation. Pathways related to osteoblast to osteocyte differentiation, such as Wnt signaling, were revealed.

Among validated miRNAs and with greatest fold change were miR-146b-5p and miR-195a-5p. The expression levels of these miRNAs were evaluated in an osteoblastic (2T3) and osteocytic (MLO-Y4) cell lines. Furthermore, primary osteoblasts and osteocytes were isolated from 2 month old murine long bones which replicated the results observed in the osteoblastic stage of IDG-SW3 cells (days 3-7) and late osteocyte stage IDG-SW3 cells (days 21-28).

To determine the potential function of miR-146b-5p and miR-195a-5p, both miRNAs were assessed for effects on osteoblast function using the IDG-SW3 cell line. The overexpression of miR-146b-5p and miR-195a-5p during the osteoblastic stage leads to a modest increase of mineralization at day 7 of differentiation. In the other hand, no effect in ALP activity was observed for any of the miRNAs tested. Unfortunately, the effects of miRNAs on osteocyte function could not be assessed due to interference by mineral surrounding the cells.

We propose that osteocytes have a unique subset of miRNAs consistent with their known properties.

CHAPTER 5
Methods

1. Bone cell line culture

IDG-SW3: This cell line was chosen for the study due to its characteristic features which mimic the transition from osteoblast to osteocyte differentiation (Figure M-1). Cells were expanded under permissive conditions (33°C in–α-MEM with 10% FBS, 100 U/ml penicillin, 50µg/ml streptomycin) (Hyclone) and 50 U/ml IFN-γ (Life Technologies) on type I collagen coated plates (0.15mg/ml; Sigma). For experiments, cells were plated at a density of $4x10^4$ cells/cm^2 in collagen coated plates. Once the cultures reached confluence, the media was replaced with osteogenic media (α-MEM with 10% FBS, 100 U/ml penicillin, 50 µg/ml streptomycin, 50µg/ml ascorbic acid and 4mM β-glycerophosphate) (both Sigma-Aldrich) in the absence of IFN-γ and cells were cultured at 37°C. Osteogenic media was changed every three days.

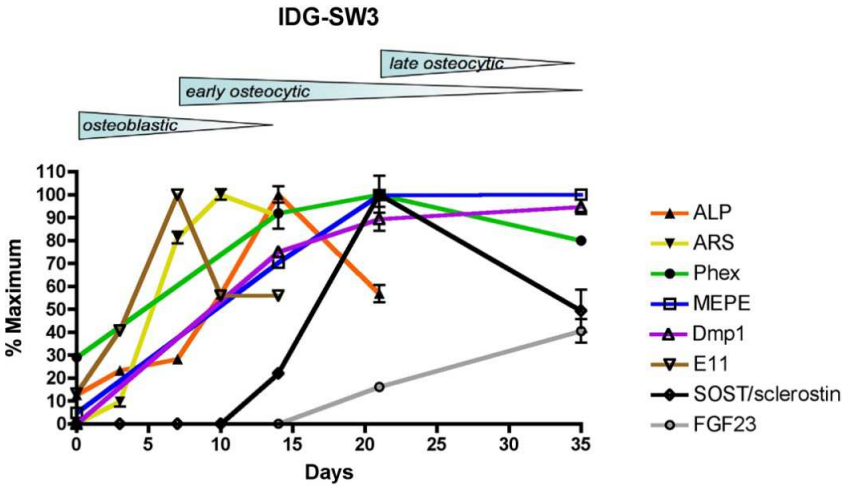

Figure M-1. Expression of markers during osteoblast-to-osteocyte ontogeny in IDG-SW3 cell line. IDG-SW3 cell line mimics the transition from osteoblast to osteocyte differentiation. Extracted from Woo *et al.* (2011)[63].

2T3: The 2T3 osteoblast-like cell line was derived from the calvarium of a transgenic mouse in which the T-antigen was expressed under control of the BMP-2 promoter, thus directing expression of the immortalizing T-antigen to the osteoblast lineage[295]. Cells were expanded in proliferation media (33°C in α-MEM with 10% FCS, 100 U/ml penicillin and 50 µg/ml streptomycin) until they reached 95 % of confluence and then harvested and seeded in 24 well plates at a density of $1x10^5$ cell/cm^2. Once the cells reached the confluence, one of the plates was harvested at proliferation stage and the others were switched to differentiation media (37°C with 50 µg/ml ascorbic acid and 4mM β-glycerophosphate). Cells were harvested 5 days after the induction of differentiation.

MLO-Y4: The osteocyte-like cell line MLO-Y4 was used as an *in vitro* osteocyte model[296]. MLO-Y4 cells were cultured on rat tail collagen type I (0.15mg/ml; Sigma) coated plates in α-MEM supplemented with 2.5 % FBS and 2.5% CS (Hyclone) in a 5% CO2 incubator at 37 ºC.

2. Primary calvaria bone cell isolation

Primary osteoblasts and osteocytes (osteoblasts, early osteocytes, late osteocytes and osteocyte enriched bone particles) were isolated from long bones (tibia, femur and humerus) of 2 months old male wild type mice C57/BL/6 (n=3) as described by Stern *et al*.[297]. Briefly, after dissection, bones were isolated and soft tissue and periosteum was removed through scraping and extensive washing. Bones were placed in fresh αMEM with 10% penicillin and streptomycin. Epiphyses were cut off, and the marrow was flushed out with HBSS using a syringe. Bones were cut into 1-2 mm lengths and placed in HBSS. Bone pieces were incubated in collagenase solution for 25 min, the solution was aspirated and kept for RNA extraction and the bone pieces were

washed in HBSS. This was repeated two more times, for a total of three digestions. Six more digestion followed using alternatively one digestion with EDTA and one digestion of collagenase for a total of 9 digestions. After the last digestion, bone pieces were collected.

Isolated cell populations and bone pieces were placed into 1 ml TRIzol Reagent (Life Technologies, Carlsbad, CA). Cells populations were stored at -80°C until total RNA extraction. Bone pieces were frozen at -80°C and then in liquid nitrogen. Frozen pieces were then smashed, homogenized and suspended in 1ml of Trizol again.

All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee at the University of Missouri, Kansas City and conformed to relevant federal guidelines.

3. MicroRNA expression profiling array

Total RNA was extracted using TRIzol reagent (Invitrogen) at 0, 3, 7, 14, 21 and 28 days of IDG-SW3 cell culture to perform miRNA qPCR based global expression profiling arrays (Fluidigim). A threshold was set at ≥2 fold change for comparison of expression levels.

4. Target prediction and pathway analysis

Gene target prediction was assessed using TargetScan database. The DAVID v6.7 database was used to identify enriched KEGG pathways involved with the genes predicted to be targeted by the 17 highly significantly ($p < 0.01$) regulated miRNAs during IDG-SW3 differentiation.

5. RNA extraction

Total RNA was extracted using *RNeasy* Mini *Kit* (Qiagen) according to manufacturer´s instructions for all bone cell lines tested: IDG-SW3, 2T3 and MLO-Y4. In the case of primary bone cells, RNA was extracted using Trizol Reagent (Invitrogen).

6. Real Time PCR (qRT-PCR)

Six miRNAs were validated by the SYBR Green qPCR approach in the IDG-SW3 cell line. MiRNA expression levels were normalized to U6. In addition, the expression levels of miR-146b and miR-195a were measured in MLO-Y4 and 2T3 cell lines as well as primary bone cells. In this case, relative expression levels and fold induction of each target were calculated using Standard Curve method. cDNA was synthesized from 500ng total RNA using miScript II RT Kit (Qiagen) according to protocol provided by manufacturer. qRT-PCR was performed on StepOnePlus (Lifetechnologies) using miScript SYBR® Green PCR kit (Qiagen). Primers were synthesized from Integrated DNA Technologies (IDT) and sequences are listed in Table M-2.

Table M-2. Nucleotide sequences of primers used for qRT-PCR

Mouse miRNAs name	Sequences of RT-qPCR Primers (5'-3')
miR-195a-5p	F: TAGCAGCACAGAAATATTGGC
miR-195a-3p	F: CCAATATTGGCTGTGCTGCTCC
miR-195b	F: TAGCAGCACAGAAATAGTAGAA
miR-351-5p	F: TCCCTGAGGAGCCCTTTGAGCCTGA
miR-351-3p	F: GGTCAAGAGGCGCCTGGGAAC
miR-146b-5p	F: TGAGAACTGAATTCCATAGGCT
miR-146b-3p	F: GCCCTAGGGACTCAGTTCTGGT
miR-497-5p	F: CAGCAGCACACTGTGGTTTGTA
miR-497-3p	F: CAAACCACACTGTGGTGTTAG
miR-224-5p	F: TAAGTCACTAGTGGTTCCGTT
miR-224-3p	F: AAATGGTGCCCTAGTGACTACA
miR-322-5p	F: CAGCAGCAATTCATGTTTTGGA
miR-322-3p	F: AAACATGAAGCGCTGCAACAC
U6	F: TGGCCCCTGCGCAAGGATG
Snord68	F: TTTGAACCCTTTTCCATCTGAT

7. Cell transfection

In order to assess the role of miR-146b and miR-195a in osteoblast to osteocyte differentiation, these microRNAs were overexpressed in IDG-SW3 cells at an early stage of differentiation representing the osteoblast stage. A single transient transfection was performed using a mmu-miR-146b-5p mimic (miRVANA mimic 5'UGAGAACUGAAUUCCAUAGGCU3'; Ambion® Life Technologies) and mmu-miR-195a-5p mimic (miRVANA mimic 5'UAGCAGCACAGAAAUAUUGGC3'; Ambion® Life Technologies) at 400 nM complexed with 3 µl of the transfection reagent Lipofectamine 2000 (Invitrogen). Cy3™ Dye-Labeled Anti-miR™ Negative Control #1 was used as a negative miRNA that is a random sequence anti-miR molecule that has no identifiable effects on known miRNA function.

9. Alizarin red quantification

The IDG-SW3 cells were cultured in 24-well plate at $4x10^4$cells/cm^2 and transfected with mmu-miR-146b-5p and mmu-miR-195a-5p mimics and the miRNA mimic control 3 days after induction of differentiation.

At day 5 and 7 media were removed from the cell monolayer and gently washed 3 times with PBS. The cells were fixed in 10% buffered formalin for 10 minutes at 4°C. After this time, fixative solution was removed, cultures were washed in PBS and plates were stored in PBS at 4°C until sample processing. PBS was removed from the stored plates and the cell layer was stained 2% Alizarin-S (Sigma-Aldrich, St Louis, MO, USA) at ~ pH 4.2 for 5 minutes. Cell preparations were washed with PBS to eliminate nonspecific staining. To quantify calcium deposition, the dye was leached from the monolayer by the addition of 10% cetylpyridinium chloride solution until all of dye had been drawn from the monolayer. 200µl of the solution (in duplicate) was transferred to a clean 96 well plate. Optical density was measured by spectrophotometry at 570nm, using 10% cetylpyridinium chloride as a blank reference.

10. Alkaline Phosphatase activity

The IDG-SW3 cells were cultured in 24-well plate at $4x10^4$cells/cm^2 and transfected with mmu-miR-146b-5p and mmu-miR-195a-5p mimic. At day 5 and 7 media were removed from the cell monolayer and gently washed 2 times with PBS. 400 µl of 0.05% Triton X-100 was added to each well and plates were stored at -20°C. The day of the assay the plates were frozen-thawed twice. Lysates were mixed and 5 µl of lysate was transferred to a 96 well assay plate. Blanks were set up containing the same amount of lysis buffer. Standard wells containing 2 to 20 nM p-nitrophenol per well and

containing equivalents amounts of triton per well were set. AMP buffer was added to bring the volume up to 100 μl and plates were incubated at 37°C for 5-20 min when the reaction was stopped by adding 100μl of 0.5M NaOH to each well. Absorbance was read at 405nm and concentrations of p-nitrophenol were calculated from the Standard Curve. Protein concentration was determined as well. Alkaline Phosphatase Activity was calculated dividing p-nitrophenol concentration value by the proteins concentration value and by the number of minutes the samples were incubated.

11. Statistical analysis

One way analysis of variance (ANOVA) with Neuman- Keuls *post hoc* tests was used to determine statistically significant changes in gene expression between groups with the statistical software package in GraphPad Prism 4.03 (corrected *p*-values < 0.05 were accepted as significant). Mann-Whitney U test was performed for comparisons between miRNA mimics and the miR transfection control in the SPSS v.12.0 for Windows.

CHAPTER 5
Results

1. Expression of miR-146b-5p and miR-195a-5p are increased with IDG-SW3 differentiation

From 133 miRNAs detected in the array, a subset of 56 miRNAs was significantly up- or downregulated during the differentiation process with ≥2 fold change. Included in the 10 most significant upregulated miRNAs ($p<0.01$) were: mmu-miR-146b, rno-miR-146b, mmu-miR-195a, mmu-miR-24, mmu-miR-218, mmu-miR-152, mmu-miR-30a, mmu-miR-676, mmu-miR-497 and mmu-miR-135-5p. The seven highly significant downregulated miRNAs ($p<0.01$) were: mmu-miR-99b, mmu-miR-322, mmu-miR-503, mmu-miR-322, mmu-miR-224, rno-miR-224 and rno-miR-351 (Figure R-10).

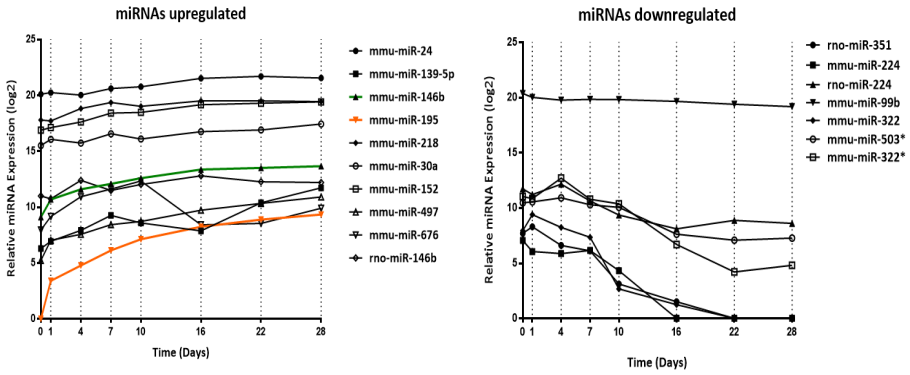

Figure R-10. MiRNAs involved in the osteoblast to osteocyte transition in IDG-SW3 cells. Left side: Representation of the miRNAs significantly upregulated with a change higher than 2 fold by ANOVA $p<0.01$, during the transition from osteoblasts to osteocytes by miRNA qPCR based global expression profiling array in IDG-SW3 cells. Right side: Representation of the miRNAs significantly downregulated. The y axis represents relative miRNA expression levels (log_2) and plotted as relative to day 0.

The miRNAs with highest fold change during differentiation (miR-146b, miR-497 and miR-195a from the upregulated group and miR-322, miR-224 and rno-miR-351 from the downregulated group) underwent validation by qPCR in IDG-SW3 cells. All of them were validated, and the results were consistent with the previous data obtained from the miRNA expression array (Figure R-11).

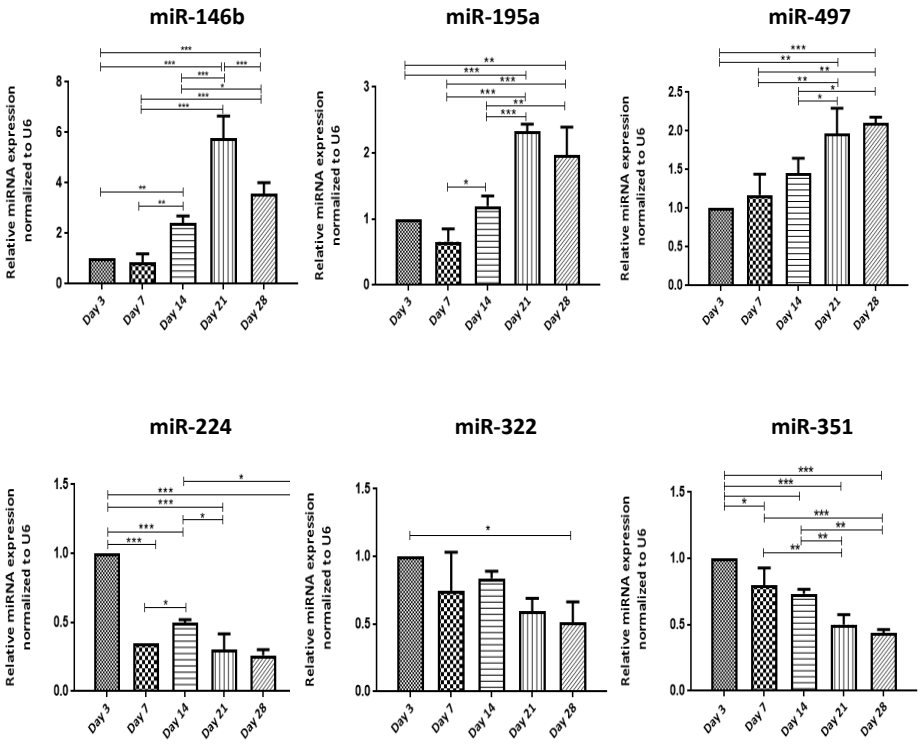

Figure R-11. qRT-PCR-validated miRNAs that reached significance in the microRNA array. The expression pattern of mmu-miR-146b-5p, mmu-miR-195-5p, mmu-miR-497-5p, mmu-miR-224-5p, mmu-miR-322-5p and rno-miR-351-5p determined in the microarray were validated by qPCR. Each miRNA expression levels were normalized by U6 expression. Normalized mean expressions of miRNAs are plotted as relative to day 3 ± SD; n=3 for each group; (*p<0.05, **p<0.01, ***p<0.001).

2. Identification of significant upregulated and downregulated miRNA target genes and pathways

An *in silico* analysis was carried out to predict the genes targeted by the 17 most significantly up or down regulated miRNAs (significant threshold was set to $p<0.01$) between day 0 and day 28 in IDG-SW3 cells. Afterwards, the DAVID v6.7 database was used to identify enriched KEGG and PANTHER pathways involved with the genes predicted to be targeted by these miRNAs (Table R-3). Pathways with established functions in osteoblast to osteocyte differentiation, such as circadian rhythm and Wnt signaling, were predicted.

Table R-3. Pathways related to significant miRNAs involved in IDG-SW3 diferentiation. Enriched pathways were identified using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7, querying for KEGG and PANTHER databases.

	Significantly **upregulated** miRNAs			
	Pathway	**Target Genes in Pathway** ***Number***	**Fold Enrichment**	**p-value**
KEGG	**Circadian rhythm**	**6**	**4,32**	**8,42E-03**
KEGG	Glycosphingolipid biosynthesis	8	3,12	1,04E-02
KEGG	Alanine, aspartate and glutamate metabolism	9	2,81	1,11E-02
KEGG	SNARE interactions in vesicular transport	11	2,71	5,24E-03
KEGG	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	21	2,62	7,46E-05
KEGG	Chondroitin sulfate biosynthesis	6	2,55	7,74E-02
KEGG	Long-term potentiation	19	2,54	2,79E-04
KEGG	Axon guidance	35	2,50	4,84E-07
KEGG	Heparan sulfate biosynthesis	7	2,43	6,07E-02
KEGG	**Wnt signaling pathway**	**37**	**2,32**	**1,46E-06**
KEGG	Type II diabetes mellitus	12	2,29	1,24E-02
KEGG	Hypertrophic cardiomyopathy (HCM)	20	2,23	1,08E-03
KEGG	Adherens junction	18	2,22	2,20E-03
KEGG	Acute myeloid leukemia	13	2,13	1,53E-02
KEGG	Amino sugar and nucleotide sugar metabolism	10	2,13	3,95E-02
KEGG	Glycerophospholipid metabolism	15	2,10	9,81E-03
KEGG	Hedgehog signaling pathway	12	2,08	2,49E-02
PANTHER	**Circadian clock system**	**7**	**3,57**	**8,41E-03**
PANTHER	Oxytocin receptor mediated signaling pathway	15	1,85	2,54E-02
PANTHER	Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway	28	1,48	3,17E-02
PANTHER	Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	31	1,42	3,79E-02
PANTHER	Dopamine receptor mediated signaling pathway	18	1,64	3,98E-02
PANTHER	Ionotropic glutamate receptor pathway	13	1,78	5,31E-02
PANTHER	5HT2 type receptor mediated signaling pathway	15	1,62	7,07E-02
PANTHER	Metabotropic glutamate receptor group III pathway	16	1,57	7,52E-02
PANTHER	Alzheimer disease-presenilin pathway	24	1,39	8,47E-02
PANTHER	B cell activation	17	1,49	9,37E-02
PANTHER	**Wnt signaling pathway**	**53**	**1,21**	**9,67E-02**
PANTHER	Histamine H1 receptor mediated signaling pathway	10	1,78	9,84E-02

	Significantly **downregulated** miRNAs			
	Pathway	**Target Genes in Pathway** ***Number***	**Fold Enrichment**	**p-value**
KEGG	Basal cell carcinoma	6	4,97	6,60E-03
KEGG	Acute myeloid leukemia	6	4,79	7,67E-03
KEGG	Amino sugar and nucleotide sugar metabolism	4	4,14	7,01E-02
KEGG	Axon guidance	8	2,78	2,37E-02
KEGG	MAPK signaling pathway	16	2,75	5,81E-04
KEGG	Melanogenesis	6	2,73	6,64E-02
KEGG	Purine metabolism	8	2,32	5,47E-02
KEGG	Insulin signaling pathway	7	2,31	7,99E-02
PANTHER	Cadherin signaling pathway	21	3,89	1,43E-07
PANTHER	**Wnt signaling pathway**	**24**	**2,07**	**6,82E-04**
PANTHER	Oxidative stress response	7	2,85	3,29E-02
PANTHER	p38 MAPK pathway	6	3,27	3,33E-02
PANTHER	Interleukin signaling pathway	10	1,79	9,98E-02

3. Expression of miR-146b and miR-195a in bone related cell lines and primary bone cells

Among the group of miRNAs validated, miR-146b-5p and miR-195a-5p were selected for further functional studies. Among the upregulated miRNAs, those two miRNAs showed the greatest fold change during the differentiation process from osteoblasts to osteocytes.

These two miRNAs were found to be expressed in 2T3 and MLO-Y4, both cell lines were used as an osteoblastic and osteocytic cell models respectively. Moreover, they were expressed in mice primary osteoblasts and osteocyte enriched bone chips, which replicated the results observed with the osteoblastic stage of IDG-SW3 cells (days 3-7) and late osteocyte stage IDG-SW3 cells (days 21-28) (Figure R-12). A higher expression of both miRNAs was found in MLO-Y4 and primary bone cells compared to IDG-SW3 cells.

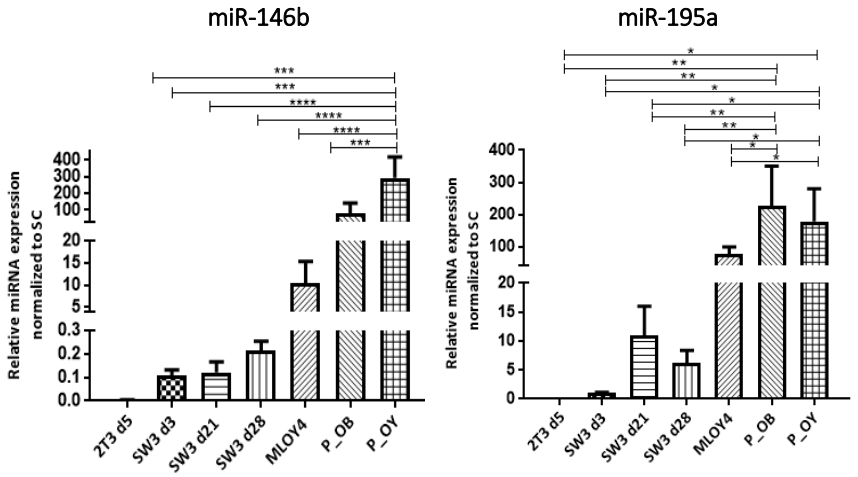

Figure R-12. Comparison of miRNA expression levels between bone cell lines and primary bone cells. Levels of miR-146b-5p and miR-195a-5p in: 2T3 day 5 differentiated (osteoblast like cells), IDG-SW3 cell line at day 3(osteoblast like cells), day 21 and day 28 differentiation (osteocyte like cells), MLO-Y4 (osteocyte like) cell line and Primary bone cells isolated from 2-month old mice femurs and tibiae (Osteoblast enriched fraction and Osteocyte enriched bone chips) normalized to Standard Curve. Bars represent the mean ± SD. n=3 for each group; (*p<0.05, **p<0.01, ***p<0.001, **** p<0.0001).

4. *In vitro* assessment of miR-146b and miR-195a on the osteoblast activity in IDG-SW3 cells

MiR-146b and miR-195a were transfected in IDG-SW3 cell line 3 days after induction of differentiation (osteoblast stage). As a first step, miRNA expression levels were measured at 24 and 48 hours after transfection in order to monitor the transfection efficiency (Figure R-13). Both miRNAs were successfully transfected increasing the miR-195a levels by more than 150-fold (p=0.002) and miR-146b levels by 2000-fold (p=0.002) after 24 hours. At 48 hours, a significant increase of miRNA levels compared to the control

was maintained (miR-195a, p=0.002; miR-146b, p=0,002) even if the relative fold change was reduced (Figure R-13).

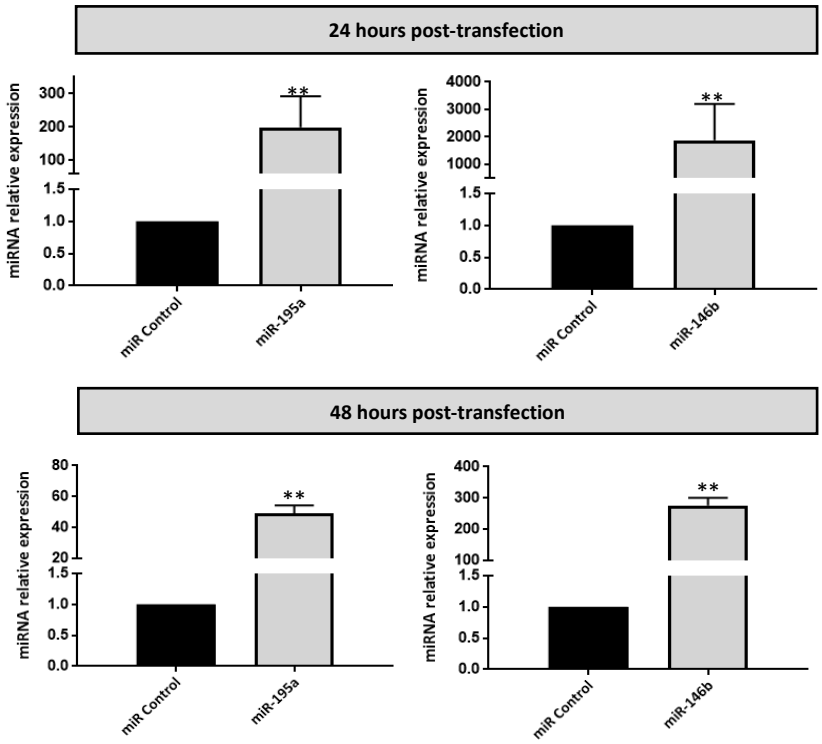

Figure R-13. Transfection efficiency of miR-195a and miR-146b in IDG-SW3 cells. IDG-SW3 cells were transfected with mimic of miR-146b and miR-195a and miR control. MiRNA levels were measured 24 and 48 hours post-transfection by qRT-PCR. miRNA expression values were normalized to U6 and expressed relative to the miR Control. Data represent the mean ± SD (n=3). **p<0.01

Thereafter, the role of miR-146b-5p and miR-195a-5p in the osteoblast function was *in vitro* assessed. At day 5 of differentiation (48 hours after transfection), mineral was not yet formed by osteoblasts. The overexpression of both miRNAs during the osteoblastic stage leads to a statistically significant but modest increase of mineralization at day 7 of differentiation (4 days after transfection) (both, p=0.002) (Figure R-14).

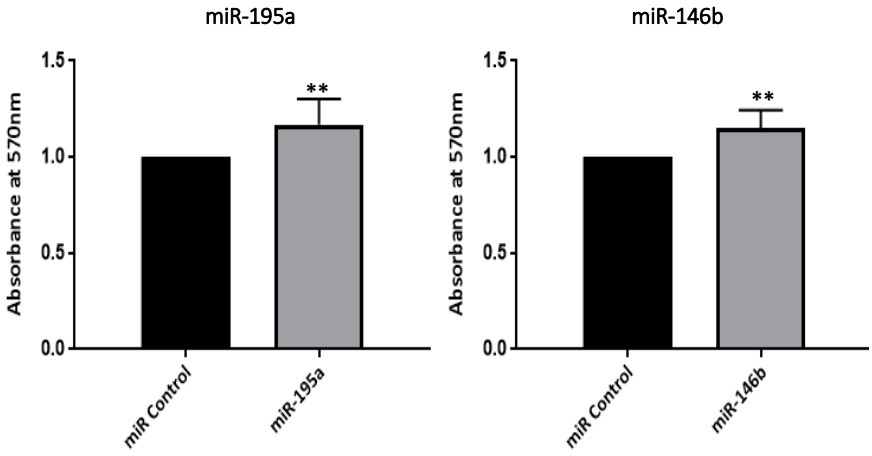

Figure R-14. Alizarin Red quantification after miR-195a and miR-146b transfection in osteoblasts. IDG-SW3 cells were transfected with mimic sequences of miR-195a-5p, miR-146b-5p and miR-control at day 3 of differentiation (osteoblast stage). Mineral quantification was evaluated at day 7 after induction of differentiation (4 days after transfection) by spectrophotometry at 570nm. Data are represented as relative to miR Control. Data represent the mean ± SD (n=3). **p<0.01

In the other hand, no significant effect in ALP activity was observed after either miR-195 transfection or miR-146b transfection (Figure R-15).

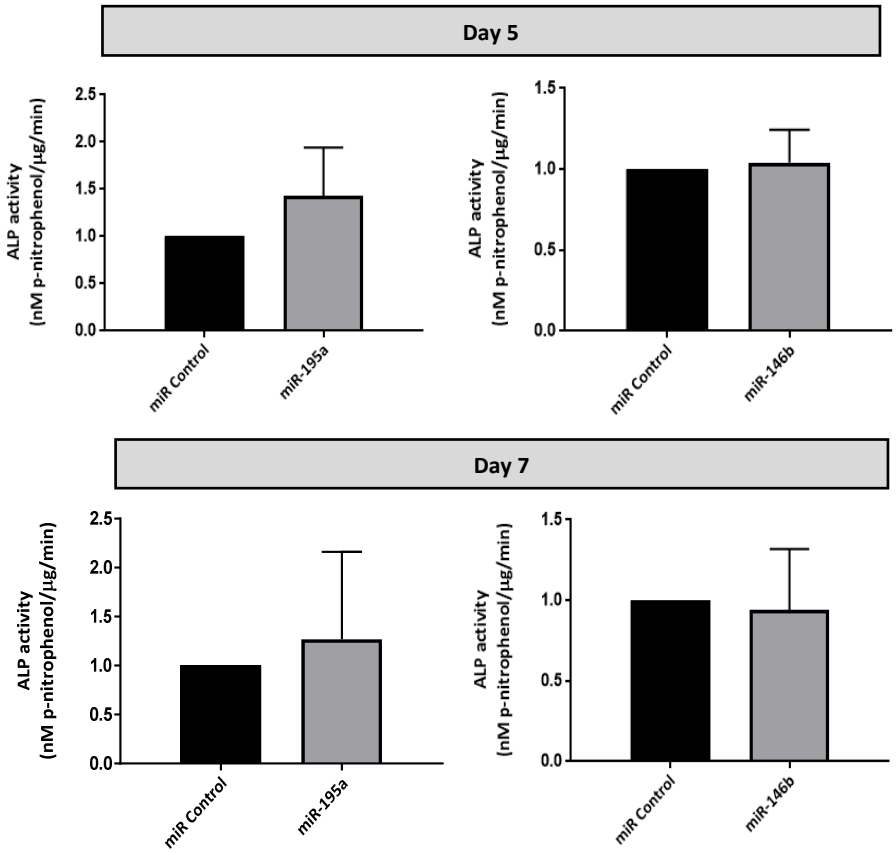

Figure R-15. ALP activity in IDG-SW3 cells after miRNA transfection. IDG-SW3 cells were transfected with mimic sequences of miR-195a-5p and miR-146b-5p and miR-control sequence. ALP activity (nM p-nitrophenol converted/ µg protein/min) was measuread at day 5 and 7 after induction of differentiation. Data represent the mean ± SD (n=3).

DISCUSSION

MicroRNAs are considered as important regulators of several cellular processes including those related to bone metabolism. Hence, a deeper investigation about the role of microRNAs in bone tissue regulation seems to be crucial to broad our knowledge on bone physiology and ultimately, to reveal unknown factors involved in bone diseases such as osteoporosis. This thesis has provided the discovery of a set of miRNAs involved in the osteoporosis fracture and a global landscape of miRNAs with promising functions in the bone tissue.

1. Identification of dysregulated miRNAs in postmenopausal osteoporosis

The first part of our study was focused on the identification of miRNAs with altered expression in the osteoporotic bone. In this regard, fresh trabecular bone samples from patients with a recent osteoporotic fracture (OP group) were compared to those from non-osteoporotic individuals (control group). A microRNA array analysis was performed in total bone tissue to detect a) all miRNAs expressed in these samples and b) to establish the miRNAs with different expression levels between biological groups.

The PCA plot showed a much more scattered distribution for OP samples compared to control samples, suggesting that the pathophysiology of the osteoporotic disease and ultimately the bone fracture has a heterogeneous etiology illustrated by different miRNA expression patterns. It is important to mention that we worked with human samples of fresh bone directly obtained from fractured sites which generate higher variability than cell lines or isogenic animal models. Thus, based on the unique miRNAs signature for each patient, the use of personalized medicine seems to be the leading path to follow for future therapies. Moreover, our study highlights the importance of working with fresh bone samples rather than using bone cell lines or *in*

vitro cell cultures, since provides a scenario closer to the pathophysiological situation and therefore, may be more suitable translating this research to the clinics.

The array analysis between osteoporotic and control samples revealed 82 miRNAs differently expressed. Apart of these miRNAs, 24 miRNAs were only expressed in one biological group and we named that as "switching" miRNAs. Unfortunately, these miRNAs could not be included in the initial analysis package performed in Exiqon due to technical reasons and thus, no statistical analyses were carried out. Analyzing deeper these miRNAs, we observed that the signals intensities of the miRNAs found in one group were just above the back-ground cut-off-limit. For this reason, we were hesitant to pursue these microRNAs. Even though, if such tightly controlled biological switches exist, it would be interesting to give further attention to this kind of regulation.

Thereafter, miRNAs with available Exiqon probes and the best hits in bone array (signal intensity and significant differences between groups), as well as predicted to target genes involved in bone metabolism were selected for validation by qRT-PCR. Two miRNAs were confirmed: miR-320a and miR-483-5p which were significantly over-expressed in osteoporotic samples. MiR-30c-1p also showed differences between OP and control groups but did not stand for multiple testing corrections and therefore, it was excluded for further analyses. Next, miR-320a and miR-483-5p were confirmed after a new set of samples were added in the analysis and moreover both miRNAs were detected in hOBs suggesting a possible role in the osteoblastic cell function. However, when the second set of samples was analyzed separately, only the miR-320a was validated as differentially expressed between groups. In this case the miR-22-3p was also validated but with a minor log fold change than miR-320a. Overall, the miR-320a showed the

highest expression difference between OP and control group and thus, it was selected for further functional experiments in hOBs.

2. Bone-related genes are regulated by miR-320a in osteoblasts

Firstly, an *in silico* target prediction was performed to get an overview of genes putatively regulated by miR-320a. Nevertheless, effective prediction of miRNA targets remains challenging due to the complexity of miRNA-mRNA interactions and the limited knowledge of the overall factors involved in those interactions. Indeed, there is a lack of consensus between miRNA target predictions programs, generating different list of targets depending of the program used. In this manner, it has been calculated that target prediction programs might provide around 24 to 70% of false positive results[298]. Moreover, some of the most common bioinformatics target prediction softwares such as TargetScan only contemplates miRNA targeting within the 3'UTR of an mRNA without incorporating interactions mediated trough the 5'UTR of an mRNA[298]. All this, underscores the necessity of performing functional experiments to demonstrate real interactions between miRNAs and their targets. A common and high-throughput method used in the experimental validation of miRNA targets is microarray approaches which enables large-scale analyses[299].

In this regard, we performed a microarray analysis in order to reveal the genes and pathways regulated by miR-320a in hOBs. As a first result it is worth noting the considerable difference in terms of gene expression among patients *per se*, independently to the transfection effect. In front of these data, we started to consider how critical might be the miRNA heterogeneity found among different patients and how this should be taken into consideration in the design of new miRNA-based therapies.

A total of 92 genes showed changes in gene expression after transfection of miR-320 mimic and/or inhibitor compared to the control transfection samples. Due to technical and economic reasons, we could just perform transfection experiments using 2 hOBs samples and therefore, we did not reach enough sample size to carry out any statistical analysis. For this reason, we established specific selection criteria to determine which genes were differently expressed between transfection and control. Transfection with mimic or inhibitor was calculated independently for each sample tested obtaining a $\log_2$FC for each transfection. In order to compare the effect of mimic and inhibitor, the order of the factors in the subtractions is inverted given that the expected effect of the mimic and the inhibitor are reverse. After sorting the groups based on the $\log_2$FC, the genes selected were those with meet the following criteria: first, the same sign of $\log_2$FC comparing mimic and inhibitor effect in both samples tested. Based on patient's heterogeneity, that selection criteria reduces the number of genes differently expressed but gives more power to the ones who have the same behavior in both samples. And second, to have at least the $|\log_2 FC| \geq 1$ in one of the groups.

Six of the top regulated genes which showed the highest fold change after miRNA transfection were *PTGS2*, *BMP2*, *STC1*, *IGFBP1* (up-regulated); *ACTA2* and *MIR296* (down-regulated).

Interestingly, these upregulated genes; *PTGS2*[281,282], *BMP2*[283-286], *STC1*[287-289] and *IGFBP1*[290-293], have been all related to biological and molecular functions on bone metabolism.

Only *MIR296* was validated in the new set of samples by qRT-PCR. So far, there is no literature relating *MIR296* to bone metabolism. Nevertheless, we

found that both miR-296-5p and miR-296-3p were expressed in bone tissue according to array's results. This miRNA has been extensively associated with several cancers such as prostate cancer, lung cancer and glioblastoma[300-302].

BMP2, STC1, and ACTA2 genes were also upregulated after overexpression of miR-320a in the new set of samples, confirming the results for mimic transfection observed in the microarray. Considering the consensus acknowledgement of the inhibitory function of gene expression by miRNAs, we can assume the existence of intermediates in the upregulation of these genes. Unfortunately, results were not validated by the inhibitor transfection and therefore we do not fully confirm the array's results. The fact that miR inhibitors displayed a much lower efficacy than miRNA mimics[257] make difficult to replicate some results in hOBs samples, due to the high biological heterogeneity of patients. However, we cannot rule out these genes as possible indirect targets of miR-320a.

Moreover, these genes have been previously described within the bone context. For example, the osteoinductive properties of BMP2 have been extensively evaluated and its biological activity in bone formation has been well-defined both *in vitro* and *in vivo*[283,286,303,304]. Hence, the use of recombinant human BMP2 (rhBMP2) has been applied in clinics to support bone formation after tibia fractures or during lower back spine fusion[305].

We observed that BMP2 was upregulated after miR-320a transfection in hOBs. Hence, the overexpression of miR-320a detected in fractured bone may alter the BMP2 levels in hOBs leading a dysregulation of osteoblastic function. For instance, an increased cell apoptosis can be induced by the BMP2 upregulation in an osteoporotic context[306].

Interestingly, the downregulation of miR-320a in BMP2 induced osteogenesis of C2C12 mesenchymal cells[307] suggests the existence of a regulatory loop between BMP2 and miR-320a.

Stanniocalcin 1 (STC1) is the mammalian homologue of STC which was identified in bony fishes as a calcium/phosphate-regulating hormone[308]. Mammalian STC1 is expressed in many tissues[309]. Interestingly, STC1 is found in chondroprogenitors, chondrocytes and osteoblasts[287] during bone development. To investigate the role of STC1 in mammals, Filvaroff *et al.*[287] generated transgenic mice expressing high levels of STC1 in skeletal muscle. They observed a lower mineral apposition rate by osteoblasts in both trabecular and cortical bone of STC1 transgenic mice, suggesting a reduced osteoblast activity. Furthermore, the gain of function of STC1 in transgenic mice had negative effects on proliferation and viability of osteoprogenitor cells and bone size and growth[288]. Hence, upregulation of *STC1* by miR-320a can be another factor for explaining the pathophysiology of bone fracture.

The ACTA2 gene encodes the alpha 2 actin cytoskeletal protein, which is a major component of the smooth muscle cell contractile apparatus. As no literature relates this gene with bone phenotype, further experiments should be performed in hOBs and hOCs to understand its role in these bone cells types.

Nonetheless, not all miRNA-mRNA interactions may be discovered by microarray analysis since miRNAs can downregulate genes by reducing protein expression levels without changes in transcript levels[299]. In fact, one limitation of our study is that we analyzed putative miRNA targets at mRNA level but not at protein level and therefore, part of the bone regulation by miR-320a is missing. Nevertheless, neither of both approaches is able to

distinguish between direct and secondary miRNA targets. Reporter assays have been commonly used to demonstrate direct interactions between miRNAs and their targets. These functional assays have, however, some disadvantages as labor intensive and the *in vitro* manipulation of the cellular systems. Moreover, the detection of only individual miRNA interactions failures the capacity of exploring miRNA-regulated gene networks, which might be identified by large scale cost-effective technologies such as microarray analysis. Indeed, in our study, the performance of a miRNA microarray analysis allowed the acquirement of a global view of gene regulation by miR-320a in the osteoblast context and thus, the identification of promising miRNA targets. Nevertheless, the combination of multiple techniques would be required to gain a complete description of gene regulation mediated by miRNAs.

In this regard, the transfection methodology used in all these approaches must be taken into consideration. In fact, the overexpression of miRNAs by using synthetic miRNA mimics would lead to an artificial supraphysiological increase of miRNA levels which may generate a number of false positive results. Furthermore, even if miRNA mimics are chemically synthetized as duplexes designed to activate only one of the two miRNA strands, it might occur that the other strand incorporates into RISC and engenders off-target effects[298]. Limitations are also present for miRNA silencing using exogenous miRNA inhibitors. The main handicap is the inhibitor specificity since members of the same miRNA families possess nearly identical sequences which may only differ for one or two nucleotides. Hence, even though our results may not properly illustrate the real situation *in vivo,* they bring us closer to them with the currently methodology available.

3. *In silico* analysis of miR-320a gene targets

Afterwards, we performed an *in silico* analysis with the aim to identify the putative pathways and functions regulated by miR-320a in hOBs. IPA revealed that miR-320a targets were significantly enriched in pathways for liver X receptor (LXR)/ retinoid X receptor (RXR) activation, agranulocyte adhesion and diapedesis, integrin linked kinase (ILK) signaling and PPAR signaling, among others.

The most significantly enriched pathway was the LXR/RXR activation, involved in the regulation of lipid metabolism, inflammation, and cholesterol[310]. The role of lipid metabolism has been extensively explored in the bone field where direct associations between osteoporosis and hyperlipidemia have been described[311]. In fact, Makovey *et al.* showed that lumbar spine and whole body BMD correlated inversely with lipid levels in postmenopausal women[312]. Additionally, lipid oxidation products enhance intracellular oxidant stress in osteoblasts and inflammatory bioactive lipids induce bone loss, by blocking the cell differentiation[311]. In this regard, fatty acid oxidation was also one of the detected significant enriched pathways in our study.

ILK is an intracellular protein whose main function is to connect integrins to the cytoskeleton. Integrins are the most common type of adhesion molecules involved in the interaction between bone cells and bone matrix[1] which suggest a role of miR-320a in cell adhesion. Interestingly, ILK signaling has been proposed as a key mechanotransduction pathways involved in bone adaptation during exercise[313].

The peroxisome proliferator-activated receptors (PPARs) signaling has also been related to bone metabolism. In this regard, different PPAR subtypes showed diverse and opposite roles in the regulation of bone homeostasis. In

fact, PPAR suppresses the differentiation of bone-forming osteoblasts, promotes osteoclast differentiation and its negatively regulated by Wnt signaling activity[314] whereas PPARβ/δ enhanced Wnt signaling activity and gene expression in osteoblasts, leading to an increased expression of OPG and in turn, attenuation of osteoblast-mediated osteoclastogenesis[315].

Taking all together, miR-320a might have a regulatory role in several key regulatory pathways needed for an appropriate bone cell function.

4. Viability and proliferation of U2OS is regulated by miR-320a

As stated previously, miR-320a was significantly associated with cell growth and proliferation in the *in silico* analysis performed by IPA.

The overexpression or inhibition of miR-320a did not cause any significant change in primary hOB proliferation at any of the miRNA concentrations tested. In contrast, a reduction in cell viability was found when miR-320a was overexpressed in the U2OS cell line. Moreover, the inhibition of miR-320a significantly increases cell growth in U2OS cells. Interestingly, this miRNA is significantly downregulated in osteosarcoma tissues compared to non-cancerous tissues and its overexpression significantly inhibited cell proliferation in U2OS and MG-63 cells[316]. However, the apoptosis rates were unaltered in both osteosarcoma cell lines. Different concentrations of miRNAs and different methodologies used to determine cell viability could explain this discrepancy with our results. In this line, miR-320a was also found to be decreased in cervical cancer[317]. Similar to our study, miR-320a also inhibited cell proliferation and induced cell apoptosis. Taking into consideration that this miRNA may reduce the viability of tumorigenic cells without affecting primary osteoblastic cells in the tested doses, suggests a higher sensitivity of osteosarcoma cells to miR-320a cell cycle regulation.

Based on these results, further investigation is encouraged for validating our data in other osteosarcoma cell lines such as MG-63 and SAOS in order to replicate the cell viability effects observed in U2OS cells.

5. Osteoblast mineralization is reduced after overexpression of miR-320a

The role of miR-320a on hOBs function was *in vitro* assessed. In our experiments, the overexpression of miR-320a in hOBs resulted in 14% reduction of matrix mineralization at 28 days of culture. Accordingly, an increase in matrix mineralization was observed after the inhibition of miR-320a even if it was non-statistically significant. These results correlates with the higher levels of this miRNA detected in the fractured osteoporotic bone, suggesting that the overexpression of this miRNA can lead to an impaired mineralization and consequently to an osteoporotic phenotype.

Due to technical and economic conditions, only two sequential transfections were performed during the whole 28 day of hOB culture: one at day 1 and, the other, at day 14 after seeding. Even if miRNAs are considered globally stable and are expected to possess long half-lives[318], there is evidence of miRNA rapid decay in some situations. In this regard, we observed a pronounced degradation of miR-146a and miR-195 from 24 hours to 48 hours post-transfection in IDG-SW3 cells (Figure R-13). Even though commercial available miRNA mimics and inhibitors are provided by chemical modifications which enhance their stability[319], we cannot rule out a certain degradation of miR-320a between transfections. Hence, the effect of miR-320a might be attenuated in the period between transfections and therefore, the inhibition of mineralization has been only partially observed. Likely, the sustained overexpression of miR-320a in the bone tissue would lead to the poor mineralization underlying to the osteoporosis.

In our study, osteosarcoma cells were used to explore the miR-320a effect on proliferation because of the accelerated cellular cycle of these cells could facilitate its detection. In contrast, factors involved in osteoblast differentiation such as mineralization and ALP activity were exclusively assessed in primary hOBs since osteosarcoma is characterized by an alteration in the usual pathways that regulate osteoblastic differentiation[320]. In this regard, Thomas *et al.* investigated the molecular basis for the connection between malignant transformation and loss of differentiation in osteosarcoma. They observed that RUNX2 protein, a key transcriptional regulator of osteogenesis, was absent or non-functional in six out of the seven osteosarcoma cell lines tested. Moreover, it has been shown that osteosarcoma often undergoes dedifferentiation[321]. For this reason, we considered more appropriate to evaluate those parameters in primary bone cells which possess a regular osteoblastic differentiation instead of osteosarcoma cell lines.

However, since miR-320a is expressed in a wide variety of cell types, we cannot rule out a wider spectrum of bone cellular dysfunction. Hence, the role of miR-320a should be further explored in other bone cells in order to fully understand the function of this miRNA in the overall bone function.

6. Overview of bone-expressed miRNAs

Bone tissue is a complex network composed of several cell types where each specific cell expresses theirs own miRNAs signature which are involved in different cell functions. The set of total miRNAs expressed for every cell type will configure the specific signature of the bone tissue. The first aim of this project was to determine the miRNA expression profile of whole bone tissue from postmenopausal women. Then, two additional miRNA profiling in

primary osteoblasts and osteoclasts were performed in order to obtain a broader view of the miRNA signature in bone.

From the 641 miRNAs detected in bone samples, a 54% (n=345) was found to be present in osteoblasts and/or osteoclasts. The other 46 % of miRNAs might correspond either to the majority bone cell type, osteocytes, or other bone resident cells. Since osteoblasts and osteoclasts represent a small fraction of the cells residing in the bone tissue, is not surprising that only half miRNAs localize in those two cell types.

The most expressed miRNAs detected in the total bone array (Table R- 2) have already been linked to the bone field. Actually, several reports described their association with osteosarcoma. For instance, the ectopic expression of miR-26b in the human U2OS cell line inhibited proliferation, migration, invasion, cell cycle arrest and induction of apoptosis[322]. MiR-20a down-regulates Fas expression in osteosarcoma, thus contributing to the metastatic potential of osteosarcoma cells[323]. Moreover, key genes related to osteogenic differentiation, such as *BMP2* and *RUNX2*, have been predicted to be targeted by miR-20a[324]. In addition, miR-27a expression was found to be significantly reduced in osteoporotic patients[325]. Interestingly, several reports have focused on the regulatory functions of miR-223 in bone metabolism. Its expression has been related to play a negative role in both, osteoblasts and osteoclasts differentiation (Figure D-1).

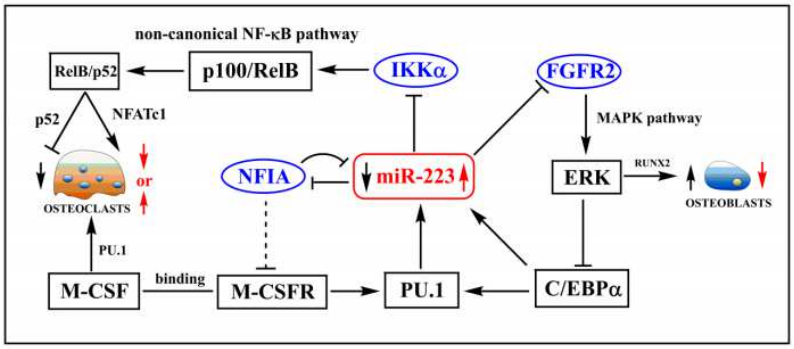

Figure D-1. Regulatory networks involving miR-223 roles in bone metabolism. MiR-223 is involved in both, osteoblast and osteoclast differentiation. Red short arrows indicate the regulation due to overexpression of miR-223, and black short arrows indicate the regulation of knockdown expression of miR-223. Extracted from Xie *et al*, 2015[326].

Among the top ten most expressed miRNAs in osteoblasts were miR-3679 and miR-4274. Both miRNAs were included in the genetic part of our investigation and will be further discussed.

The miRNA expression profiling in hOCs detected 340 miRNAs, of which 196 (58 %) was also present in the bone tissue array, representing a 31 % of all miRNAs detected in total bone tissue. An important proportion of osteoclast-related miRNAs was not found in the bone tissue array. This fact might be explained by the marked low percentage of osteoclasts in bone, making difficult the detection of osteoclast-specific miRNAs when total bone is analyzed. In accordance with this hypothesis, the top 20 most expressed osteoclast-related miRNAs were found in the bone array. Hence, only high expressed miRNAs in osteoclasts are detected in the bone tissue array which is a limitation of whole tissue studies. On the other hand, as osteoclasts cells were *in vitro* differentiated from blood mononuclear cells instead of isolating them *in-situ* from bone tissue, the detection of some true miRNAs expressed in human osteoclasts might be lost due to the procedure. Moreover, some

miRNAs detected in hOC array can be artefacts due to the artificial laboratory cell differentiation explaining their absence in bone array. Unfortunately, due to the relatively small number of osteoclasts in human bone tissues, the *in vitro* differentiation from PBMC is the most common current methodology used to obtain hOCs.

We then explored the current literature related to miRNAs involved in osteoclast differentiation. Recently, a group of researchers published a miRNA expression profile during human osteoclastogenesis[327]. In this study, osteoclasts were differentiated from primary monocytes obtained from three human donors, and mature osteoclasts were obtained 21 days after RANKL/M-CSF stimulation. In our analysis, miR-146a-5p, miR-378a-3p and miR-342-3p were three of the ten most expressed miRNAs found in mature osteoclasts. Interestingly, these 3 miRNAs were detected as significantly upregulated during osteoclast differentiation by de la Rica *et al.*[327]. Hence, the implication of these miRNAs in osteoclast regulation should be fully explored.

Interestingly, we observed that miR-146b-5p, which is closely related to miR-146a-5p, was significantly upregulated during osteoblast to osteocyte differentiation in the murine IDG-SW3 cell line. Moreover, it was also present in the osteocyte-like MLOY4 cell line and primary murine osteocytes.

The involvement of miR-146a-5p with osteoclastogenesis has been investigated by some authors. It has been observed that miR-146a levels increase during human and murine osteoclast differentiation[327,328], which is in accordance with the high expression found in our mature hOCs. As observed by Nakasa *et al.*[258], the overexpression of miR-146a in PBMCs inhibited osteoclastogenesis and prevented joint destruction in arthritic mice. The

same effect was observed by Yao *et al.*[329] in which the up-regulation of miR-146a in PBMCs resulted in dramatic inhibition of osteoclastogenesis and bone resorption. These results suggest a complex feedback regulation that controls osteoclast differentiation. Moreover, some authors have described the involvement of miR-146a as an important regulator on osteoarthritis[330,331] likely by down-regulation of SMAD2 and SMAD3 protein translation, which are known to be positively involved in chondrogenesis[257]. The authors observed that this downregulation was coupled with a down-regulation of SOX9 expression and upregulation of RUNX2 mRNA expression and thus suggesting miR-146a as a positive regulator on osteogenesis. MiR-146a was up-regulated during hMSC osteogenic differentiation[332]. Indeed, the inhibition of miR-146a function suppressed the osteogenic differentiation of MC3T3 preosteoblasts.

MiR-378 is preferentially expressed in skeletal muscle, cardiac muscle, and brown adipose tissue[333]. Recently, it has been reported that skeletal muscle mass was significantly reduced in transgenic mice globally overexpressing miR-378[334]. Thereafter the same research group demonstrated that miR-378 regulates skeletal development and regeneration by targeting the gene encoding insulin-like growth factor 1 receptor (IGF1R)[333]. Interestingly, miR-378 was found to promote BMP2-induced osteogenic differentiation of C2C12 cells[335]. In another study performed by Novello *et al.*[336], a lower expression of miR-378a-5p was found in osteosarcoma compared to normal bone. The function of miR-378 in hOCs is still unknown and therefore, needs to be explored.

Even though the role of miR-342-3p in hOCs is unknown, this miRNA has been related to the hematopoietic lineage differentiation[337]; its expression reduces the viability of macrophages by inducing their apoptosis.

As we observed during our study and described in the literature, the vast majority of miRNAs are non-restrictive to an exclusive cell type. Thus, a wide view of miRNA function in one tissue would be a fundamental requisite to understand the whole regulation mechanism of miRNAs in the explored tissue.

Primary osteoblasts and osteoclasts are the most common explored bone cells because of the availability of cell isolation protocols. Although several techniques have been published for the isolation of osteocytes from mouse bone, no such technique was designated for human osteocytes till now. Recently, Prideaux *et al.*[338] developed a protocol for the isolation of osteocytes from human trabecular bone samples obtained during surgery. This discovery would contribute to enhance our knowledge about the role of these cells in the bone. Thanks to this amazing innovative protocol, in the next future we would be able to obtain human osteocytes from postmenopausal women and therefore, to provide a complete view of the human miRNA signature in the human bone tissue.

7. MiRNA signature involved in osteoblast to osteocyte differentiation

In 2011, Woo & coworkers described and developed a new novel cell line that recapitulated osteoblast to osteocyte differentiation[63]. This cell line, IDG-SW3, was derived from long bones of mice as was the MLO-Y4 cell line but, unlike MLO-Y4, the IDG-SW3 expresses green fluorescent protein under control of the DMP1 promoter and has the capability to go through a complete differentiation from late osteoblast to late osteocyte. Another interesting feature about this new cell line is that it has the capability to produce an extracellular matrix that undergoes mineralization. The

availability of such an interesting cell model gave us the opportunity to deepen the knowledge beyond microRNAs during bone cell differentiation.
We observed that the relative expression of 56 miRNAs changed significantly during osteoblast to osteocyte differentiation in IDG-SW3 cells, according to array data. Expression of three of the most highly upregulated miRNAs (miR-146b, miR-195a and miR-497) and three of the most downregulated miRNAs (miR-322, miR-224 and rno-miR-351) were validated using qRT-PCR in IDG-SW3 cells.

We chose the upregulated miR-146b and miR-195a from among the validated miRNAs for further investigations due to their greater fold increase during differentiation and because these miRNAs are known to target genes related to bone metabolism. For example, miR-146b-5p is known to be elevated during osteogenic differentiation of muscle derived progenitor cells[339]. Additionally, three polymorphisms in the *FGF2* gene that are potential binding sites for miR-146b-5p were found to be significantly associated with FN BMD[260]. Interestingly, the expression of miR-195a-5p was dysregulated in osteosarcoma patients[340,341]. In agreement with our data, miR-195 was found to be upregulated during the last stages of osteoblast differentiation[342]. Moreover, our *in silico* analyses demonstrated that those two miRNAs were shown to influence genes involved in key bone-related pathways such as circadian rhythm[343] and Wnt signaling pathway[18] and thus, highlighting the potential role of these miRNAs in bone metabolism.

In order to validate the observations using the IDG-SW3 cells, we decided to compare the expression pattern of these miRNAs in IDG-SW3 cells with other osteoblastic and terminally differentiated osteocytic cell lines and in primary cells. For this purpose, the expression levels of both miRNAs were assessed in the osteocytic MLO-Y4 and osteoblastic 2T3 cell lines and in murine primary

bone cells in order to have a complete expression profile of these miRNAs in the osteogenic context. Genes such as *U6* and small nucleolar RNA, C/D box 68 (*Snord68*) have often been used as endogenous controls or 'housekeeping' genes that enable gene normalization in miRNA quantification studies. Surprisingly, we observed significantly lower expression levels of U6 and Snord68 in primary murine osteocytes compared to the primary osteoblast population. Indeed, other authors have reported that U6 is unsuitable as an internal reference control in circulating miRNAs studies[344,345]. Therefore, it is of fundamental importance to determine a proper normalizing gene for primary murine bone cells. For this reason, we were not able to use these genes as normalizers in primary bone cells. Instead, gene normalization was performed using a standard curve method in order to compare the expression levels of both miRNAs in all the bone cells tested.

Consistent with our observations using IDG-SW3 cells, a higher expression of both miRNAs was found in MLO-Y4 osteocytes compared to 2T3 osteoblast cells and higher expression in osteocyte enriched bone chips when compared to primary osteoblasts. These data confirm our preliminary results using IDG-SW3 cells showing an upregulation of both miRNAs during osteoblast to osteocyte differentiation.

Our initial theory was that the overexpression of these miRNAs, which were found to be most highly expressed in osteocytes, in the osteoblasts cells would accelerate their differentiation to osteocytes. Consequently, the role of miR-146b-5p and miR-195a-5p in osteoblast function was *in vitro* assessed in the IDG-SW3 cell line. The overexpression of both miRNAs led to a statistically significant but modest increase of mineralization at day 7 of differentiation (4 days after transfection), giving support to our theory;

meanwhile their overexpression did not cause any significant effect in ALP activity even if we observed a trend of increase in ALP levels relative to miR control. In contrast to our observations, miR-195 mimic transfection in primary calvaria osteoblasts resulted in significantly reduced levels of ALP and alizarin red staining[342]. These discrepancies might be attributable to the different concentrations of miRNA and the different cell sources used. Moreover, in the study performed by Grunhagen *et al.* cells were stimulated with BMP2, which might lead to some cell alterations which in turn, might affect miRNAs regulation.

The potential crucial role of those miRNAs in the osteoblast to osteocyte differentiation and lastly, to the functions of osteocytes, are most likely not depicted by the osteoblast parameters evaluated. Regulation of osteocyte differentiation by these two miRNAs might be identified by other approaches such as expression of early osteocyte genes such as *Dmp1, Phex* and *E11/gp38*, by expression of late osteocyte genes such as *Sost, FGF23* and *MEPE* or by maintenance of osteocyte viability.

As mentioned in the introduction, osteocytes are the most long-lived cells in bone, able to reside within the mineralized bone matrix up to decades[60]. Therefore, viability and survival are fundamental to ensure an ideal osteocyte function. This suggests that osteocytes have developed specific mechanisms which protect them against cell death. Future goals include investigating whether those miRNAs that are upregulated in the osteocytic stage are also protective against apoptosis with the aim to reveal one of the mechanisms underlying high osteocyte longevity. As a higher expression of miR-146b and miR-195a was found in MLO-Y4 than those detected in osteocytes in IDG-SW3 cells, our future plan is to knockdown these miRNAs in MLO-Y4 cells to see whether cell death by external apoptotic agents is elevated.

Taking all together, miR-146b and miR-195a might have important roles in osteocyte function and further investigation of both miRNAs will shed light on the mechanisms responsible for osteoblast to osteocyte differentiation.

8. Genetic variants in bone-related miRNAs are associated with the osteoporotic phenotype

Epigenetics factors such as microRNAs have recently appeared as one of the potential mechanisms able to unravel the missing heritability in complex diseases like osteoporosis. The miRNA expression signatures in patients with osteoporosis resulted from miRNA arrays do not provide evidence of causality because the altered pattern could be a consequence of the disease or even unrelated to the pathogenesis. Another approach in miRNAs studies is the association analysis between one SNP within a candidate miRNA (miR-SNP) or in a miRNA target site, and one disease related-outcome. In this case, the associated variant is likely involved in the pathophysiology or confers susceptibility to develop the disease.

The potential impact of miRNA-related SNPs on skeletal phenotype has already been described[253]. In our study we investigated the role of SNPs present in bone-related pre-miRNA sequences. For this purpose we performed an association study between miR-SNPs and BMD in our extensive cohort of postmenopausal women. We have identified two SNPs, rs6430498 in the miR-3679 and rs12512664 in the miR-4274, significantly associated with FN BMD. Unfortunately, we were not able to evaluate the association of these variants with the fracture outcome, because an important portion of the patients included in the cohort did not have records of the fracture history. However, both BMD-associated microRNAs were found overexpressed in fractured bone.

Moreover, our results demonstrated that the allele that generated higher microRNA expression levels in osteoblastic cells was associated with lower BMD values. As these variants fall into a low linkage disequilibrium region according to HapMap, lead us to speculate that they could be functional. Finally, we observed an increase in matrix mineralization after miR-3679-3p inhibition in human osteoblastic cells, which is in accordance with the miR-3679-3p overexpression detected in osteoporotic fractured bone. Taking all together, we can affirm that excessive expression levels of both miRNAs is harmful for bone leading, among other factors, the osteoporosis onset.

Up to now, little is known about the link of these two miRNAs with bone metabolism.

Thus, validating the function of miR-SNPs has the potential to enhance our understanding of the miRNA regulation of bone metabolism and to find a new piece on the genetic predisposition to skeletal diseases such as osteoporosis.

9. The promising role of miRNAs in bone disorders

In summary, we have depicted an overview of bone-related miRNAs with encouraging applications for the diagnosis and treatment of bone metabolic diseases such as osteoporosis. This study highlights the relevance of both, miRNA profiling and genetics assays as approaches for the study of fracture etiology. Both should be explored in parallel in order to obtain a global and complete overview of the miRNA function in bone tissue. Also the existing possibility of using miRNAs as biomarkers and treatment on bone diseases, convert them as appealing factors for their further investigation. Our study has provided our grain of sand on the miRNA field knowledge.

CONCLUDING REMARKS

1. Osteoporotic bone samples have an altered miRNA signature compared to non-osteoporotic samples. Moreover, the miRNA expression profile of osteoporotic samples shows a more dispersed distribution than non-osteoporotic samples, suggesting a heterogeneous etiology for bone fracture.

2. We have identified several miRNAs differently expressed between osteoporotic and control samples. Among them were miR-320a, miR-22-3p and miR-483-5p, which were over-expressed in bone samples from patients with osteoporosis and detected in primary hOBs.

3. Bone-expressed genes as *BMP2, STC1* and *ACTA2* were found differently expressed after miR-320a transfection in hOBs cells. *MIR296* was also regulated by miR-320a even though its role in bone is unknown.

4. *In-silico* analysis revealed that miR-320a targets were significantly enriched in pathways such as LXR/RXR activation and ILK signaling, and involved in cellular functions including cellular movement, cellular development and cell growth and proliferation.

5. Inhibition of miR-320a in U2OS cells increased osteoblast cell proliferation while no effect in cell proliferation and viability was observed in primary hOBs.

6. Overexpression of miR-320a reduces matrix mineralization of hOBs cells, that correlates with the increased miR-320a levels detected in the fractured samples.

7. We have depicted an overview about the miRNA signature of whole bone tissue from postmenopausal women. From the 641 miRNAs detected in bone samples, a 54% was found to be present in osteoblasts and/or osteoclasts. The most miRNAs detected in hOBs cultures were also present in total bone samples. In contrast, only a 58% of miRNAs detected in hOCs were in the bone array samples.

8. Two miR-SNPs, rs6430498 and rs12512664, within the pre-miRNA sequence of miR-3679 and miR-4274, respectively, were associated with FN BMD in the OSTEOMED2 cohort. The A alleles for both variants were associated with lower BMD values and with higher microRNA expression levels in human osteoblastic cells. Both microRNAs were found overexpressed in fractured bone. Additionally, miR-3679-3p negatively regulates matrix mineralization by osteoblasts.

9. A subset of miRNAs varies their expression during osteoblasts to osteocyte differentiation in IDG-SW3 cells. The overexpression of miR-146b-5p and miR-195a-5p during the osteoblastic stage leads to a modest increase of matrix mineralization in IDG-SW3 cells. Both miRNAs were highly expressed in the osteocyte-like cell line MLOY4 and in primary murine osteocytes.

10. Our study provides new insights for the understanding of miRNA involvement in bone metabolism and highlights the importance of miRNAs as potential therapeutic targets in osteoporosis.

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