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Identification of a Novel Transcription Factor Required for Osteogenic Differentiation of Mesenchymal Stem Cells

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1. Abstract

Osteogenic differentiation is a complex and still poorly understood biological process regulated by intrinsic cellular signals and extrinsic micro-environmental cues. Following appropriate stimuli, mesenchymal stem cells (MSCs) differentiate into osteoblasts through a tightly regulated multi-step process driven by several transcription factors and characterized by the expression of a number of bone-specific proteins. Here, we describe a novel transcription factor that we named Osteoblast Inducer (ObI)-1, involved in MSC differentiation towards the osteogenic lineage. ObI-1 encodes for a nuclear protein subjected to proteasomal degradation and expressed during osteoblast differentiation both in a murine multipotent mesenchymal cell line (W20-17) and in primary murine MSCs. RNAi-mediated knockdown of ObI-1 expression significantly impairs osteoblast differentiation and matrix mineralization with reduced expression of the osteogenic markers Runx2 and osteopontin. Conversely, ObI-1 over-expression enhances osteogenic differentiation and bone-specific markers expression. ObI-1 stimulates bone-morphogenetic protein (BMP)-4 expression and the consequent activation of the Smad pathway; treatment with a BMP receptor-type I antagonist completely abolishes ObI-1-mediated stimulation of osteogenic differentiation. Collectively, our findings suggest that ObI-1 modulates osteogenic differentiation, at least in part, through the BMP signaling pathway, increasing Runx2 activation and leading to osteoblast commitment and maturation.

2. Introduction

2.1 Bone tissue

Bone tissue is an essential component of the body with a number of functions: bones act as structural scaffolding, serves as storage for inorganic matrix and provides also protection to the soft tissues and vital internal organs (Remedios. 1999)

Bone is composed for only 5% of cells whereas the largest part is constituted by the extracellular organic and inorganic matrix. The organic matrix is mainly composed of type I collagen forming fibrils, glycosaminoglycans, osteocalcin, bone sialo-proteins, osteopontin and osteonectin. The inorganic mineral matrix is constituted by hydroxyapatite that is largely responsible for the strength and density of the bone, composed mainly of calcium and other minerals. Therefore, in order to maintain bone density and strength, the body requires an adequate supply of calcium, other minerals and vitamin D.

Bone is a dynamic and constantly changing tissue that undergoes a continuous process of remodeling characterized by bone resorption and deposition of new bone tissue (bone neoformation); indeed, every bone of the body is completely reformed about every 10 years.

A characteristic of all connective tissues, including bone and cartilage, is that they contain a large amount of intercellular substance surrounding cells. There are four important types of cells associated with bone tissue: osteogenic cells, osteoblasts, osteocytes, and osteoclasts (Fig.1).

- Osteogenic cells are pre-osteoblast cells derived from mesenchyme, which is the precursor of all forms of connective tissue. When osteogenic cells undergo mitosis, the resulting daughter cells can differentiate into osteoblasts in relation to local or systemic needs.

- Osteoblasts are mononucleate bone-forming cells descending from osteoprogenitor cells. They are fully differentiated cells that do not undergo further divisions and are responsible for bone formation, secreting both organic matrix and mineral salts of the inorganic matrix. Osteoblasts express receptors for hormones regulating bone growth.
- Osteocytes are typical mature bone cells. They are osteoblasts that have become isolated in the intercellular substance they have deposited around themselves during bone neosynthesis. The spaces they occupy are known as lacunae. They are cells that have stopped laying down new bone, but they play an important role in maintaining the cellular activities of bone tissue in their immediate area by secreting enzymes and controlling the bone mineral content and the calcium release from the bone into the blood. Furthermore, osteocytes have been shown to act as mechano-sensory receptors, regulating the bone response to stress and mechanical load.
- Osteoclasts are cells responsible for bone resorption and play an important role in bone growth, healing and remodelling. They belong to the monocyte/macrophage cell lineage and are giant multinucleated cells (up to 50 nuclei per cell depending on the species) located in contact with calcified bone surfaces in Howship's lacunae. Osteoclasts produce enzymes, such as the tartrate resistant acid phosphatase secreted against the mineral substrate and express receptors for various hormones that regulate their activity.

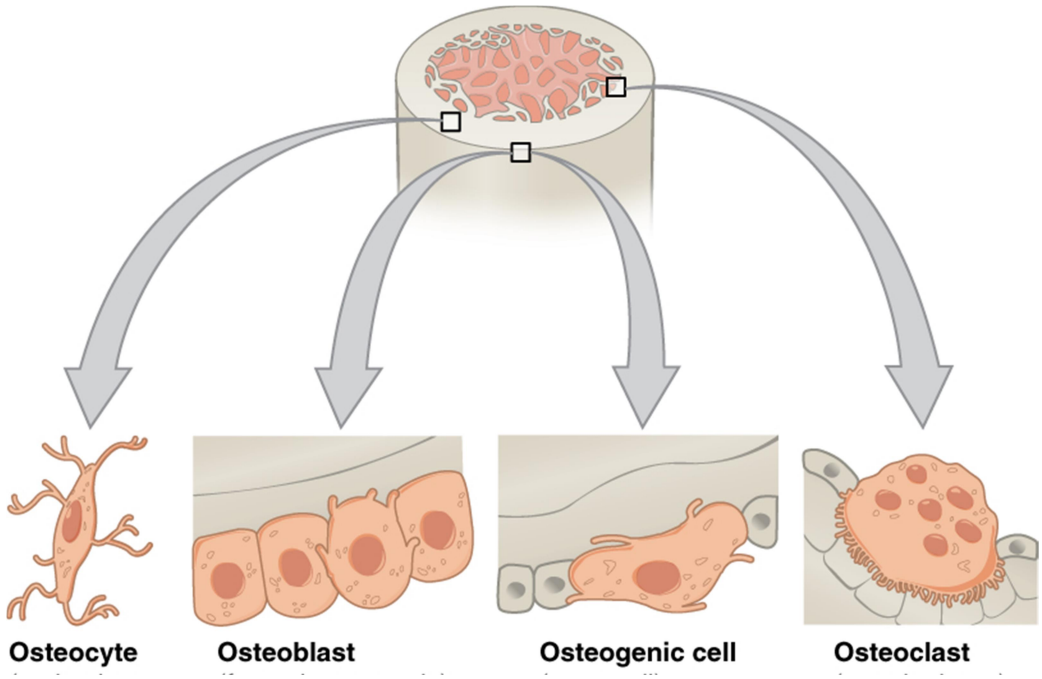


Figure 1: Bone cell types.

2.1.1 Bone tissue development and ossification

In mammals, bone development is the last event of skeleton development. During the first phase of skeletogenesis, mesenchymal cells aggregate at the sites where skeletal elements will develop (skeletal patterning); these mesenchymal condensations, prefiguring the future skeletal elements, are evident between 10.5 and 12.5 days postcoitum (dpc) in mouse.

Bone formation (or ossification) can occur through two different mechanisms:

- Intramembranous ossification
- Endochondral ossification

In the skeletal elements prefiguring the clavicles and some bones of the skull, cells of the mesenchymal condensations differentiate directly into osteoblasts that appear at 15.5 dpc of mouse development, without a cartilage template through a process known as intramembranous ossification (Huang et al. 1997). Development of the majority of skeletal elements, instead, contemplates the formation of a hyaline cartilage intermediate model of the bone that is eventually replaced and ossified (Fig.2). In this process, called endochondral ossification, cells present in the mesenchymal condensations differentiate into chondrocytes whereas cells at the periphery differentiate into osteoblasts and form the perichondrium. Chondrocytes located in the center of mesenchymal condensations stop proliferating, become hypertrophic and start producing a mineral matrix rich in type X collagen. Afterwards, while hypertrophic chondrocytes die through apoptosis, the vascular invasion from the perichondrium brings in cells of the osteoblastic lineage, at about 14.5-15.5 dpc in mouse, that differentiate into osteoblasts and produce an extracellular matrix rich in type I collagen (Erlebacher et al. 1995). Endochondral ossification is the process associated with fetal development of long bones, such as the femur and humerus, bone growth, and fracture repair.

Endochondral ossification begins in points in the cartilage called primary ossification centers. They mostly appear during fetal development, although a few short bones begin their primary ossification after birth. They are responsible for the formation of the diaphyses of long bones, short bones and certain parts of irregular bones. Secondary ossification occurs after birth, and forms the epiphyses of long bones and the extremities of irregular and flat bones. The diaphysis and both epiphyses of a long bone are separated by a growing zone of cartilage.

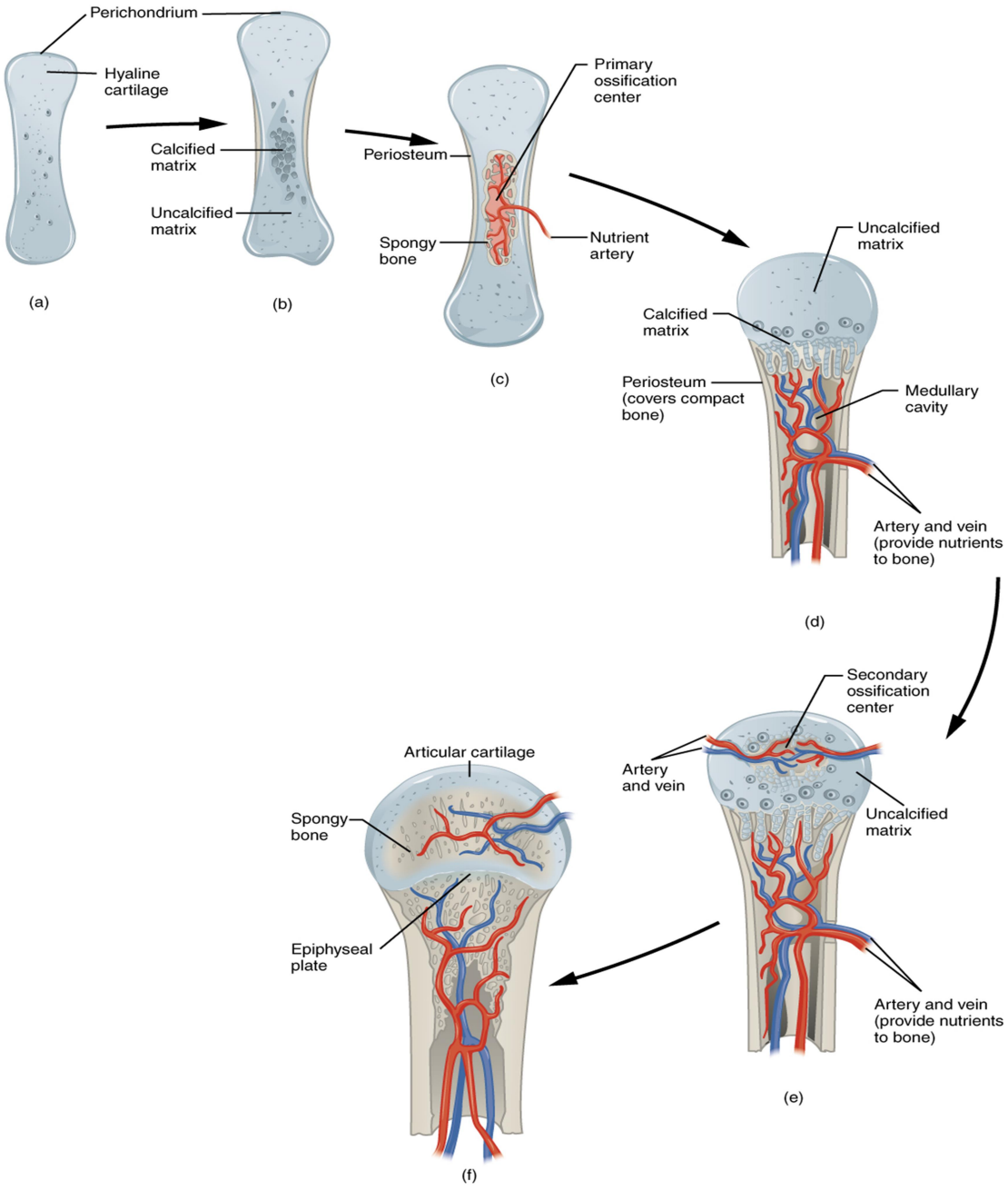


Figure 2: Endochondral ossification.

2.1.2 Main bone-related pathologies

2.1.2.1 Bone fractures

A fracture is defined as a partial or complete break in continuity of the bone that occurs under mechanical pressure. Most fractures are caused by traumatic injury; however, bone cancer or metabolic disorders can also cause fractures by weakening the bone. Fractures may result in loss of function in the affected limb, guarded movements, pain, soft tissue swelling (edema), and deformity. Fractures are classed anatomically, by the extent of the injury, or by the angle of the fracture.

Fracture repair is required to restore the normal alignment and function of a broken bone. Throughout the stages of fracture healing, the bone must be held firmly in the correct position. In the event that a fracture is not properly repaired, misalignment of the bone may occur, resulting in possible physical dysfunction of the bone. There are four phases in fracture healing:

1 **Hematoma formation**

This stage is characterized by inflammation and the formation of a granulation tissue. When a bone breaks it causes also the rupture of the blood vessels in the bone, periosteoum and surrounding tissues. This results, within a few hours after fracture, in the formation of a mass of clotted blood, or hematoma, at the fracture site.

2 **Fibrocartilagineous callus formation**

This phase is characterized by the formation of a soft tissue called cartilaginous callus, necessary to keep the broken bones together. Capillaries grow into hematoma and phagocytic cells digest the cellular debris.

3 **Bone callus formation**

Blood vessels bring in osteoblasts that gradually replace the hyaline cartilage with the formation of a spongy bone callus.

4 **Remodeling phase**

In the following weeks or months, depending on the location and extent of the injury, osteoblasts and osteoclasts continue to migrate into the site of the fracture remodeling the existing callus.

2.1.2.2 Osteoporosis

Osteoporosis is the most common metabolic disorder characterized by low bone mass and microarchitectural deterioration of bone tissue leading to enhanced bone fragility and a consequent risk in fractures.

Osteoporosis has a multifactorial etiology and it can be primary, postmenopausal and senile, or secondary; the underlying mechanism in all cases is an imbalance between bone resorption by osteoclasts and bone formation by osteoblasts during physiological bone remodeling. As bone resorption overwhelms bone formation, bones become weaker, fragile and more susceptible to fracture.

Estrogens have been found to play a major role in osteoporosis pathogenesis, since it maintains bone homeostasis by slowing the rate of bone erosion. Indeed, it inhibits osteoclasts activity, and when estrogens levels decrease, osteoclasts activity increases to erode the bone matrix. Other factors contributing to osteoporosis can include an inactive lifestyle, a diet low in calcium and protein, or as a consequence of other diseases such as hyperthyroidism.

2.1.2.3 Osteoarthritis

Osteoarthritis, or degenerative joint disease, is the most common form of chronic arthritis. In this disease, the articular cartilage degenerates and bone grows into the joint space. As the joint space decreases, movement becomes more difficult and painful. Osteoarthritis occurs as a consequence of aging and “wear and tear” on a joint over the year.

Treatment includes exercise, efforts to decrease joint stress, support groups, and pain medications. Efforts to decrease joint stress include resting and the use of a cane. Weight loss may help in those who are overweight.

Pain medications may include paracetamol (acetaminophen) as well as NSAIDs such as naproxen or ibuprofen. Long-term opioid use is generally discouraged due to lack of information on benefits as well as risks of addiction and other side effects. If pain interferes with normal life despite other treatments, joint replacement surgery may help. An artificial joint typically lasts 10 to 15 years (Di Puccio et al. 2015).

2.2 Stem cells

Stem cells are undifferentiated cells that have the ability to self-renew and the potential to differentiate into several different cell lineages in response to appropriate stimuli. Stem cells can be classified according to their origin, in embryonic (ESCs) and adult stem cells (ASCs), and their differentiation potential, in totipotent, pluripotent, multipotent, oligopotent and unipotent stem cells. Totipotent stem cells, i.e. the zygote, are the most versatile stem cells in that they can differentiate into embryonic and extra-embryonic tissues, giving rise to a complete and viable organism. Cells isolated from the inner cell mass of embryos at the blastocyst stage (ESCs) are pluripotent stem cells; they can differentiate into nearly all cell types, namely cells derived from any of the three germ layers, mesoderm, endoderm and ectoderm, but they are not able to differentiate into extra-embryonic tissues. Multipotent stem cells are stem cells that have the potential to differentiate into a quite large number of cell types but usually deriving from a unique germ layer. Finally, oligopotent stem cells can differentiate into only a few cell types, while unipotent stem cells give rise to one specific cell lineage.

More recently, an exciting new source of stem cells has been developed by Takahashi and Yamanaka (2006), the induced pluripotent stem (iPS) cells. iPS cells are derived by genetic reprogramming of differentiated somatic cells using a cocktail of factors, originally Oct4, Sox2, Klf4 and c-Myc. They are regarded as a promising stem cell source for regenerative medicine, to repair damaged tissues or organs, since iPS cells are pluripotent but, unlike ESCs, they do not pose ethical issues. Furthermore, iPSs can be established from the somatic cells of the patient, thereby avoiding issues of histhocompatibility. However, like ESCs, iPS can easily form teratomas *in vivo* and great efforts have been made in order to develop protocols that allow a complete and reproducible differentiation to appropriate cell lineages.

2.2.1 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are adult, multipotent stem cells able to self-renew and generate, after appropriate stimuli, several mesodermal cell lineages, including adipocytes, osteoblasts, chondrocytes, myoblasts, tenocytes and haematopoiesis-supporting stroma. MSCs were identified by Alexander Friedenstein and co-workers in the 1960s. Bone marrow cells plated at low density form a population of fibroblastoid, clonogenic plastic-adherent cells named colony-forming unit-fibroblasts (CFU-f). Each colony, seeded under the kidney capsule of mice, is able to give rise in few weeks to fibrous tissue, bone and bone marrow; bone cells were of donor origin, whereas the hematopoietic tissue was of recipient origin, probably due to the homing of host circulating blood hematopoietic stem cells (HSCs) (Friedenstein et al. 1966). These data showed that in the bone marrow there is a population of cells distinct from hematopoietic precursors, able to generate bone tissue and to provide an appropriate microenvironment for HSCs homing and haematopoiesis. In the following years, further studies demonstrated that the inherently osteogenic cells discovered by Friedenstein and named osteogenic stem cells were actually multipotent and capable of generating not only osteoblasts, but also adipocytes, chondrocytes and myoblasts (Prockop 1997).

In the last few years, several studies have shown that MSCs could exhibit a broad degree of plasticity, since they seem to be able to differentiate into non-mesodermal cell types, including neural cell lineages, endothelial cells, hepatocytes and pancreatic progenitor cells (Ibraheim et al. 2017) (Fig.3). However, there are still controversies regarding the true plasticity of MSCs; in fact, differentiations observed *in vivo* may be due to fusion of MSCs with endogenous cells, whereas *in vitro* non-standardized culture conditions may lead to the selection of rare cell populations with greater differentiation potential. Furthermore, recent reports have shown that, at least in some circumstances, the ability

of MSCs to promote *in vivo* tissue regeneration in response to disease or injury is unlikely due to their ability to differentiate, since they exhibit very low or transient levels of engraftment (Iso et al. 2007). These observations suggest that MSCs could promote tissue repair through the secretion of factors that modify tissue microenvironment and stimulate the regeneration.

Although MSCs were originally isolated from bone marrow, these cells are widely distributed throughout tissues, including adipose tissue, peripheral blood, skeletal muscle, tendon and several fetal tissues. However, several studies have reported that MSCs isolated from different sources are not equivalent and, indeed, they show differences in many aspects, including proliferation rate, differentiation potential and expression of some specific markers (Barlow et al. 2008). Therefore, further studies are required to better characterize MSCs populations from different sources in order to be able to select the most suitable ones for the specific purpose of the study as well as for clinical applications.

During the last years several techniques for MSCs isolation and culture-expansion have been developed and MSCs have been successfully harvested from several tissues of multiple species; so far, bone marrow-derived MSCs are the most intensely studied. The conventional isolation protocol, widely used for the purification of MSCs, is based on the ability of these cells to adhere to tissue culture plates (Esposito et al. 2009). However, in the last years, great efforts have been made to characterize MSCs immunophenotype in order to identify a surface antigen panel that could be useful for the isolation and purification of MSCs.

The definition of MSCs is extremely complicated as such cells represent a morphologically, phenotypically and functionally heterogeneous population of cells. For this reason, minimal criteria for the definition of human MSCs have been established:

MSC have to be plastic-adherent, express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules and, finally, they have to be able to differentiate at least into osteoblasts, adipocytes and chondroblasts *in vitro* (Dominici et al. 2006).

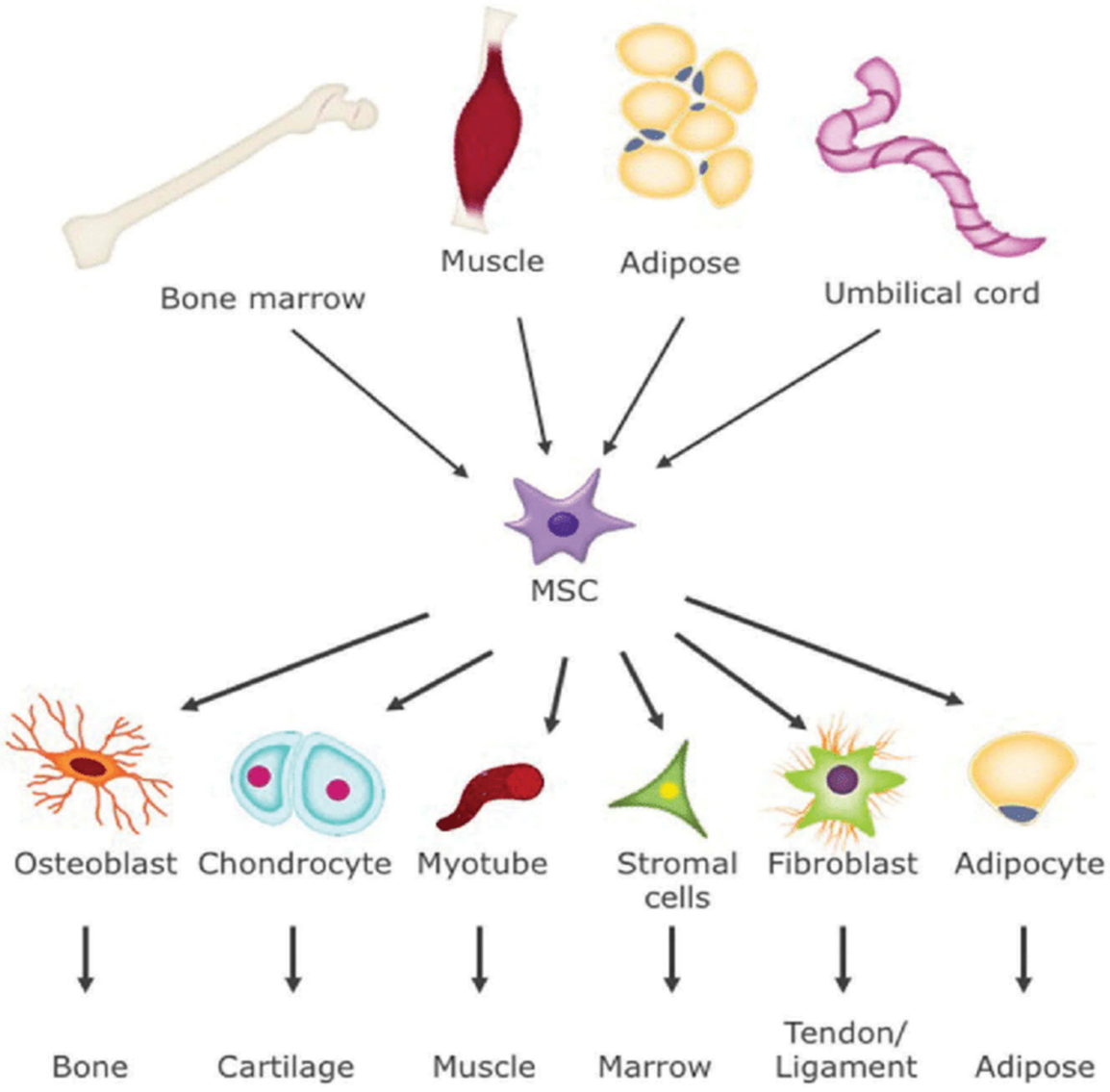


Figure 3: Sources and multilineage differentiation of MSCs.

2.3 Mesenchymal stem cells differentiation

MSCs can differentiate into mesodermal lineages such as osteoblasts, chondrocytes and adipocytes.

2.3.1 Adipogenesis

Adipogenesis, or the development of fat cells from preadipocytes, has been one of the most intensely studied models of cellular differentiation. The nuclear hormone receptor peroxisome proliferation activated receptor gamma, (PPARγ), is a critical adipogenic regulator that promotes MSC adipogenesis while repressing osteogenesis (Rosen et al., 2006). The bipotent co-regulator TAZ was recently discovered to function as a coactivator of Runx2 and as a co-repressor of PPARγ, thus promoting osteogenesis while blocking adipogenesis. A similar role is played by Wnt signaling and suppression of Wnt signalling is required for adipogenesis (Hong et al., 2005).

2.3.2 Chondrogenesis

Chondrogenic differentiation of MSCs in vitro mimics that of cartilage development *in vivo*. The transcription factor Sox-9 is considered the master gene of chondrogenesis and is responsible for the expression of extra cellular matrix (ECM) genes such as collagen types II and IX, aggrecan, biglycan, decorin and cartilage oligomeric matrix protein. However, the specific signaling pathways that induce the expression of these chondrogenic genes remain largely unknown. Naturally occurring human mutations and molecular genetic studies have identified several relevant signaling molecules, including various transforming growth factor β (TGF-β), bone morphogenetic protein (BMP), growth and differentiation factor, FGF and Wnt ligand (Griffin et al. 2012).

2.3.3 Osteogenesis

Osteogenesis is a complex and not entirely elucidated biological process regulated by intrinsic cellular signals and extrinsic micro-environmental stimuli from the surrounding stem cell-niche. Cells differentiate in a multi-step process in which the progression from one stage to the following is regulated by a complex network of growth factors, signaling molecules and hormones, and it is characterized by the activation and subsequent inactivation of transcription factors and the expression of bone specific marker genes (Fig.4).

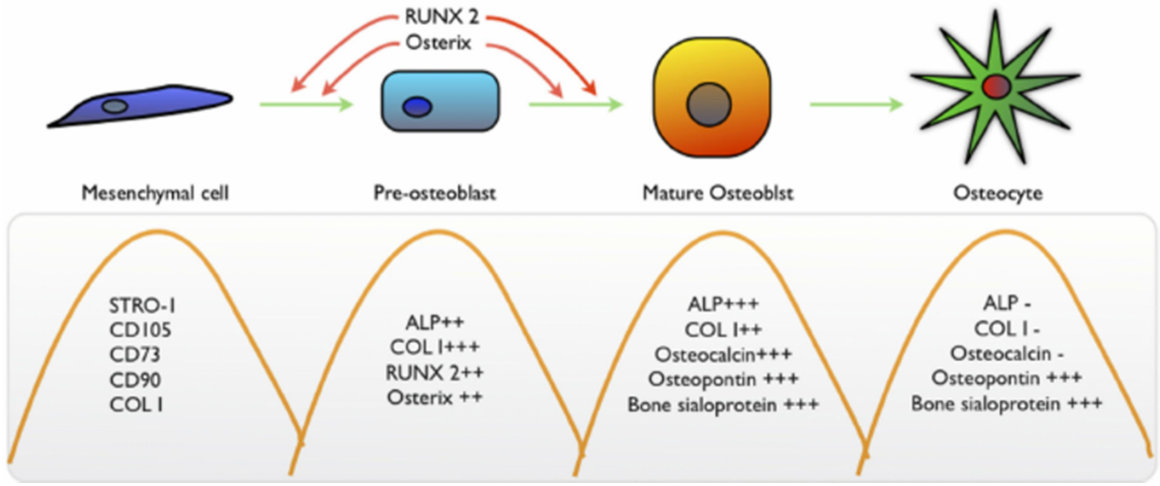


Figure 4: **Differentiation of MSCs towards osteoblasts.**

Regulators and markers of the different stages of osteogenic differentiation

In vitro experiments have shown that osteogenesis proceeds through three different steps:

- a proliferation phase, characterized by the proliferation of MSCs and pre-osteoblasts

- A differentiation phase, characterized by the differentiation of osteoprogenitors into osteoblasts, mature cells able to produce new bone matrix. During this stage the cells gain the expression of bone specific markers, as alkaline phosphatase (ALP)
- A final mineralization phase that represents the last step of osteogenesis. The osteoblasts start to produce calcium and phosphate mineralized deposits and to synthesize late-phase proteins, like osteocalcin

Although some determinants of osteogenesis have been already identified, further studies are required to better elucidate this biological process. Some of the most important regulators of osteogenesis, including growth factors and transcriptional factors, are described in this section (Fig.5).

Transcription factors: the earliest and most specific marker of osteoblast differentiation is Runt-related transcription factor (Runx)-2 or Core binding factor (Cbfa)-1, member of the RUNX family of transcription factors that stimulates MSCs commitment towards the osteogenic lineage and inhibits adipogenic differentiation. Indeed, Runx2 deletion in mice results in a skeleton constituted only by chondrocytes and in a complete lack of bone formation due to absence of osteoblasts (Ducy et al. 1997; Komori et al. 1997). Runx2 also plays an important role in mature osteoblasts and in matrix formation and mineralization, regulating the expression of osteoblast-specific genes, such as ALP, type I collagen, osteopontin and osteocalcin. However, its function in later stages of differentiation is less clear since Runx2 over-expression under the control of type I collagen promoter results in osteopenia due to the lack of terminal maturation of osteoblasts (Liu W et al. 2001). Osterix is a zinc finger transcription factor expressed by osteoblast and required for endochondral and intramembranous bone formation. Osterix null mice show normal cartilage development but fail to develop mineralized bone (Nakashima et al. 2002).

Osteoblast differentiation is arrested, and histological analysis reveals absence of trabecular bone. Furthermore, Osterix-null mice have reduced or absent expression of several bone matrix proteins, such as osteocalcin, ALP, type I collagen and osteopontin, confirming a key role of this transcription regulator in the induction of osteoblast differentiation. In contrast to Runx2 null mice (that do not form osteoblasts), osterix null mice form cells of the osteoblastic lineage expressing Runx2; however these cells fail to mature.

The Msh family of homeobox genes includes three transcription factors: Msx1, 2, and 3. Msx3 is expressed in the central nervous system while Msx 1 and 2 are expressed in skeletal tissue and modulate osteogenesis. Msx2 null mice show defects in skull ossification, enhanced in double Msx1/Msx2 mutants, and impaired chondrogenesis and osteogenesis due to a decreased number of osteoprogenitor cells (Satokata et al. 2000). The skeletal abnormalities are associated with a decreased expression of Runx2 indicating that Msx2 is necessary for osteogenesis and acts upstream of Runx2. Furthermore, Msx2 could play a role also in late differentiation events since it is able to repress the transcription of osteocalcin, a marker expressed late in osteoblast differentiation, binding directly its promoter (Dodig et al. 1999).

The mammalian homolog of *Drosophila* Distalless (Dlx)-5 is a homeobox gene essential for craniofacial and skeletal development. Indeed, targeted gene inactivation of Dlx5 results in severe skeletal abnormalities, whereas Runx2 expression is unaltered (Robledo et al. 2002). Dlx5 mRNA is expressed in osteoblasts after differentiation, concomitant with a decline in Msx2 mRNA and with the appearance of osteocalcin transcript, and it is able to induce the expression of ALP and osteocalcin and thereby the mineralization of the extracellular matrix (Miyama et al. 1999).

Growth Factors: Bone morphogenetic proteins (BMPs) are members of the Transforming Growth Factor (TGF) family of polypeptides (Wozney et al. 1988) and were originally identified for their ability to induce endochondral bone formation.

To date, around 20 BMP family members have been identified and characterized. BMPs signal explicates through serine/threonine kinase receptors composed of type I and type II subtypes. After ligand binding they form a heterotetrameric-activated receptor complex and interact with SMAD proteins, that play a key role in the signal transduction from the receptor to target genes in the nucleus. Furthermore, BMPs can activate SMAD-independent pathways, such as Ras/MAPK pathway (Fig.5). BMPs are able to increase commitment of bone marrow mesenchymal stem cells to osteoblasts phenotype by increasing Runx2 mRNA expression (Lee et al. 2002).

Wnt proteins are signalling proteins that act on target cells by binding to Frizzleds (Fzs), seven span transmembrane receptor proteins, and LRP-5/6, single span transmembrane co-receptor proteins. Wnt pathway is involved in BMPs-mediated osteogenesis (Chen et al. 2007), and is able to induce the osteoblastic transcription factors Runx2, Dlx-5, and Osterix, indicating that this pathway is intimately associated with bone regenerative process.

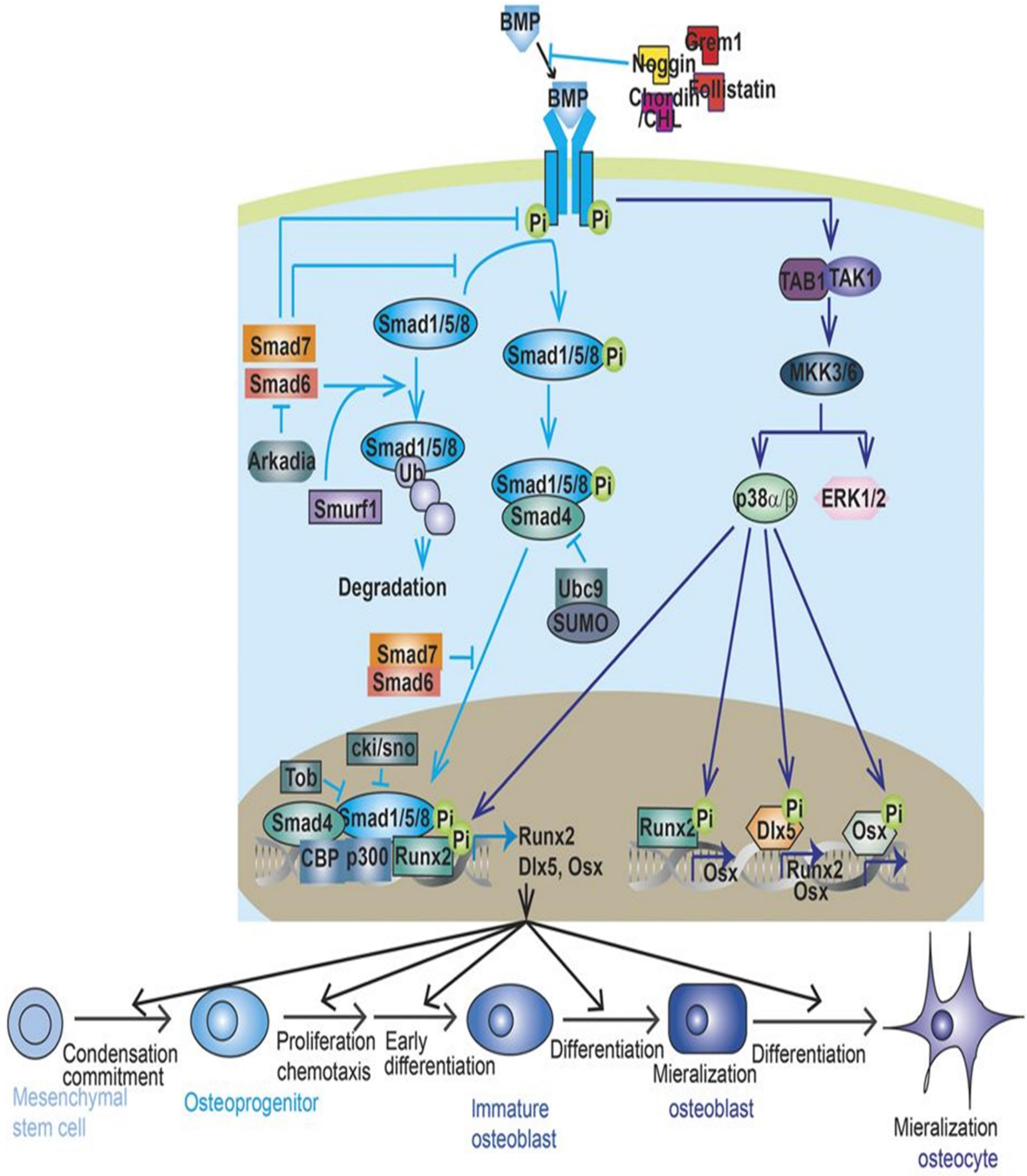


Figure 5: Signalling and transcription factors regulation in osteogenic differentiation. BMP and Wnt pathways induce osteogenic differentiation through the up-regulation of some osteo-specific transcription factors such as Runx2, Osterix, Dlx5 and Msx2.

2.3.4 Applications of MSCs in developmental research

MSCs are considered a promising source for cell-based therapy and regenerative medicine, since they can be extensively expanded *in vitro* retaining their undifferentiated phenotype and differentiation potential, they do not cause teratomas formation *in vivo* and are well-tolerated in clinical trials. Moreover, they show unique features that largely contribute to their attractiveness: MSCs are immune-privileged, have the ability to migrate to injured tissues after systemic administration, produce several trophic factors and are able to regulate inflammatory and immune responses. For these reasons MSCs were largely employed in preclinical and clinical studies to treat a broad spectrum of diseases, including auto-immune and inflammatory diseases, graft versus host disease, myocardial infarction and spinal cord injuries (Ren et al. 2012).

The MSCs potential to differentiate into several cell types has opened a variety of tissue engineering applications. In fact, undifferentiated or differentiated MSCs seeded into a variety of natural or synthetic biomaterial scaffolds have been successfully applied in repair of cartilage and bone defects (Ren et al. 2012).

Most interestingly, MSCs and their derived cell lines represent a valid *in vitro* differentiation system and a valuable tool for the identification of the molecular mechanisms underlying cellular differentiation; the identification of genes that regulate osteoblast differentiation, for instance, can directly provide new pharmacological targets for the induction of bone neosynthesis and, therefore, the treatment of bone-related pathologies, such as osteoporosis and bone fractures.

Recently a number of automated procedures for cell culture and manipulation have been developed (high-throughput screenings, HTS) making it possible to conduct a large number of tests with reduced manpower and more affordable costs. These screenings allow the identification of genes involved in a number of biological processes or

compounds able to alter them. Recently, RNA interference-based approaches have become particularly popular for their ability to silence specific genes and, therefore, study the effect of the absence of a single gene product in a particular biological phenomenon. In addition HTS can be used to screen compounds libraries to directly identify chemical compounds able to induce or block a biological process in order to discover potential drugs.

2.4 RNA interference

RNA interference (RNAi) is a form of post-transcriptional gene silencing mediated by a little double-strand molecule of RNA (dsRNA, double strand RNA) capable of blocking mRNA target molecules that will not be able to be translated.

This is a natural process preserved during the evolution and it is involved in several mechanisms:

- Protection against genome instability
- Defence against viral attacks
- Gene expression regulation
- Developmental regulation

RNAi can be activated by a double strand of RNA brought into the cell (siRNA, small interfering RNA) that leads to the degradation of mRNA target. RNA interference can be also activated by small noncoding transcripts synthesized within the cell (miRNAs, microRNA): in this case not always the mRNA target is degradated through cleavage.

Main effectors of RNA interference process are:

- **miRNA**: miRNA are short single-strand molecules of RNA of about 20-22 nucleotides with a 5'P Cap and 2 nucleotides 3' protruding. Their biogenesis starts in the nucleus: their corresponding genes are located in the intergenic regions and in transcriptional units both in the sense and anti-sense orientation.

  miRNA are first synthesized as primary miRNA (pri-miRNA) containing the 5' Cap and a polyadenilation sequence at 3'; then they are processed by Drosha RNA polymerase III that generates molecules of 70-80 nucleotides with a stem and loop structure, precursor miRNA (pre-miRNA). Pre-miRNAs are translocated from

nucleus to cytoplasm by a complex of receptors on the nuclear membrane, such as exportin5 (Bohnsack et al., 2004).

Pre-miRNAs are processed in miRNA by Dicer, a ribonucleasic enzyme of the RNA polymerase III family with two catalytic domains and one dsRNA-binding domain.

Each miRNA binds the enzymatic complex RISC (RNA-interference silencing complex), forming a RNA-protein complex named miRISC (Fig. 6).

Small RNA molecules are then separated: the sense strand is degraded while the antisense strand is driven toward the mRNA target by a RISC complex subunit, Argonaute 2 (Ago2) (Liu et al., 2004). If miRNA and mRNA target strands are highly complementary, RNA is cleaved; otherwise they are partly mismatched, RISC complex linked to mRNA does not allow the normal ribosomal translation. In both cases no protein is made.

- **siRNA**: siRNA are short molecules of double-strand RNA of 21-23 nucleotides with two phosphate groups at 5' and 2 nucleotides 3' protruding generated by Dicer. They can be introduced into the cells by both transfection and infection.

  The antisense strand binds the RISC complex forming the RNA-protein complex siRISC which recognize the mRNA target and cleave it (Fig. 6).

- **shRNA**: short hairpin RNA (shRNA) are small RNAs with a stem and loop structure: they mimic the pre-miRNA structure and so follow their same processing. shRNA are expressed by plasmids or viral vectors and for this reason they present the advantage to perform a stable silencing of their mRNA targets.

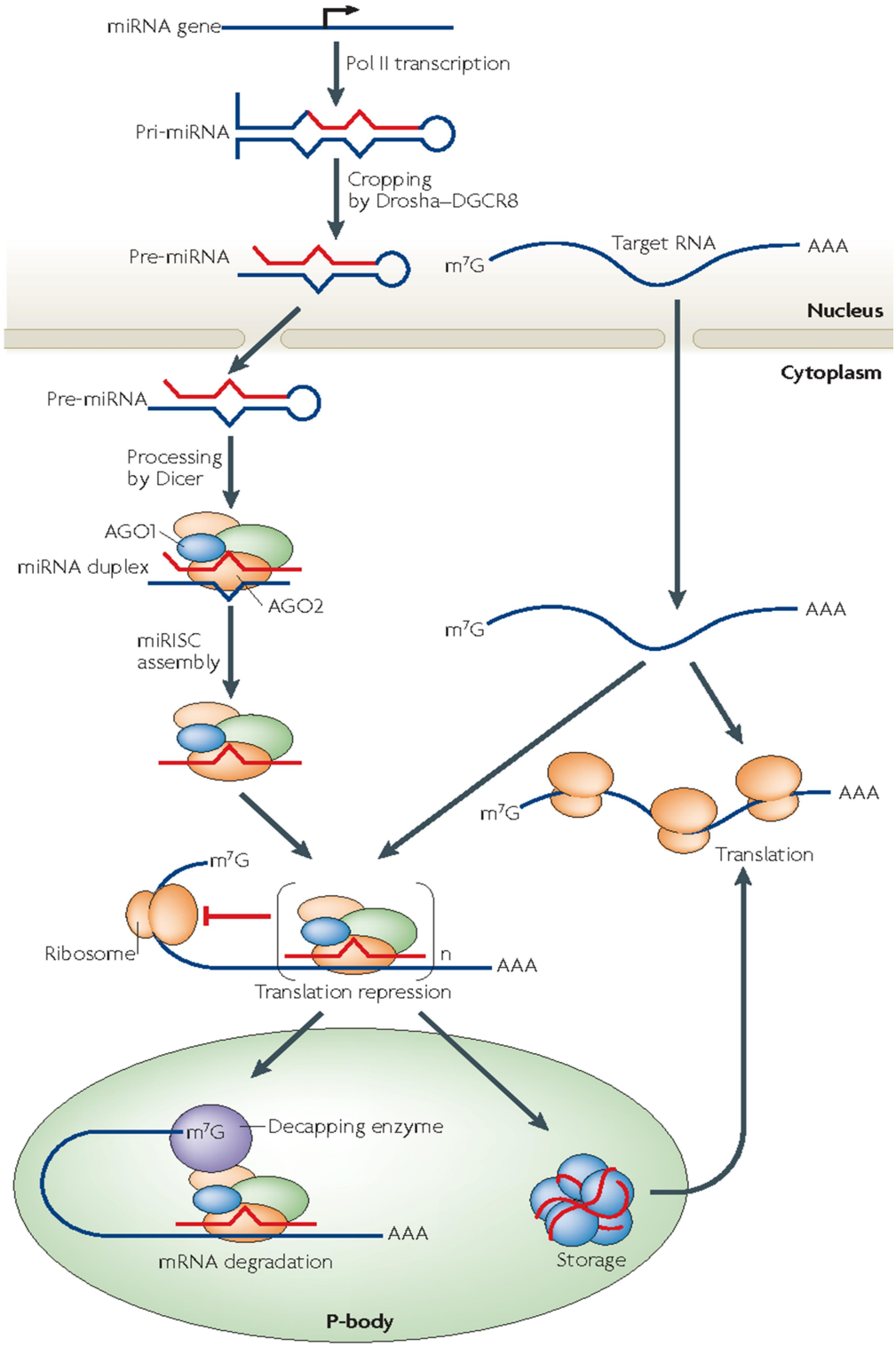


Figure 6: Functional model of the miRISC complex in human
miRNA are synthesized by RNA polymerase II in primary miRNA (pri-miRNA) of 60-100 nucleotides. Pri-miRNAs are cleaved by Drosha to form precursor miRNA (pre-miRNA) of 60-70 nucleotides containing 2 nucleotides 3' protruding. Pre-miRNAs are traslocated into cytoplasm by exportin5-RanGTP complex and by other adapter proteins. In cytoplasm, pre-miRNA are processed in short molecules (~22- nt), miRNAs, by Dicer. After, miRNA bind protein complex RISC containing several known proteins such as AGO2, AGO1, Dicer, TRBP e RCK and unknown proteins. RISC complex drive antisense strand towards mRNA target.

2.4.1 Off target effects

Off-target effects are defined as the consequences of unspecific interactions between RNAi molecules and cellular components or unrelated target mRNAs and can be classified in sequence-independent and sequence-dependent effects**.**

Sequence-independent effects include the transfection conditions and the inhibition of the activity of endogenous miRNAs that may be caused by RNA-interference machinery saturation, for example the saturation of exportin 5 (Yi et al. 2005).

Among the sequence-dependent effects there are immune response stimulation and interaction with no mRNA targets. The immune response can be provoked by type I interferons when siRNAs are longer than 30 base pairs or by the recognition of single or double strand RNA molecules by Toll-like receptors (TLRs). It is possible to reduce the interferon-mediated response avoiding the presence of some nucleotides motifs, such as GU traits, in the RNA sequence. However, the main cause of sequence-dependent effects is the interaction between RNA interference effectors and unspecific target mRNAs; in order to overcome this issue it is fundamental that the RNA-interference molecule has the minimal homology with mRNA sequences other than its target. In addition, it is recommended to perform redundancy or phenotype rescue experiments. In redundancy experiments different effectors having the same mRNA target are employed. In this way, the probability that at least two different effectors with distinct sequences share the same sequence-dependent off target effects is minor. In phenotype rescue experiments it is used a functional version of the target gene resistant to RNA interference effectors.

2.5 Ubiquitin system and mesenchymal stem cells

The proteins in a eukaryotic cell are subjected to a huge variety of post-translational modifications, which greatly extend the functional diversity and dynamics of the proteome. Proteins can be modified by attaching to small molecules such as phosphate groups, methyl groups or acetyl groups, or, usually only transiently, to certain proteins. The first such protein-based modification to be described was ubiquitination which has been extensively characterized. Ubiquitin is a small protein that is extremely well conserved among eukaryotes. Effect of ubiquitin-protein modification can differ and depends upon the attachment of either a monomer or polyubiquitin chains. Several ubiquitin like proteins have been identified including NEDD8, SUMO, ISG15 which share the same three-dimensional fold (Bedford L et al., 2011). When a substrate protein is coupled to a polyubiquitin chain, it binds to the 26S proteasome a large multisubunit protease complex that degrades the substrate into small peptides and recycles the ubiquitin tag. Proteasome are a large protein complex located in the cytoplasm and nucleus that degrade unneeded or damaged proteins by proteolysis (Jan-Michael P et al., 1994).

Ubiquitin-Proteasome (UPS) pathway has been identified as the main tool in cells for extralysosomal cytosolic and nuclear protein degradation and characterization of its multiple biological functions has paved the way to understand the regulation of basic cellular processes by controlled and limited proteolysis of cell proteins (Ciechanover A, 2006). UPS is responsible for the degradation and proteolytic processing of cell proteins essential for the regulation of development, proliferation, cell cycle, apoptosis, gene regulation, signal transduction, senescence, antigen presentation and stress response and thereby regulates the cellular processes.

Protein degradation by UPS takes place in a multistep process and requires three classes of enzymes (Fig. 7A): ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin-protein ligases (E3). E1 activates ubiquitin by forming a high-energy thiol ester bond between an E1 active site-located cysteine residue and the C-terminal glycine residue of ubiquitin in a reaction that requires the hydrolysis of ATP. This activated ubiquitin moiety is then transferred to one of the cell's approximately 30 E2s via the formation of an additional thiol ester bond and finally is transferred to one of hundreds of distinct E3s, which catalyze the covalent attachment of ubiquitin to the target protein by the formation of isopeptide bonds. A single E2 may function with multiple E3s to provide specificity in a combinatorial fashion. There are several mechanistically distinct classes of E3 enzymes, including the HECT (homologous to E6-associated protein carboxyl terminus) domain family, RING (really interesting new gene) finger proteins, and a U-box protein family. E3s are the key determinants of substrate specificity and are capable of recognizing a few or multiple substrates through specific degradation signals. Multiple cycles of ubiquitylation finally result in the synthesis and attachment of polyubiquitin chains that serve as a recognition signal for the degradation of the target protein by the 26S proteasome.

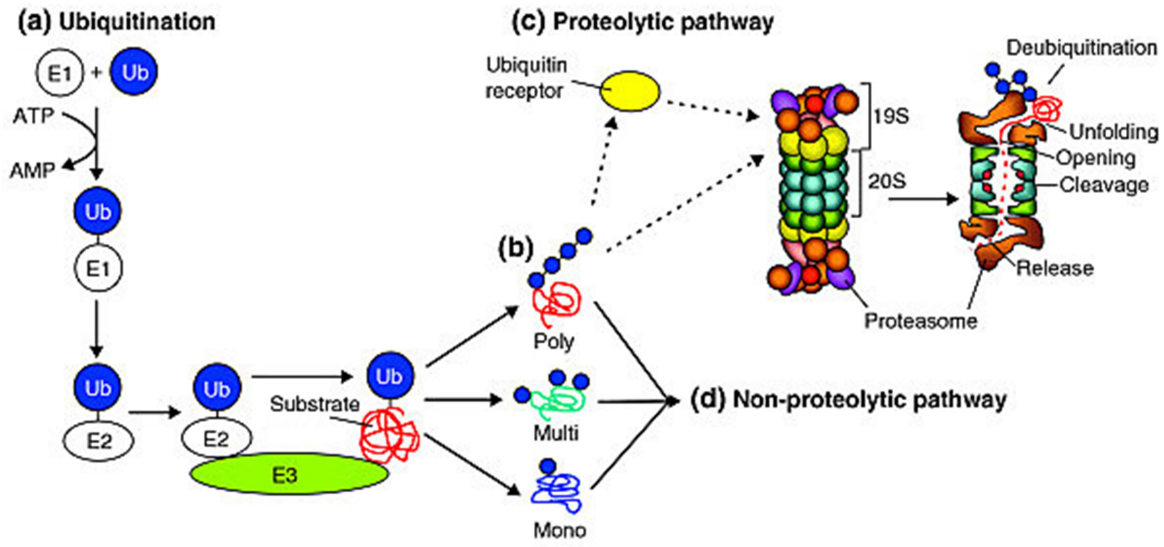


Figure7: The ubiquitin proteasome system.
(a) Ubiquitin is activated by an ubiquitin-activating enzyme (E1) and transferred onto substrate proteins by ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3), resulting in **(b)** either attachment of a single ubiquitin molecule (mono-ubiquitylation), attachment of multiple ubiquitin units to several substrate lysine residues on the same protein (multi-ubiquitylation) or synthesis of ubiquitin chains (poly-ubiquitylation). **(c)** Many poly- ubiquitylated proteins are subsequently degraded by the 26S proteasome, which consists of the catalytic 20S complex and the regulatory 19S particles. Degradation substrates are either delivered to the proteasome by soluble ubiquitin receptors or recognized by the intrinsic ubiquitin-binding activity of the 19S particle. At the 19S proteasome the ubiquitin chain is disassembled, and the substrate is unfolded before it can enter the cavity of the 20S subunit where proteolysis takes place. Finally, proteolytic fragments exit the proteasome in a poorly understood way. **(d)** Ubiquitylation can also directly regulate protein function in a proteolysis-independent manner, via mono-, multi- or poly-ubiquitylated proteins. (Kaiser and Huang., 2005).

Wnt/β-catenin, BMP and Notch pathways including transcription factors NFkB, Sp7/Osterix, and runt-related transcription factor 2 (Runx2) have been implicated in osteoblast differentiation, bone formation, and chondrogenesis (Satija NK et al., 2007), and the UPS has been recognized as an important regulator of these pathways. The UPS pathway interacts with these processes by regulating the stability of key signaling molecules and transcription factors in the cell. For example, β-catenin levels and its transcriptional activity are controlled by the UPS. The physiological consequence of proteasome inhibition is the accumulation of β-catenin in the cell followed by the dedifferentiation of chondrocytes. The stability of β-catenin is regulated by diverse extracellular Wnt ligands. In the absence of Wnt, intracellular β-catenin is constitutively phosphorylated and maintained at low levels by degradation in the UPS. PPARγ2 is a key element for the differentiation of MSCs into adipocytes and is also subject to regulation by the UPS. Several E3 ubiquitin ligases of the Smad ubiquitin regulatory factor (Smurf) family, such as Smurf1 and Smurf2, ubiquitylate specific Smad molecules and target them to the proteasome for degradation (Chen D et al., 2004) Smurf1 also recognizes bone-specific transcription factor Runx2 and mediates Runx2 degradation.

Ubiquitin mediated signaling and UPS has been shown in Paget's disease of bone (PDB) (Layfield R and Shaw B, 2007).

3. Material and Methods

3.1 Mouse shRNA library

shRNA library (Open Biosystems) consists in 64.000 bacterial stocks (PirPlus competent bacteria) containing plasmid DNA expressing shRNA constructs that cover the entire mouse transcriptome. Bacterial stocks are stored in single wells in 96 well plates in glycerol stock at -80° C.

The plasmid DNA is pShag Magic Version 2.0 (pSM2) retroviral vector (Open Biosystems). This plasmid contains the following features (Fig. 8):

1) Murine Stem Cell Virus (MSCV) backbone with Retroviral Signaling Sequences;

2) U6 promoter;

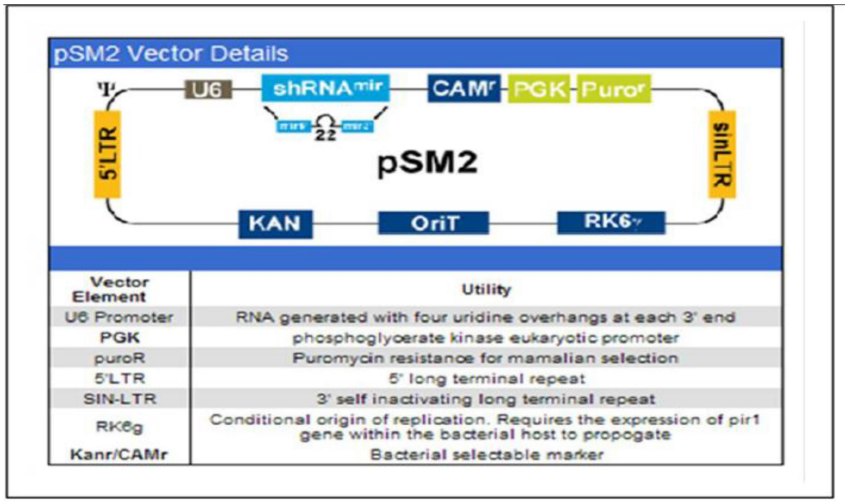
3) RK6γ conditional origin of replication. It requires the expression of Pir1 gene within the bacterial host to propagate;

4) Kanamycine and Chloramphenicol resistance as bacterial selection markers;

5) Puromycine resistance mammalian selection marker driven by the PGK promoter;

6) Sequence encoding the shRNA construct.

These shRNA expressing plasmids were grown in 2X LB medium for 14-16 hours at 37°C. After bacterial growth the plasmid DNA was purified with MIDI prep kit (Qiagen, Milan, Italy) according to the manufacturer's procedure. The purified plasmid DNA was used for further experiments.



Vector Element	Utility
U6 Promoter	RNA generated with four uridine overhangs at each 3' end
PGK	phosphoglycerate kinase eukaryotic promoter
puroR	Puromycin resistance for mamalian selection
5'LTR	5' long terminal repeat
SIN-LTR	3' self inactivating long terminal repeat
RK6g	Conditional origin of replication. Requires the expression of pir1 gene within the bacterial host to propogate
Kanr/CAMr	Bacterial selectable marker

Figure 8: pShag Magic Version 2.0 features.
Structure and functional elements of the DNA plasmid of the mouse shRNA library.

3.2 Cells and culture conditions

W20-17 cell line was obtained from American Type Culture Collection (ATCC, Cat. No. CRL-2623 /LGC standards, Milan, Italy). Primary mMSCs have been previously isolated from bone marrow of C57Bl/6 mice and characterized (Esposito et al. 2009). Human MSCs (hMSCs) were isolated from bone marrow of healthy adult donors after informed consent according to the procedure established by the local Bioethics Institutional Committee (Mariotti et al. 2008) mMSCs and W20-17 cells were seeded at a density of 6,000 cells/cm^2 in regular medium consisting of D-MEM (Euroclone, Siziano, Italy) supplemented with 10% FBS (HyClone, Northumberland, UK) and 4 mM L-glutamine (Gibco, Paisley, UK) in absence of antibiotics, and cultured for a limited number of passages (≤10-15). hMSCs were seeded at a density of 5,000 cells/cm2 and cultured in regular medium consisting of D-MEM supplemented with 20% FBS and 4 mM L-glutamine in absence of antibiotics for a limited number of passages (≤5). For osteoblastic differentiation, cells were grown until they reached 80-90% confluence and then cultured in osteogenic medium consisting of D-MEM with 10% FBS, 4 mM L-glutamine, 50 µM ascorbic acid 2-phosphate, 10 mM glycerol 2-phosphate and 1 µM dexamethasone (all from Sigma-Aldrich, St. Louis, MO, USA) for the times indicated in the Results section.

For adipogenic differentiation of W20-17 cells, cells were grown until they reached 80-90% confluence and then cultured in D-MEM with 10% FBS, 4 mM L-glutamine, 1 µM dexamethasone, 100 µM indometacin, 10 µg/mL insulin, and 0.5 mM 3-isobutyl-1-methylxanthine (all from Sigma-Aldrich, St. Louis, MO, USA) for 10 days.

3.3 shRNA-mediated screening

The high-throughput screening procedure used to identify novel genes potentially relevant for osteoblast differentiation has been previously described (Querques et al. 2015). In brief, W20-17 cells were plated in 96-well plates and transfected with different small hairpin RNA (shRNA)-expressing plasmids (Open Biosystem, Huntsville, AL, USA) using Lipofectamine 2000 (Invitrogen, Paisley, UK) according to manufacturer's instructions. Cells were then cultured in osteogenic differentiation medium for 21 days and mineral deposition was evaluated with alizarin red staining. Genes that, when silenced, impaired the ability of W20-17 cells to produce a mineralized matrix were considered potential candidate genes and further analyzed.

3.4 Alkaline phosphatase (ALP) staining

For ALP staining, cells were washed with PBS and fixed in 10% cold neutral formalin buffer (10% formalin, 0.1M Na2HPO4, 0.029M NaH2PO4 (Sigma-Aldrich, St. Louis, MO). Cells were then rinsed with distilled water and stained with a substrate solution containing 0.24 mM naphthol AS MX-PO4 (Sigma-Aldrich, St. Louis, MO), 0.4% N,N-dimethylformamide (Sigma-Aldrich, St. Louis, MO), 1.6 µM red violet LB salt (Sigma-Aldrich, St. Louis, MO S) in 0.2 M Tris-HCl pH 8.3 (Carlo Erba Reagenti, Milano, Italy) for 45 minutes at room temperature. Excess staining was removed washing twice with distilled water and ALP-positive cells visualized under microscope.

3.5 Alizarin Red-S staining

For alizarine red staining, cells were washed with PBS and fixed in 10% formaldehyde (Sigma-Aldrich, St. Louis, MO) for 1 hour; after rinsing with distilled water, they were incubated with 2% alizarin red S (Sigma-Aldrich, St. Louis, MO) at pH 4.1 for 10 minutes. Excess staining was removed using water. For W20-17 cell cultures, after visual examination the mineralized deposit-bound dye was extracted adding 100 µl of 4 M guanidine–hydrochloric acid (Sigma-Aldrich, St. Louis, MO) and incubating them over night at room temperature. A semi-quantitative assay was used to determine the amount of cell-bound dye assessing absorbance at 490 nm of a 10-times dilution of the resulting supernatant.

3.6 Lipofection

Cells were plated in the plastic support, allowed to grow until they reach the optimal confluence for transfection (80-90%) and then transfected with shRNA plasmids using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Co-transfection with a plasmid for the expression of a reporter gene is performed to supervise transfection efficiency. Two days after transfection, the cells were culture in selective medium containing puromycin in order to enrich for cells that incorporated the plasmid DNA. Puromycin amount was established on the basis of kill-curve experiments (3 µg/ml for W2017, 2 µg/ml for primary mMSCs). The medium was replaced every 2 days. A plate of untransfected cells was used as a control for the enrichment.

3.7 RNA extraction

Total RNA was extracted from cells using TriReagent (Sigma-Aldrich) according to manufacturer's instructions. C57Bl/6 mice were euthanized with carbon dioxide vapors and organs and tissues were immediately collected in ice and homogenized in TriReagent.

3.8 Real-time PCR

cDNAs were amplified from 2 µg of RNA using M-MuLV reverse transcriptase (New England Biolabs). RNA was incubated with dNTPs (Amersham Biosciences) and Random Examers (Promega) at 70°C for 10 minutes and in ice for 1 minute; then, after the addition of M-MuLV and the specific buffer, the incubation was performed at 42°C for 1 hour followed by enzyme denaturation at 90°C for 10 minutes.

Real time PCR was performed using the SYBR Green PCR master mixture in an Applied Biosystems 7900HT apparatus (Applied Biosystems). Levels of target genes were quantified using specific oligonucleotide primers and normalized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

3.9 Construction of a plasmid for the expression of ObI-1 with a C-terminal Flag

RNA was extracted from mouse lungs and retro-transcribed in cDNA as described before. The following primers were used to amplify ObI-1 (≈2 Kb): Forward (BamHI restriction site-containing) 5'- GGCCACGGGATCCAGCCATGGTGAGAAGACG-3' and Reverse (NotI restriction site-containing) 5'GGGCTCGCGGCCGCCAAATCCCAGATAGGGTTTG -3'. We used for PCR amplification Phusion Taq polymerase (New England Biolabs, Euroclone, Pero, Italy) with the following protocol: 98°C for 3 min, 15 cycles at 98°C for 1 min, 49°C for 20 sec and 72°C for 2 min, 5 cycles at 98°C for 1 min, 60°C for 20 sec and 72°C for 2 min, 15 cycles at 98°C for 1 min and 72°C for 2 min and a final extension at 72°C for 10 min.

The amplified product was resolved by agarose gel electrophoresis (1% agarose with 0.5 µg/ml ethidium bromide, Sigma-Aldrich, St. Louis, MO, USA); the band was purified using Wizard SV Gel and PCR Clean-up system (Promega Italia Srl, Milano, Italy). PCR product and pcDNA3.1 vector (Invitrogen, Paisley, UK) were digested for 2 hours at 37°C with BamHI and NotI (New England Biolabs, Euroclone, Pero, Italy), resolved by agarose gel electrophoresis and extracted.

Vector dephosphorilation and ligation reactions were performed using the Rapid Dephosphorilation and Ligation kit (Roche, Milano, Italy) according to manufacturer's instructions. The ligation mixture was transformed into calcium chloride competent DH5α cells (Invitrogen, Paisley, UK). Plasmids that after BamHI-NotI digestion showed the presence of a 2 Kb insert were validated by sequencing (Ceinge Sequencing Service).

mMSCs were grown until they reached 90% confluence and then transfected with either ObI-1 Flag-expressing plasmid or pcDNA3.1 empty vector using Lipofectamine 2000 and, after 2 days, plated in selective media containing 400 µg/ml G418 (Gibco, Paisley, UK); the

medium was replaced every 3 days. A plate of untransfected cells was used as a control for the selection.

3.10 Immunofluorescence

mMSCs were grown until they reached 60-70% confluence and then transfected with either pcDNA3.1 expressing ObI-1 Flag or an empty vector using Lipofectamine 2000. After 24 hours, cells were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA), permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) and blocked in 10% BSA in PBS for 30 minutes at room temperature. The samples were incubated with primary anti-Flag antibody (mouse monoclonal, Sigma-Aldrich, Cat. No. F1804, 1:250 dilution) and then with an anti-mouse IgG conjugated with Alexa Fluor 488 (Molecular Probes, Cat. No. A-21202, Eugene, OR, USA, 1:500 dilution). Both antibodies were diluted in 3% BSA/PBS. Cells nuclei were counterstained with DAPI diluted 1:5,000 in PBS. Images were captured using a confocal microscope (LSM 510 Meta, Zeiss).

3.11 BrdU incorporation assay

mMSCs were plated at 50% confluence and the following day transfected with either stealth siRNAs against ObI-1 or scramble negative control siRNA (20nM, NS# 462001, 1# 291188A08, 2# 291188A10, Invitrogen, Paisley, UK) using Lipofectamine 2000 (Invitrogen, Paisley, UK) according to manufacturer's instructions. 24 hours after transfection, cells were cultured in regular media or complete osteogenic medium for the next 24 hours. Cell proliferation was then assessed performing a Bromo-2'-deoxyuridine (BrdU) incorporation assay (BrdU labeling and detection kit I, Roche) according to manufacturer's instructions; cells were incubated in the BrdU labeling medium for 40 minutes at 37˚C.

Cells nuclei were counterstained with DAPI diluted 1:5,000 in PBS and the percentage of BrdU positive nuclei was determined by counting 30 fields (10X magnification).

3.12 Proteasomal inhibition

mMSCs, stably transfected with either pcDNA3.1 empty vector or the ObI-1 Flag-expressing plasmid, were seeded at a density of 19,000 cells/cm2 and the following day treated with either DMSO (control) or proteasome inhibitor MG 132 (Sigma-Aldrich, St. Louis, MO, USA) at 5 or 10 µM concentration. After 6 hours, cells were lysed and protein collected for further analyses.

3.13 Cycloheximide chase assay

mMSCs were grown until they reached 70-80% confluence and then transfected with either pcDNA3.1 expressing ObI-1 Flag or the empty vector using Lipofectamine 2000. After 24 hours, cells were treated with either DMSO (control) or 10 µg/ml cycloheximide (Sigma-Aldrich, St. Louis, MO, USA) and proteins collected at different time points and subjected to Western Blot analysis.

3.14 BMP-4 stimulation analysis

mMSCs, stably transfected with either pcDNA3.1 empty vector or ObI-1 Flag-encoding plasmid, were seeded at a density of 19,000 cells/cm^2 and starved overnight in D-MEM supplemented with 2% FBS to reduce cell proliferation. The following day, cells were treated with 40 ng/ml recombinant BMP-4 (Sigma-Aldrich, St. Louis, MO, USA) and proteins collected at different time points and subjected to Western Blot analysis. Unstimulated cells were used as control.

3.15 BMPR-I inhibition analysis

mMSCs, stably transfected with either pcDNA3.1 empty vector or ObI-1 Flag-encoding plasmid, were grown until they reached 80-90% confluence and then were cultured in regular medium, osteogenic medium alone or supplemented with the BMPR-I antagonist LDN193189 (Sigma-Aldrich) at a concentration of 100 nM, chosen on the basis of previous reports (Boergermann et al 2010; Lee et al 2011). LDN193189 was added to the culture medium every 48 hours during osteogenic differentiation. After 14 days of differentiation, alizarin red staining was performed to evaluate mineral matrix deposition.

3.16 Western Blot assays

Total cell lysates were obtained by treatment with lysis buffer (1mM EDTA, 50 mM Tris-HCl, pH 7.5, 70 mM NaCl, 1% Triton X-100), protease inhibitors (Complete Protease Inhibitor Cocktail, Roche, Milano, Italy) and phosphatase inhibitors sodium fluoride (50 mM) and sodium orthovanadate (1 mM) (Sigma-Aldrich, St. Louis, MO, USA). Proteins were quantified with the Bradford solution according to manufacturer's instructions (AppliChem, Darmstadt, Germany).

Protein extracts were electrophoresed on 10% SDS-PAGE gels transferred on Immuno-Blot PVDF membranes (Bio-Rad, Hercules, CA, USA) and analyzed using the following antibodies: anti-Flag (mouse monoclonal, Sigma-Aldrich, Cat. No. F1804, 1:1,000 dilution), anti-GAPDH (mouse monoclonal, Santa Cruz Biotechnology, Cat. No. sc-32233, CA, USA, 1:1,000 dilution), anti-Ubiquitin (Ub, rabbit polyclonal, Santa Cruz Biotechnology, Cat. No. sc-9133, 1:700 dilution), anti-p53 (rabbit polyclonal, Santa Cruz Biotechnology, Cat. No. sc-6243, 1:1,000 dilution), anti-pSmad1/5/8 (rabbit monoclonal, Cell Signaling, Cat. No. 9516, Danvers, MA, USA, 1:1,000 dilution), anti-Smad1 (rabbit polyclonal, Cell Signaling, Cat. No. 9743, 1:1,000 dilution), anti-HA (mouse monoclonal, Santa Cruz Biotechnology,

Cat. No. sc-7392, 1:1,000 dilution) and secondary HRP-conjugated antibody (Amsterdam Bioscience, Uppsala, Sweden, 1:5,000 dilution). Proteins were detected by chemiluminescence (ECL or ECL plus, GE Healthcare, UK). Quantitative analysis was performed using ChemiDoc (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. pSmad1/5/8 levels were normalized to Smad1 levels and results are expressed as fold change of normalized pSmad1/5/8 levels in BMP4-treated cells at different time points versus untreated cells.

3.17 Immunoprecipitation

mMSCs, stably transfected with the ObI-1 Flag expressing vector or pcDNA3.1 empty vector, were grown until they reached 90% confluence and then transiently transfected with HA-Ubiquitin-encoding pcDNA3 plasmid (HA-Ub). HA-Ub (Addgene plasmid # 18712) was a gift from Edward Yeh (Kamitani et al 1997). Twenty-four hours after transfection cells were treated with a 5 µM concentration of MG 132. Six hours after treatment cell extracts were collected and subjected to Western blot analysis. HA-Ub-untransfected mMSCs and mMSCs treated with either DMSO or MG 132 were included as controls. 1.5 mg of protein lysate was subjected to immunoprecipitation (IP). A pre-clearing step with an irrelevant antibody (mouse IgG1 isotype control, Abcam, Cat. No. ab81032, Cambridge, UK) was performed to reduce non-specific binding. Cell lysates were then incubated with 3.75 µg of anti-Flag antibody (Sigma-Aldrich) for 3 hours at 4°C and with protein A/G-agarose (Santa Cruz Biotechnology, Cat. No. sc-2003, CA, USA) overnight at 4°C. The following day, samples were washed 3 times and analyzed by Western blot as previously described.

4 Results and Discussion

4.1 Identification of genes involved in osteoblast differentiation with an shRNA-based approach

In order to identify genes that regulate osteoblast differentiation, we have used single clones from a library of shRNA-expressing plasmids able to silence specific murine genes in the multipotent murine cell line W20-17. With this approach, we identified 650 candidate genes that, when silenced, impair the ability of W20-17 cells to differentiate in osteoblasts and produce mineralized matrix (Querques et al. 2015). In-silico analysis of the data obtained from screening results was performed using bioinformatics tools such as GENECODIS to obtain gene ontology classification of the candidate genes on the basis of their function. About 30% of these genes identified had no known function at the time of the screening, and many of them belonged to Riken collection.

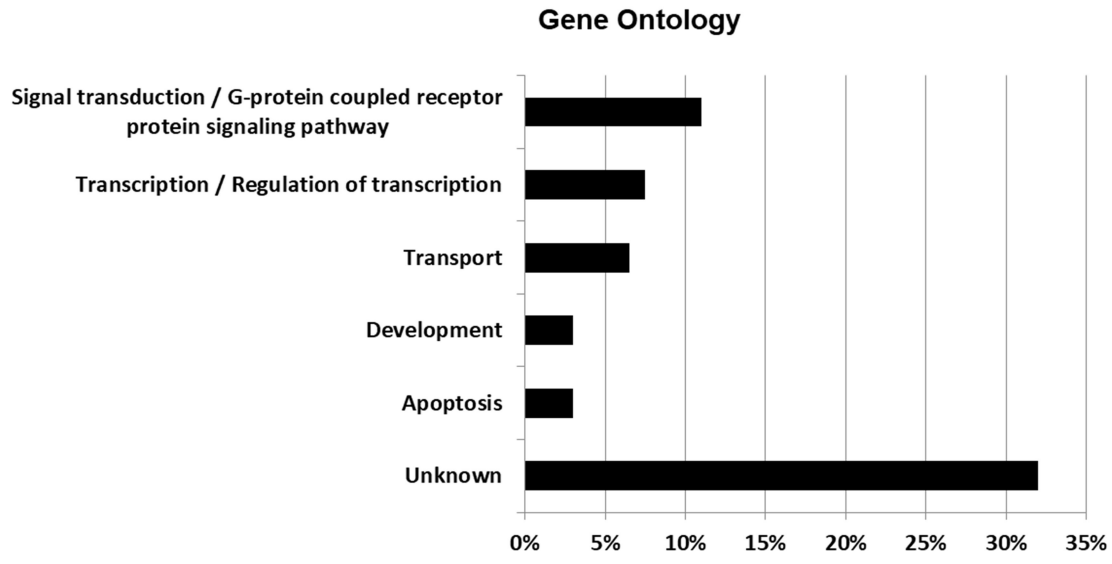


Figure 9: Gene Ontology classification of screening candidate genes.
Results of Gene Ontology classification of the candidate genes from the first part of our screening.

4.2 Osteoblast Inducer-1 (Obi-1) is necessary for osteoblast differentiation of MSCs

4.2.1 In silico characterization of ObI-1 and phylogenetic analysis

Among the identified genes with unknown function, we focused on the characterization of a previously undescribed gene (that we named ObI-1), and its role in osteogenic differentiation.

In silico analysis was performed to better understand ObI-1 structure and function. ObI-1 (A430033k04Rik) belongs to the Riken family of transcripts and is predicted to encode for 2 short and 2 long isoforms originating from alternative splicing.

The shortest isoforms are predicted to be approximately 100 aa in length (Fig. 10B and C) and contain a Kruppel-associated box (KRAB) domain usually associated to transcriptional repression (Urrutia R. 2003). The long isoforms contain, in addition to the amino-terminal KRAB domain, several zinc-finger domains at the C-terminus (Fig. 10D and E). The presence of both an effector and a DNA binding domain suggests that ObI-1 long isoforms could play a role as transcriptional regulators.

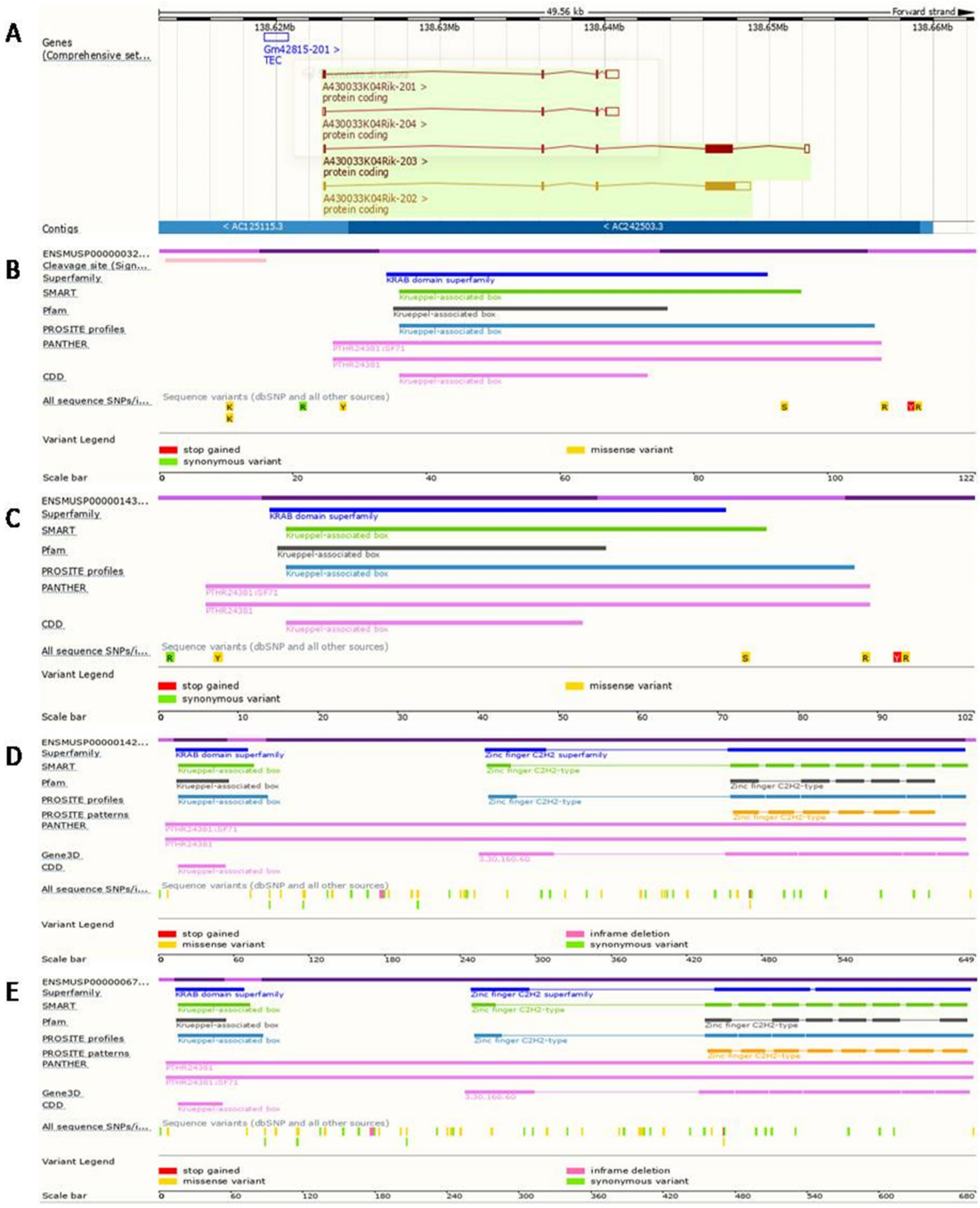
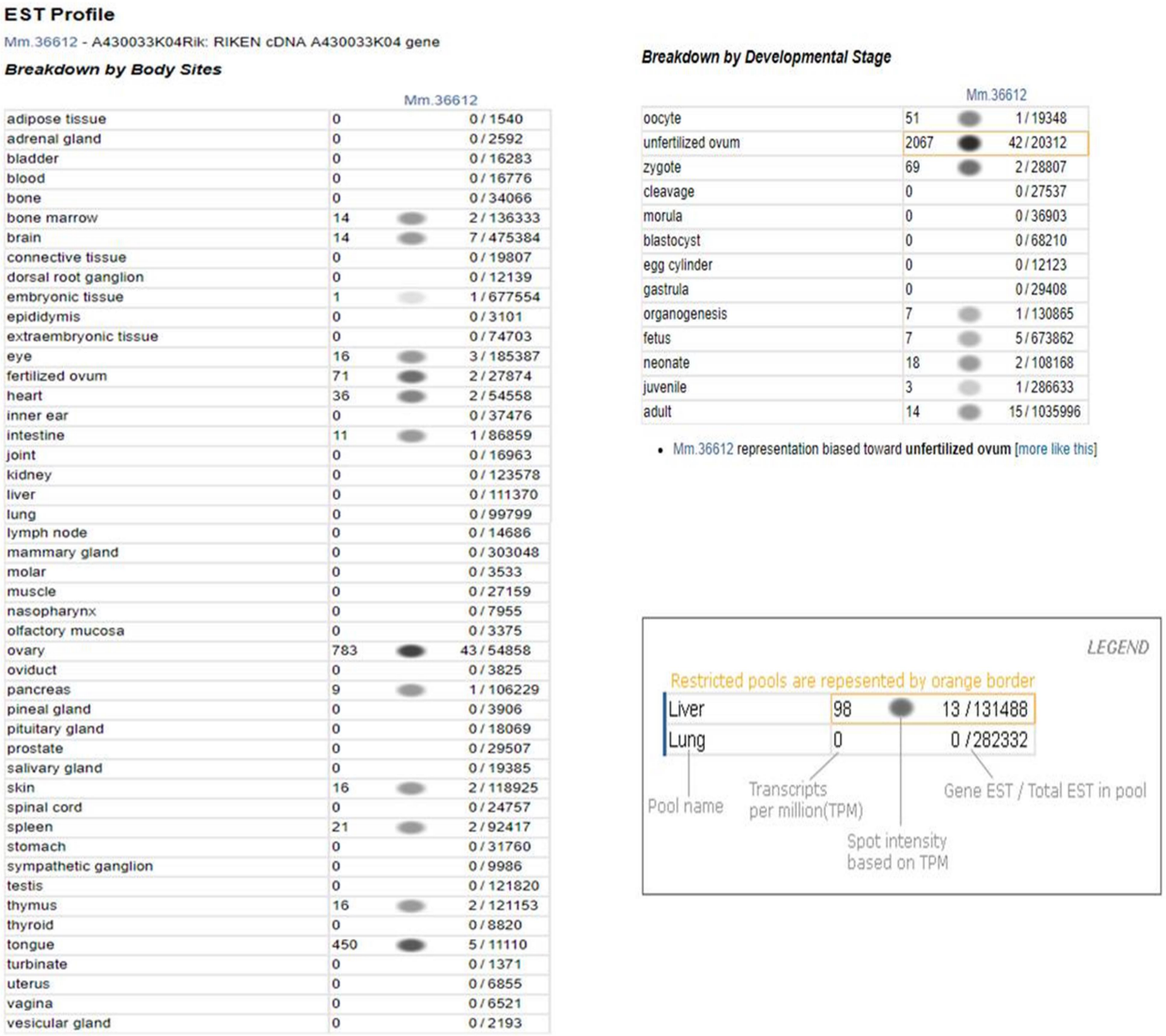


Figure 10: ObI-1 predicted transcripts and proteic domains.
Bioinformatic analysis (Ensembl) of ObI-1 transcripts (A). Bioinformatic analysis to evaluate the presence of functional domains in ObI-1 proteins: the short isoforms presents a KRAB domain (B, C); whereas the two long isoforms contain a KRAB domain and several Zinc finger domains (D, E).

EST profiles (**Expressed Sequence Tag**) were evaluated and revealed ObI-1 expression in several murine adult tissues as well as during embryonic development.



EST Profile

Mm.36612 - A430033K04Rik: RIKEN cDNA A430033K04 gene

Breakdown by Body Sites

	Mm.36612	
adipose tissue	0	0 / 1540
adrenal gland	0	0 / 2592
bladder	0	0 / 16283
blood	0	0 / 16776
bone	0	0 / 34066
bone marrow	14	2 / 136333
brain	14	7 / 475384
connective tissue	0	0 / 19807
dorsal root ganglion	0	0 / 12139
embryonic tissue	1	1 / 677554
epididymis	0	0 / 3101
extraembryonic tissue	0	0 / 74703
eye	16	3 / 185387
fertilized ovum	71	2 / 27874
heart	36	2 / 54558
inner ear	0	0 / 37476
intestine	11	1 / 86859
joint	0	0 / 16963
kidney	0	0 / 123578
liver	0	0 / 111370
lung	0	0 / 99799
lymph node	0	0 / 14686
mammary gland	0	0 / 303048
molar	0	0 / 3533
muscle	0	0 / 27159
nasopharynx	0	0 / 7955
olfactory mucosa	0	0 / 3375
ovary	783	43 / 54858
oviduct	0	0 / 3825
pancreas	9	1 / 106229
pineal gland	0	0 / 3906
pituitary gland	0	0 / 18069
prostate	0	0 / 29507
salivary gland	0	0 / 19385
skin	16	2 / 118925
spinal cord	0	0 / 24757
spleen	21	2 / 92417
stomach	0	0 / 31760
sympathetic ganglion	0	0 / 9986
testis	0	0 / 121820
thymus	16	2 / 121153
thyroid	0	0 / 8820
tongue	450	5 / 11110
turbinate	0	0 / 1371
uterus	0	0 / 6855
vagina	0	0 / 6521
vesicular gland	0	0 / 2193

Breakdown by Developmental Stage

	Mm.36612	
oocyte	51	1 / 19348
unfertilized ovum	2067	42 / 20312
zygote	69	2 / 28807
cleavage	0	0 / 27537
morula	0	0 / 36903
blastocyst	0	0 / 68210
egg cylinder	0	0 / 12123
gastrula	0	0 / 29408
organogenesis	7	1 / 130865
fetus	7	5 / 673862
neonate	18	2 / 108168
juvenile	3	1 / 286633
adult	14	15 / 1035996

- Mm.36612 representation biased toward **unfertilized ovum** [more like this]

LEGEND

Restricted pools are repesented by orange border

Liver	98	13 / 131488
Lung	0	0 / 282332

Pool name

Transcripts per million(TPM)

Spot intensity based on TPM

Gene EST / Total EST in pool

Figure 11: In silico analysis of ObI-1 expression.
EST profile of ObI-1 transcript in different mouse tissues and developmental stages. (https://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Mm.36612)

We performed also a phylogenetic analysis of the murine ObI-1 gene to identify potential orthologous genes in other species using MetaPhOrs, a public repository of phylogeny-based orthology and paralogy predictions computed using resources available in seven different homology prediction services (Pryszcz et al. 2011). Potential ObI-1 orthologs were identified in several species including Human (Homo sapiens), Macaque (Macaca mulatta), Orangutan (Pongo abelii) and Rat (Rattus Norvegicus). In particular, the predicted human ortholog corresponds to the zinc finger protein-717 (ZNF717) gene. Bioinformatic analysis to evaluate the presence of functional domains was performed also for ZNF717 and this gene is predicted to encode for 7 transcripts originating from alternative splicing. Similarly to ObI-1, ZNF717 contains KRAB and zinc-finger domains.

4.2.2 Obl-1 silencing impairs formation of mineralized deposits

In order to ensure the specificity of the data and to overcome off-target effects, we confirmed the screening results using two additional shRNA constructs interfering with Obl-1 gene available in the library; these shRNAs recognize different regions of Obl-1 mRNA, thereby minimizing the possibility of sequence-dependent off-target effects. Indeed, in RNA-interference experiments, a molecule can cause off-target effects, independently by its activity on the putative mRNA target (Jackson and Linsley 2010). However, since we obtained the same results with different RNA molecules interfering with the same gene, we could exclude off-target effects.

We therefore, stably transfected W20-17 cells with 3 independent shRNAs that recognize different region of the Obl-1 transcript, and observed an almost complete absence of matrix mineralization, as assessed by alizarin Red staining after 21 days of osteogenic differentiation (Fig. 12A-E).

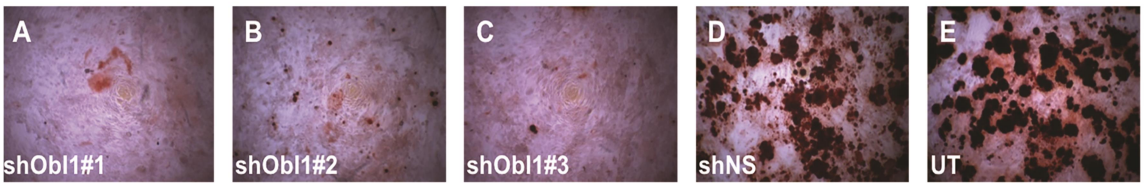


Figure 12: Obl-1 gene silencing impairs osteoblast differentiation.
W20-17 cells transfected with 3 different shRNAs against Obl-1 transcript, (**A**) shObl1#1, (**B**) shObl1#2 and (**C**) shObl1#3, show a reduction of alizarin red staining compared to (**D**) cells transfected with non-silencing shRNA (shNS) and (**E**) untransfected (UT) cells 21 days after differentiation induction (magnification 10X).

Staining quantification showed a significant reduction of mineralized deposits in ObI-1 silenced cells compared to untransfected cells and W20-17 cells transfected with shNS, used as controls (Fig. 13a). Effective reduction of ObI-1 levels were confirmed by Real Time PCR analysis (Fig. 13b).

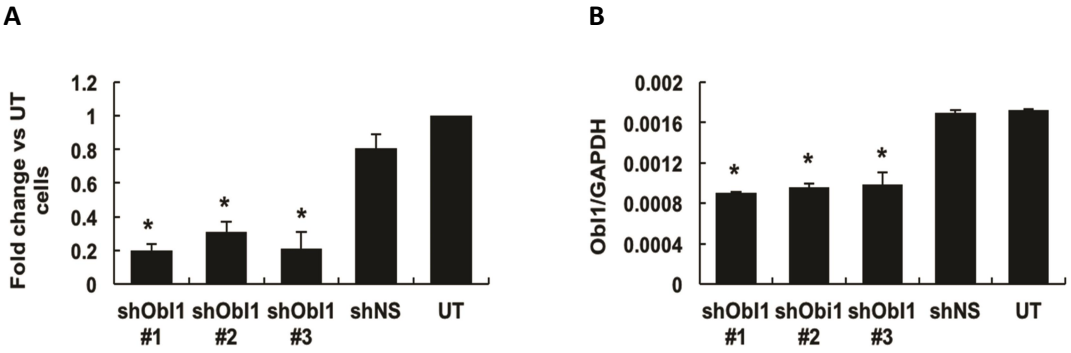


Figure 13: ObI-1 gene silencing impairs osteoblast differentiation.
Alizarin red dye quantification confirms microscopy data that mineral deposition is significantly impaired in ObI-1 silenced cells (A). Effective silencing has been confirmed by Real-time PCR analysis that shows a significant decrease in ObI-1 transcript levels in W20-17 cells transfected with shRNAs against ObI-1 compared to control cells (B). Results are expressed as ObI-1/GAPDH levels ratio.

4.2.3 ObI-1 silencing affects osteogenic differentiation in primary mMSCs

Next we evaluated ObI-1 involvement in osteogenic regulation also in primary mMSCs. As observed in W20-17 cells, ObI-1 silencing significantly impairs the ability of cells to produce mineralized nodules 18 days after osteogenic induction (Fig. 14E-H). Subsequently we evaluated ALP (Alkaline Phosphatase) expression in order to better characterize the phase in which ObI-1 function is crucial for correct osteogenic differentiation of mMSCs. ALP is a protein expressed by mature osteoblasts that promotes extra-cellular matrix mineralization and is considered an intermediate marker of osteogenic differentiation. ObI-1 silencing resulted in a reduced expression of ALP, as assessed by ALP staining after 12 days of differentiation (Fig.14A-D). This finding indicates that ObI-1 is not involved only in the mineralization phase, but that osteoblasts differentiation and/or proliferation of osteogenic precursors may be dependent on the expression of this gene.

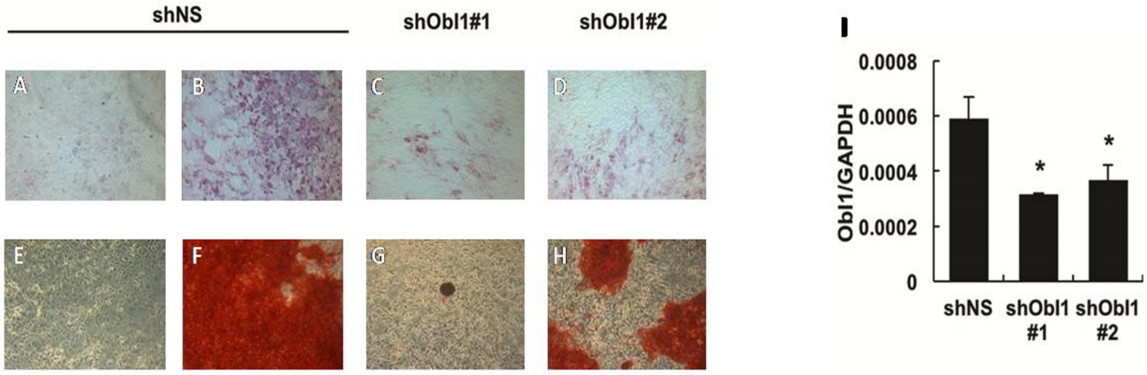


Figure 14: ObI-1 silencing impairs osteogenic differentiation of mMSCs
cells transfected with (**C** and **G**) shObI1#1 and (**D** and **H**) shObI1#2 and cultured with osteogenic medium show a reduction of ALP staining (**C** and **D**) after 12 days as well as a decrease of mineral deposition (**G** and **H**) assessed by alizarin red staining after 18 days of differentiation. mMSCs cells transfected with shNS and cultured with regular (**A** and **E**) and osteogenic (**B** and **F**) medium were used as controls. Magnification 10X. Pictures are representative of 3 independent experiments. (**I**) ObI1 silencing efficacy was confirmed by Real-time PCR analysis: ObI-1 transcript levels are significantly reduced in mMSCs transfected with shObI1#1 or shObI1#2 compared to cells transfected with shNS. Results are expressed as ObI-1/GAPDH levels ratio.
*$p<0.05$.

4.2.4 ObI-1 levels increase during osteogenic differentiation of W20-17 cells and mMSCs

In order to investigate ObI-1 expression during osteogenic differentiation of mMSCs Real Time PCR analysis of the 4 transcript variants was performed. Real-time PCR analysis in W20-17 cells indicates that the ObI-1 long isoform (A430033K04Rik-002) transcript levels increase from the first days of differentiation and remain high during the whole process. On the other hand, both the short forms and the other long isoform (A430033K04Rik-001) were almost undetectable, indicating that they may represent only a bioinformatic prediction or they are tissue-specific isoforms not expressed in MSCs nor involved in osteogenic differentiation (Fig. 15A). Therefore, these isoforms were not further investigated.

Subsequently, ObI-1 long form (A430033K04Rik-002) expression was analyzed also during osteogenic differentiation of primary murine MSCs, isolated from bone marrow. Similar expression profile was observed as compared to W20-17 cells; wherein the levels were low at T0 and increased at T3 with the highest expression at T21 (Fig. 15B).

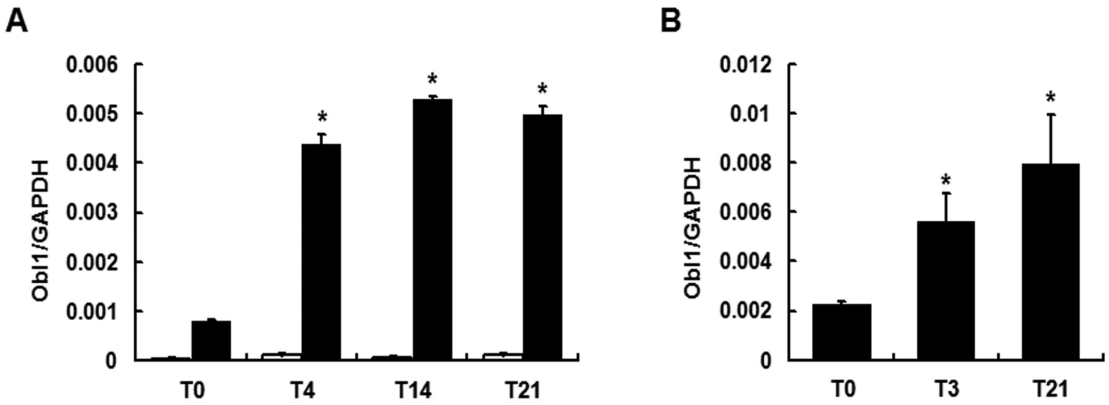


Figure 15: Expression analysis of ObI-1 during osteogenic differentiation.
Expression profile of ObI-1 transcripts during osteogenic differentiation of W20-17 cells at day 0, 4, 14 and 21 (A) and of mMSC cells at day 0, 3 and 21 (B). Data are means ± SD of 3 experiments.

Moreover, ObI-1 transcription appears to be selectively up-regulated during osteogenic differentiation of MSCs compared to other differentiation fates, as its transcript levels decrease during adipogenic differentiation of W20-17 cells (Fig.16).

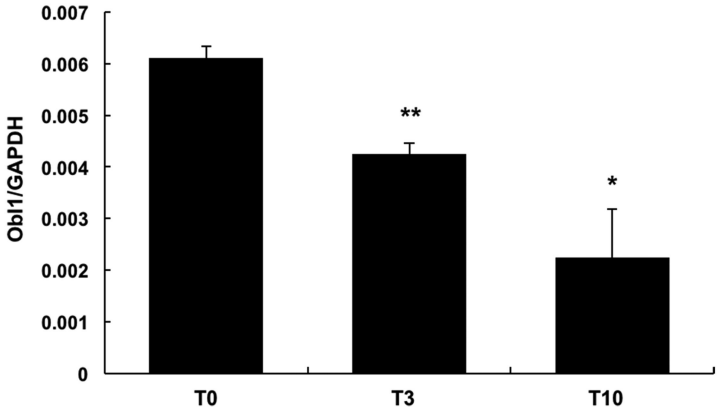


Figure 16: ObI-1 expression decreases during adipogenic differentiation.
Expression profile of ObI-1transcript during adipogenic differentiation of W20-17 cells at day 0, 3, and 10. ObI-1 levels significantly decrease from day 3. Results are expressed as ObI-1/GAPDH levels ratio. *p< 0.01; **p<0.001

Interestingly, we observed that ObI-1 expression is not restricted to osteoblasts; Real-time PCR analysis in several murine tissues showed a broad expression pattern since ObI-1 transcript could be detected at variable levels in all the tissues examined. Surprisingly, we observed the highest levels of expression in lung (Fig.17). This finding confirms previous EST data and suggests that ObI-1 could play a role in different biological processes in a number of tissues.

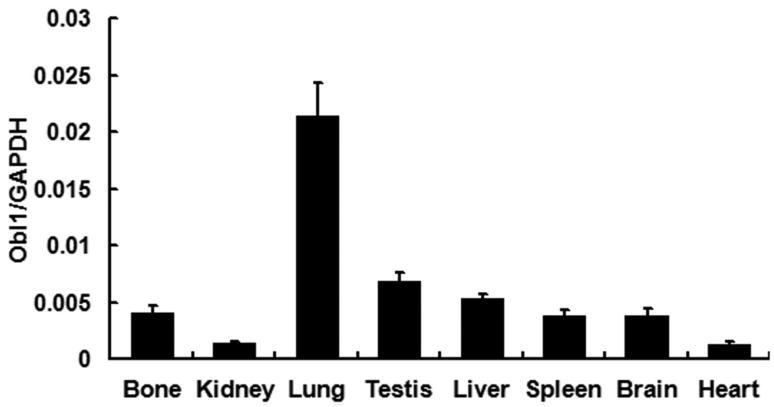


Figure 17: Expression analysis of ObI-1 transcript in tissues
Real-time PCR analysis shows that ObI-1 is expressed in several mouse tissues and organs, with highest levels in lung. Results are expressed as ObI-1/GAPDH levels ratio (*p<0.05).

4.2.5 ObI-1 silencing affects expression profile of osteogenic differentiation markers

To study the effect of ObI-1 silencing on bone differentiation markers, real time PCR was performed on either shNS or ObI-1 silenced cells and expression of Runx2 and osteopontin (OPN) was analyzed throughout the differentiation process. Indeed, the transcription factor Runx2 is an early marker of osteogenesis and it is absolutely required for the commitment of MSCs towards the osteogenic lineage as well as for the function of mature osteoblasts, whereas OPN is a bone matrix sialoprotein expressed throughout osteogenic differentiation, from the first days of the process, reaching a peak during the mineralization phase (Ducy et al. 1997; Komori et al. 1997; Chen et al. 1993). We found that the transcript level of both the markers was significantly reduced in ObI-1 silenced cells while, in cells transfected with shNS, the transcript level was comparable to untransfected cells (Fig.18).

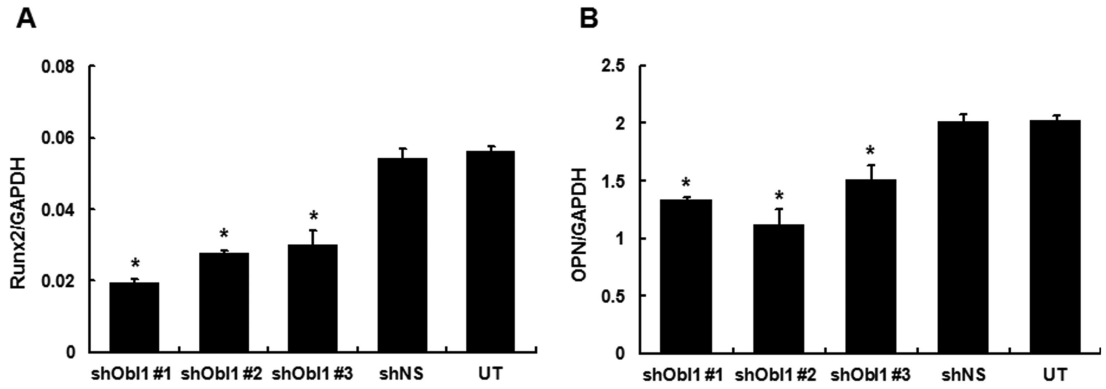


Figure 18: ObI-1 silencing reduces the expression of osteogenic differentiation markers. Real-time PCR analysis was performed to evaluate expression profile of early osteogenic differentiation markers in W20-17 cells and reveals a significant decrease in (**A**) Runx2 and (**B**) osteopontin (OPN) transcript levels in ObI-1 silenced cells compared to untransfected (UT) cells and cells transfected with non-silencing shRNA (shNS). Runx2 and OPN levels were evaluated at day 4 and day 7 of differentiation, respectively. Results are expressed as Runx2/GAPDH and OPN/GAPDH levels ratio (*p<0.05).

4.2.6 ObI-1 silencing impairs osteoprogenitors proliferation

We then evaluated the effects of ObI-1 silencing on osteoprogenitors proliferation, which represents the first step of osteogenic differentiation. Interestingly, ObI-1 silencing selectively impaired the proliferation of pre-osteoblasts, with a decreased BrdU incorporation 24 hours after osteogenic induction compared to control cells, whereas the proliferation of undifferentiated MSCs was not affected (Fig. 19A and B). Silencing efficacy was confirmed by Real-time PCR analysis 48 hours after siRNA transfection (Fig. 19C). These findings suggest that ObI-1 specifically stimulates the proliferation of cells committed towards the osteogenic lineage, rather than being a general regulator of cell proliferation.

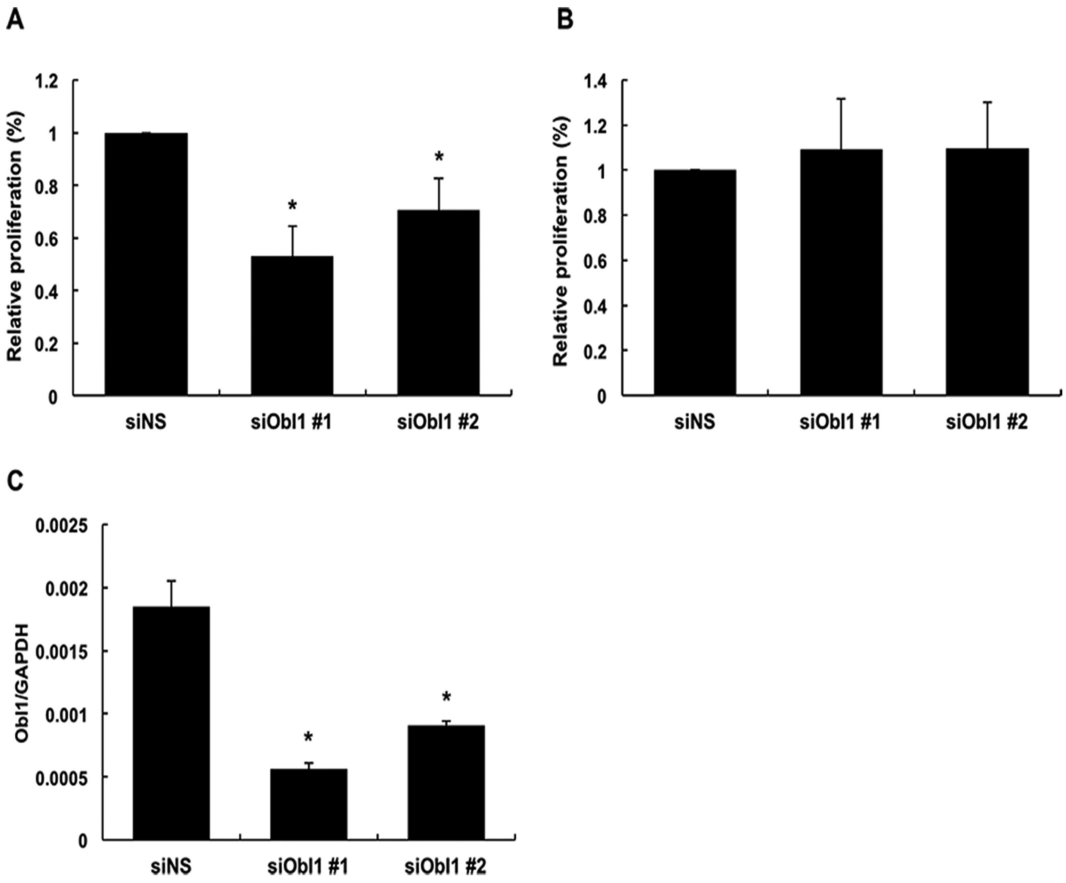


Figure 19: Obl-1 silencing selectively impairs osteoprogenitors proliferation.
mMSCs were transfected with either non-silencing siRNA (siNS) or siRNAs targeting Obl-1 (siObl1#1 or siObl-1#2). **(A and B)** BrdU incorporation assay was performed to evaluate the proliferation of cells transfected with the siRNAs for 24 hours and then either stimulated with osteogenic medium (pre-osteoblasts, **A**) or cultured in regular media (undifferentiated mMSCs, **B**) for the following 24 hours (48 hours post-transfection). Obl-1 silencing significantly decreases osteoprogenitor proliferation (**A**), while does not alter mMSC proliferation (**B**). Cell proliferation is calculated as the percentage of BrdU- over DAPI-positive nuclei and expressed as fold change of the proliferation of control cells (shNS), and was determined by counting 30 fields per condition (10X magnification, *p<0.01).
(**C**) Silencing efficacy was confirmed by Real-time PCR analysis 48 hours after siRNA transfection: Obl-1 transcript levels are significantly reduced in mMSCs transfected with siObl1#1 or siObl1#2 compared to cells transfected with siNS. Results are expressed as Obl-1/GAPDH levels ratio.

4.2.7 ObI-1 over-expression stimulates osteoblast differentiation

Subsequently, we evaluated whether over-expression of ObI-1 could enhance osteoblast differentiation in mMSCs. At this aim, we induced the osteogenic differentiation in mMSCs stably transfected with a vector expressing ObI-1 fused to a Flag tag (ObI-1 Flag) and observed an increased mineralized matrix deposition compared to cells stably transfected with pcDNA3.1 (empty vector) (Fig. 20A-F). Similar results were obtained when cells were cultured in medium supplemented only with ascorbic acid 2-phosphate and glycerol 2-phosphate without the osteogenic inducer dexamethasone, further indicating that ObI-1 expression enhances mMSCs commitment and/or differentiation towards the osteogenic lineage (Fig. 20B and E).

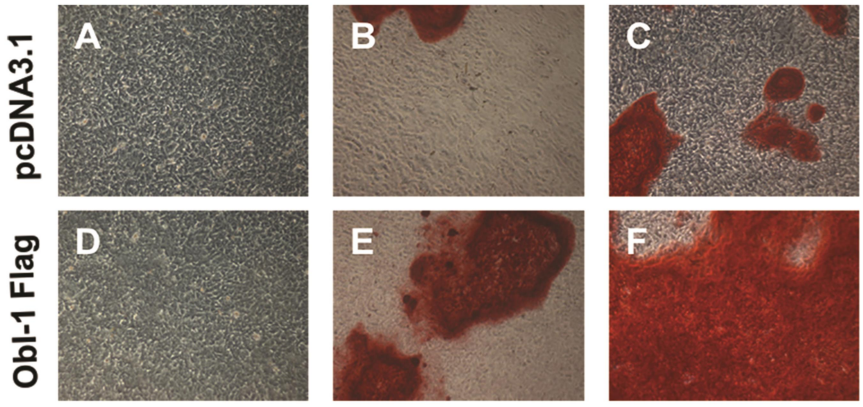


Figure 20: ObI-1 over-expression enhances osteoblastogenesis of mMSCs
ObI-1 over-expression increases osteoblast-mediated calcium deposition after 14 days of differentiation. mMSCs stably transfected with (**A**-**C**) pcDNA3.1 or (**D**-**E**) ObI-1 Flag were cultured for 2 weeks in (**A** and **D**) regular medium, (**B** and **E**) medium supplemented with ascorbic acid 2-phosphate and glycerol 2-phosphate or (**C** and **F**) complete osteogenic medium. Alizarin red staining shows that ObI-1 over-expression enhances matrix mineralization compared to controls. Magnification 10X. Pictures are representative of 3 independent experiments.

In agreement with this finding, mMSCs transfected with the ObI-1 Flag encoding plasmid showed increased levels of Runx2 and OPN compared to controls (Fig.21). Therefore, it is conceivable that ObI-1 favors osteoblastogenesis by directly or indirectly stimulating Runx2 expression.

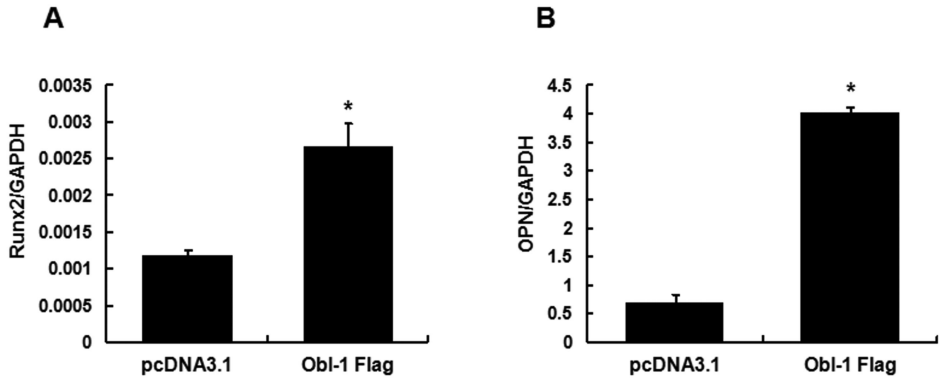


Figure 21: ObI-1 over-expression increases the expression of osteogenic differentiation markers.

Real-time PCR analysis reveals a significant increase in (A) Runx2 and (B) osteopontin (OPN) transcript levels in mMSCs transfected with ObI-1 Flag-expressing plasmid compared to cells transfected with the empty vector (pcDNA3.1). Runx2 and OPN levels were evaluated at day 3 and day 7 of differentiation, respectively. Results are expressed as Runx2/GAPDH and OPN/GAPDH levels ratio.*p<0.0

4.2.8 ObI-1 is localized in the nucleus

In order to evaluate the sub-cellular localization of the protein product of ObI-1 gene, we performed an immunofluorescence analysis of cells transiently transfected with either pcDNA3.1 or the ObI-1 expressing plasmid. As previously described by Jin et al. in mouse gonads, we confirmed that the ObI-1 protein is exclusively localized into the nucleus, thereby supporting a possible role in transcriptional regulation (Fig.22 B). Western Blot analysis was performed to confirm ObI-1 over expression (Fig.22 A).

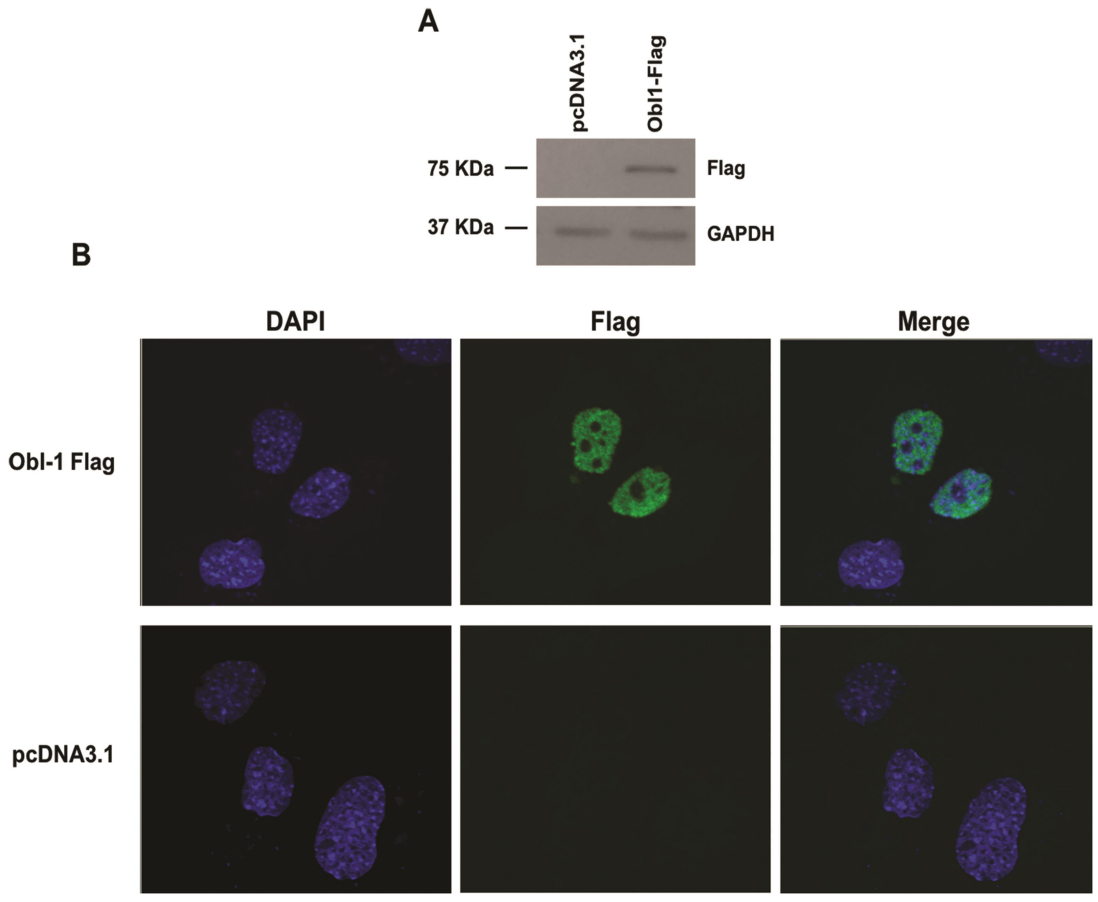


Figure 22: ObI-1 is localized in the nucleus.
(A) Western Blot analysis confirms ObI-1 expression in mMSCs transfected with the pcDNA3.1 ObI-1 Flag-expressing plasmid 24 hours after transfection. Cells transfected with the empty vector (pcDNA3.1) were used as negative control. GAPDH was used as control for protein loading. (B) ObI-1 sub-cellular localization was investigated in mMSCs transfected with either the ObI-1 Flag-expressing plasmid or the empty vector by immunofluorescence analysis with an anti-Flag antibody 24 hours after cell transfection. Merging between the nuclear dye DAPI and the anti-Flag antibody indicates that the protein is localized in the cell nucleus. Magnification 63X.

4.2.9 ObI-1 protein is subjected to proteasomal degradation

We then attempted to generate mMSCs stably expressing the ObI-1 Flag protein. However, in spite of a robust expression of the ObI-1 transcript (Fig.23A), we were not able to detect the corresponding protein through Western Blot analysis. This result suggested that ObI-1, as most transcriptional factors, could be subjected to post-translational modifications that regulate its stability and/or function (Filtz et al. 2014). In order to assess whether mechanisms such as ubiquitin (Ub)-mediated proteasomal degradation may be involved, we evaluated the presence of the ObI-1 Flag protein after inhibition of proteasomal function. Consistently with our hypothesis, treatment with the proteasome inhibitor MG 132 results in ObI-1 Flag protein accumulation in mMSCs (Figure 23B).

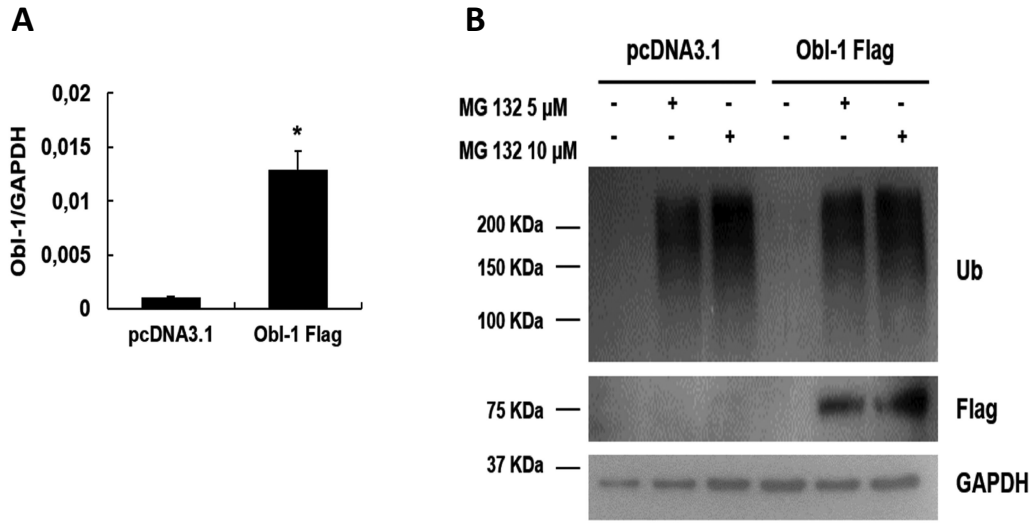


Figure 23: ObI-1 protein is subjected to proteasomal degradation.
(**a**) Real-time PCR analysis shows a significant increase in ObI-1 transcript levels in mMSCs stably transfected with ObI-1 Flag compared to cells transfected with the empty vector (pcDNA3.1). Results are expressed as ObI-1/GAPDH levels ratio (*p< 0.01). (**b**) Western blot analysis of cells transfected with either ObI-1 Flag or empty vector. Cells were treated with DMSO (control) or the proteasome inhibitor MG 132 (5 or 10 µM) for 6 hours. Whole cell extracts were probed with anti-Flag, anti-ubiquitin (Ub) and anti-GAPDH antibodies. GAPDH levels were included as loading control. Proteasomal inhibition allows ObI-1 Flag protein accumulation, indicating that the protein is subject to proteasomal degradation. The accumulation of high molecular weight proteins reactive to anti-Ub indicates that MG 132 treatment was effective in reducing the degradation of ubiquitin-conjugated proteins.

We then performed an immunoprecipitation analysis in mMSCs stably expressing ObI-1 Flag and transiently transfected with a vector expressing HA-Ubiquitin (HA-Ub) in order to increase the cellular pool of free ubiquitin. Efficiency of both HA-Ub transfection and MG 132 proteasomal inhibition were confirmed by Western blot analysis (Fig.24A).

Subsequently, the same cell lysates were subjected to immunoprecipitation with an anti-Flag antibody and Western blot analysis confirmed that the ObI-1 protein, stabilized following MG 132 treatment, is polyubiquitylated (Fig.24B) These results indicate that ObI-1 protein levels, and therefore its function, are finely regulated by Ub-mediated proteasomal degradation, a mechanism commonly used by eukaryotic cells to tightly control the activity of transcription factors and other chromatin-associated proteins (Hammond-Martel et al. 2012).

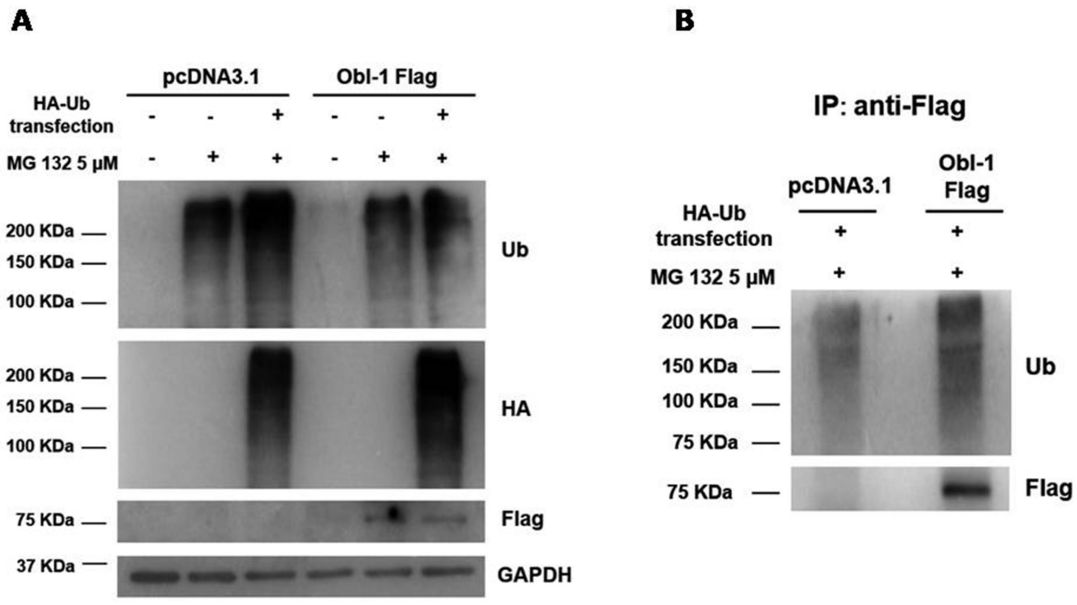


Figure 24: ObI-1 protein is subjected to proteasomal degradation.
(A) Cells stably transfected with either ObI-1 Flag or empty vector were transiently transfected with a vector expressing HA-Ubiquitin (HA-Ub) and treated with DMSO or 5µM MG132. Efficiency of HA-Ub transfection and MG 132 proteasomal inhibition were confirmed by Western blot analysis on whole cell extracts probed with anti-Ub, anti-HA, anti-Flag and anti-GAPDH antibodies. (**B**) The same protein extracts were immunoprecipitated with anti-Flag antibody and probed with anti-Flag and anti-Ub antibodies. ObI-1 protein, accumulated following MG 132 treatment, is ubiquitylated.

4.2.10 ObI-1 encodes for a highly unstable, rapidly degraded protein

In order to further characterize Obi-1 protein product stability, we then performed a cycloheximide chase experiment in mMSCs transiently transfected with ObI-1 Flag or the empty vector to evaluate ObI-1 stability when translation, and therefore de novo protein synthesis, is inhibited. Consistently, we showed that ObI-1 encodes for a highly unstable, rapidly degraded protein (Fig.25).

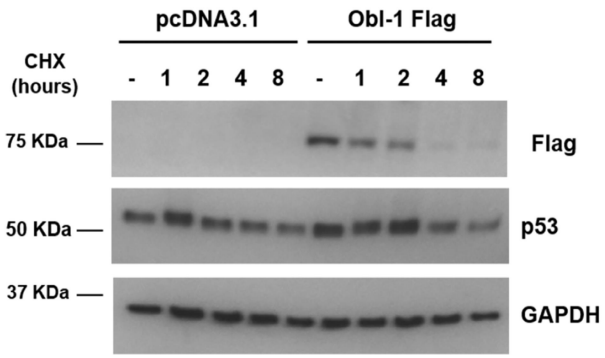


Figure 25: ObI-1 encodes for a highly unstable, rapidly degraded protein.
Western blot analysis of mMSCs transiently transfected with either ObI-1 Flag or empty vector. 24 hours after transfection, cells were treated with DMSO (control) or the translation inhibitor cycloheximide (CHX, 10 µg/ml) for 1, 2, 4 or 8 hours. Whole cell extracts were probed with anti-Flag, anti-p53 and anti-GAPDH antibodies. GAPDH was employed as loading control; p53 was included as a known unstable protein control. ObI-1 Flag protein levels rapidly decrease after CHX-induced inhibition of protein synthesis.

4.2.11 ObI-1 modulates osteoblastogenesis through a BMP-mediated pathway

Since ObI-1 knockdown strongly reduces Runx2 expression while its over-expression increases it, the notion that ObI-1 may act upstream of Runx2 is convincible.

BMPs play a crucial role in bone development and are able to induce, among other target genes, Runx2 expression (Canalis et al., 2003; Lee K.S. et al., 2000). To investigate whether ObI-1 affects the BMP pathway, we evaluated BMP-4 expression by Real Time PCR analysis and we found that its transcript levels are significantly up-regulated in ObI-1 Flag transfected mMSCs compared to control cells (Fig.26A). On the other hand ObI-1 silencing induced a decreased expression of BMP-4 (Fig.26B).

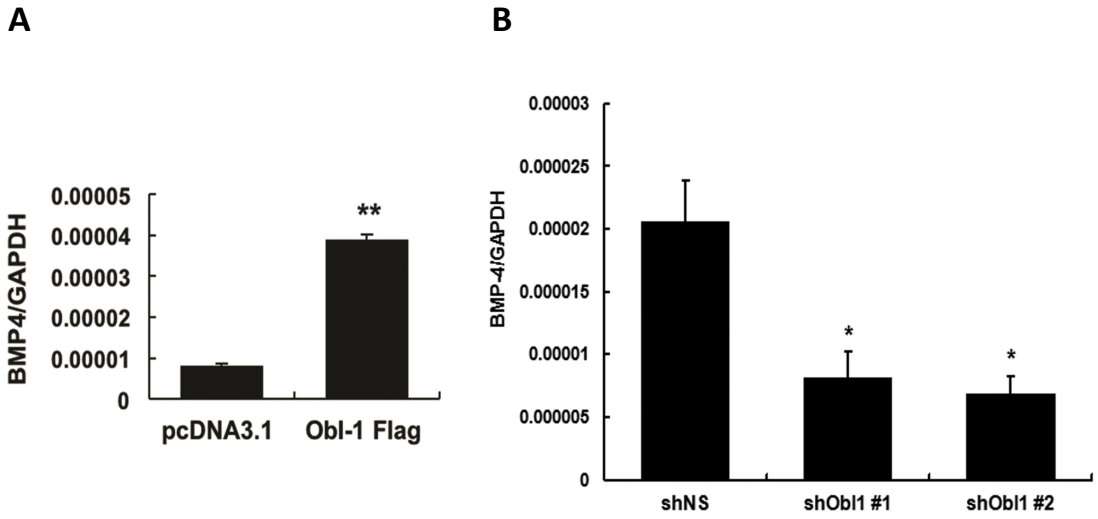


Figure 26: ObI-1 affects the BMP pathway
(**A**) Real-time PCR analysis shows that BMP-4 transcript levels are significantly increased in mMSCs transfected with ObI-1 Flag compared to control cells. Results are expressed as BMP-4/GAPDH levels ratio (**p<0.01). (**B**) ObI-1 silencing impairs BMP-4 expression. Real-time PCR analysis shows a reduction in BMP-4 transcript levels in ObI-1-silenced mMSCs compared to cells transfected with non-silencing shRNA (shNS), as control. Results are expressed as BMP-4/GAPDH levels ratio.*p<0.01

We then evaluated whether ObI-1 expression influences BMPs signaling and Smad1/5/8 phosphorylation and, therefore, activation. mMSCs stably transfected with either ObI-1 Flag or pcDNA3.1 empty vector were treated with 40 ng/ml BMP-4 and Smad1 and phospho(p)Smad1/5/8 levels were assessed after 1, 3 or 6 hours of stimulation. Western blot analysis showed that ObI-1 over-expression induces a more pronounced activation of Smad pathway at all-time points examined (Fig.27A and B).

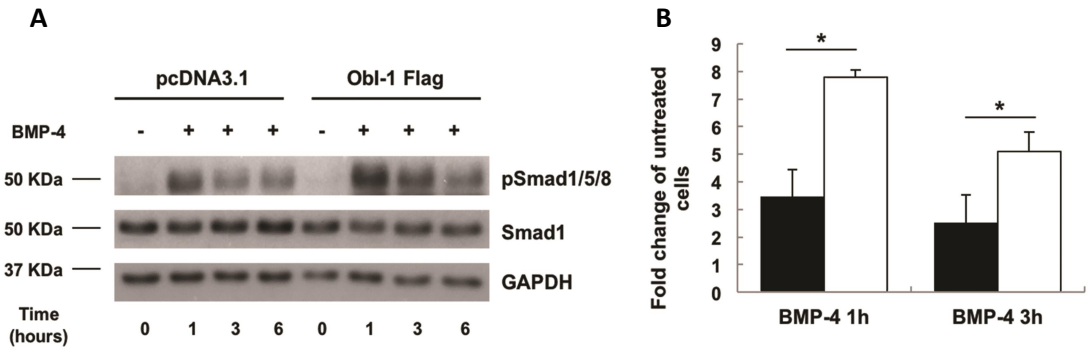


Figure 27: ObI-1 expression influences BMPs signaling
(**A**) ObI-1 expression potentiates BMP-mediated Smad1/5/8 activation (phosphorylation). mMSCs stably transfected with either ObI-1 Flag or empty pcDNA3.1 were treated with 40 ng/ml BMP-4 and total Smad1 and phosphorylated (p)Smad1/5/8 levels were evaluated at different time points by Western blot analysis. As controls, cells were maintained in medium only. ObI-1 Flag transfected cells show increased levels of pSmad1/5/8. GAPDH levels were included as loading control.
(**B**) Densitometric analysis of the immunoreactive bands confirms an increased phosphorylation of Smads in cells transfected with ObI-1 Flag (white bars) compared to empty vector (black bars); results are reported as fold change of pSmad1/5/8 levels (previously normalized to Smad1 levels) in BMP4-stimulated cells versus untreated cells (*$p<0.05$).

This hypothesis was further corroborated by the finding that BMP signaling inhibition counteracts Obl-1 stimulation of osteogenic differentiation. Indeed, treatment with the BMP Receptor type I (BMPRI) antagonist LDN193189 during osteogenic differentiation of mMSCs transfected with either pcDNA3.1 or Obl-1 Flag markedly reduced bone mineralization, as expected, and completely abolished Obl-1 enhancement of differentiation (Fig.28A-F).

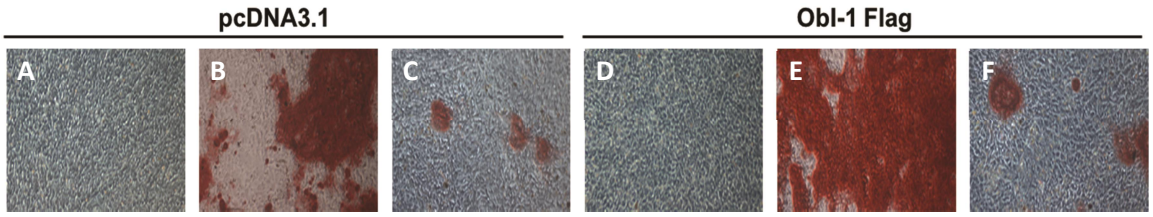


Figure 28: Obl-1 expression influences BMPs signaling
mMSCs transfected with (**A**-**C**) pcDNA3.1 or (**D**-**F**) Obl-1 Flag were culture for 2 weeks in (**A** and **D**) regular medium, (**B** and **E**) osteogenic medium alone or (**C** and **F**) supplemented with 100 nM of the BMPR-I antagonist LDN193189. Alizarin red staining shows that BMP inhibition strongly reduces matrix mineralization and abolishes Obl-1-mediated stimulation of differentiation. Magnification 10X. Pictures are representative of 3 independent experiments.

4.2.12 Expression analysis of ZNF717 during osteogenic differentiation

Finally we evaluated the expression profile of ZNF717, putative human ortholog of the murine Obl-1, during osteogenic differentiation of primary hMSCs. Interestingly, ZNF717 expression also increases during osteoblast differentiation of hMSCs from day 3 (Fig.29); this observation supports the predicted orthology and, therefore, suggests a possible role of ZNF717 in human osteoblastogenesis.

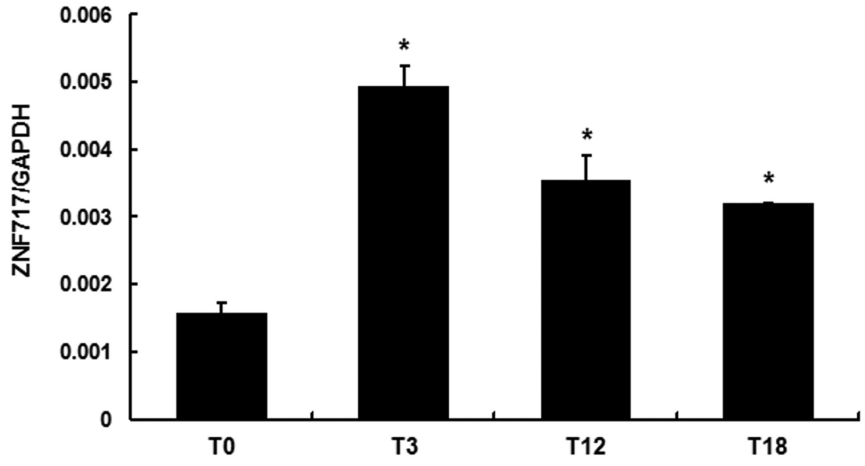


Figure 29: ZNF717 expression increases during osteogenic differentiation
Expression profile of ZNF717 transcript during osteogenic differentiation of hMSCs at day 0, 3, 12 and 18. ZNF717 levels significantly increase from day 3. Results are expressed as ZNF717/GAPDH levels ratio. *p< 0.01

5. Discussion

Despite intensive investigation, our knowledge of the molecular mechanisms driving MSCs differentiation towards the osteogenic lineage is still incomplete. A better comprehension of this biological process is of crucial importance since the increasing healthcare burden represented by osteopenia/osteoporosis in industrialized countries that demand the implementation of safer and more effective therapeutic strategies (Cauley, 2013). Furthermore, MSCs represent a promising cell source for regenerative medicine applications. These cells, indeed, present several advantages over other adult stem cells: they can be extensively expanded in vitro without losing their differentiation potential, they are immune-privileged and able to produce several anti-inflammatory and immunomodulatory cytokines (Bruder et al., 1997; Nauta et al., 2007). Therefore, the identification of the molecular bases of osteogenic differentiation is mandatory to regulate and optimize the process and to exploit MSC therapeutic potential fully.

In order to identify genes involved in osteoblast differentiation, we have previously performed a high-throughput screening based on an RNA-interference (RNAi) approach. The murine bone marrow-derived stromal cell line W20-17 was used as a cellular model and differentiated into osteoblasts, and mineral matrix deposition was chosen as read-out (Querques et al. 2015).

We then analyzed the Gene Ontology classification of candidate genes, and we found a significant fraction (30%) of genes with unknown function at the time of the screening. Hence, we focused our attention on one of these genes, predicted to be a transcription factor, that we named Osteoblasts Inducer-1 (ObI-1).

ObI-1 belongs to the family of KRAB-containing zinc-finger transcription repressors, that represents the largest single-family of transcription factors in mammals. These

transcription factors are present exclusively in tetrapod vertebrates and have been involved in the regulation of several biological processes, as cell proliferation and differentiation, including osteogenic differentiation, apoptosis and neoplastic transformation (Urrutia, 2003; Jheon et al., 2001). Its nuclear localization also supports the hypothesis that ObI-1 is a transcription factor. This finding is consistent with a report by Jin et al. identifying ObI-1 as a transcriptional repressor expressed in mouse gonads. The authors demonstrated that the protein is localized into the nucleus and showed that its KRAB domain fused to GAL4 DNA binding domain could repress transcription (Jin et al., 2015).

As most regulatory proteins, ObI-1 levels and function seem to be tightly regulated by Ub-mediated proteasomal degradation. Proteasomal degradation, in addition to its role in protein turnover and degradation of misfolded products, plays an essential regulatory role in fine-tuning the activity of proteins involved in a myriad of processes, from cell cycle regulation to DNA repair (Kirkin et al. 2007). Since gene expression needs to be finely modulated in time and space and in response to external signals, it is not surprising that transcription factors and chromatin-modifying proteins constitute major targets for the Ub-proteasome system (Muratani et al., 2003; Hammond-Martel et al., 2012). The proteasomal degradation of ObI-1, as well as its very short half-life, could indicate the requirement of tightly regulated levels of this protein within cells. Although we could not detect the ObI-1 protein by Western blot analysis in stably transfected mMSCs, we were clearly able to appreciate the positive effects of its overexpression on osteogenic differentiation in ObI-1 transfected cells compared to the control. This could indicate that this protein is rapidly degraded after stabilization in response to a stimulus, or undergoes cycles of stabilization and destabilization, hampering our ability to detect it. Further studies are required to elucidate the mechanisms regulating ObI-1 stability, the E3

ubiquitin ligases and deubiquitinating enzymes involved, as well as the specific stimuli inducing its stabilization.

Our data suggest that ObI-1 is required in the early steps of osteoblast differentiation: in fact, its silencing impairs osteoprogenitors proliferation resulting in a reduction of both ALP expressing mature osteoblasts and mineral matrix deposition. Conversely, its overexpression enhances the differentiation process.

Osteoblast differentiation requires the early expression of the transcription factor Runx2, considered the master gene of bone formation (Ducy et al. 1997; Komori et al. 1997). It is expressed early during mouse embryonic development in the cells prefiguring the future skeleton and can regulate the expression of most bone-specific proteins (Ducy et al., 1999). Similarly, Runx2 expression and function are highly regulated at transcriptional and translational levels as well as through post-translational modifications, including phosphorylation, ubiquitination, and acetylation (Jonason et al., 2009). We observed that ObI-1 knockdown sharply reduces Runx2 early expression while its overexpression increases it; these data suggest that Runx2 partly mediates ObI-1 role in osteoblastogenesis and that ObI-1 may directly or indirectly regulate Runx2 levels. Indeed, ObI-1 function is required for osteoblast differentiation since its silencing impairs ALP expression and mineral matrix deposition, whereas its overexpression enhances the differentiation process. The notion that ObI-1 may act upstream of Runx2 is supported by the findings that ObI-1 knockdown sharply reduces Runx2 expression while its overexpression increases it.

BMPs play a crucial role in cartilage and bone development; these growth factors, locally synthesized by the skeleton, play an autocrine role in osteoblastic differentiation stimulating the expression and function of a plethora of osteogenic inductors and proteins necessary for osteoblast maturation and bone matrix deposition (Canalis et al.,

2003). In particular, BMPs can induce Runx2 expression that in turn activates osteoblast-specific genes, and Runx2-Smads cooperation is thought to mediate BMP action in modulating gene transcription in cells of the osteoblastic lineage (Lee KS at al., 2000; Zaidi et al., 2002).

Our findings are consistent with the hypothesis that ObI-1 role in osteogenic differentiation is mediated by the BMPs pathway. The following lines of evidence support this contention: i) silencing of endogenous ObI-1 reduces and its overexpression enhances BMP-4 transcript levels, respectively; ii) ObI-1 over-expression potentiates BMP signaling and increases Smad1/5/8 phosphorylation; and iii) the treatment with a BMPRI antagonist completely abrogates ObI-1 stimulation of osteogenic differentiation. The BMP pathway is subjected to multiple levels of regulation by extracellular and intracellular molecules, such as antagonists, pseudoreceptors, inhibitory Smads and miRNAs (Canalis et al., 2003; Brazil et al., 2015). Therefore, further studies will be required to characterize better ObI-1 role and mechanism of action in modulating BMP pathway.

Despite their name, BMP expression is not limited to bone. In fact, these proteins constitute a group of morphogenes that play a pivotal role during embryonic development and organogenesis as well as in adult tissue homeostasis (Wagner et al., 2010). These multifunctional growth factors regulate proliferation, differentiation and function of a wide variety of cell types and are involved in processes as diversified as fracture repair, vascular remodeling, spermatogenesis and iron metabolism (Tsuji et al., 2006; Huang et al., 2009; Puglisi et al., 2004; Babitt et al., 2006). Concordantly, we found that ObI-1 is expressed in several mouse adult tissues and organs and, therefore, it may serve other biological functions besides its role in osteogenic regulation. In particular, we showed that ObI-1 is expressed at very high levels in the lung, although the significance of

this finding has to be determined. BMP signaling has been implicated in the regulation of lung morphogenesis, lung stem cell differentiation, adult lung homeostasis and tissue-injury repair (Weaver et al., 1999; Lee J.H. et al., 2014; Rosendahl et al., 2002; Song et al., 2008). Therefore, although further studies are required, it is tempting to speculate that ObI-1 is a novel modulator of BMP signaling capable of affecting a variety of cell types and processes beyond bone and osteogenic differentiation.

According to bioinformatics analysis, ZNF717 is the best candidate as ObI-1 ortholog in humans. ZNF717 is present in humans as well as in other non-human primates and encodes a KRAB zinc-finger transcription factor.

Our preliminary results are consistent with the hypothesis that ZNF717 could represent ObI-1 human ortholog since this gene is expressed in hMSCs and its levels increase during osteogenic differentiation with an expression profile comparable to that of ObI-1. However, further studies are mandatory to confirm a role for ZNF717 in human osteogenic differentiation as well as to elucidate its mechanisms of action and possible modulation of BMP signaling.

6. Conclusion

In conclusion, we have identified a novel transcriptional regulator of osteogenic differentiation able to stimulate MSC commitment towards the osteogenic lineage and osteoblast maturation, at least in part, through BMP-mediated activation of Runx2 and other proteins required for bone formation. Our findings shed new light on the transcriptional regulation of osteogenic differentiation and identify a novel player of the BMP pathway in the bone.

Moreover, we have demonstrated that ObI-1 transcript reached the highest levels of expression in lung, suggesting that ObI-1 could play a role not only in osteogenic differentiation.

Further studies will be required to fully elucidate ObI-1 mechanism of action in osteoblast development and other biological processes.

7. Bibliography

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