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**Identification of genes that regulate bone
marrow stromal cell differentiation by RNA
interference**

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ABSTRACT

Mesenchymal stem cells (MSCs) can be isolated from bone marrow and can differentiate in a variety of cell types, including osteoblasts, adipocytes, chondrocytes, myoblasts, hepatocytes, and neural cells. For this reason, MSCs are very promising for the development of cell-based therapy for degenerative diseases. Understanding the mechanisms behind MSC cell fate determination is not easy, since the molecular processes that drive differentiation are complex and poorly understood. Even though in the recent years many improvements have been done, many mechanisms need to be elucidated. We planned to investigate on differentiation mechanisms of MSC and, in particular, we focused our activities towards osteoblast differentiation; at this aim, we have silenced specific mRNAs using a mouse shRNA library in a 96 well plate-based screening strategy. With this methodology we identified several genes that are possible candidates for a role in osteoblast differentiation.

Among the candidate genes, a consistent fraction was represented by genes whose function is still unknown: we focused our attention on a short number of these genes that we named ObI (Osteoblast inducer). In this thesis project we focused our experiments on a particular gene, that we named ObI-1.

We also considered genes identified during the screening with a known

function but an unknown role during the osteogenic differentiation process. Among these genes we focused our attention on Angiotensin receptor 1 (AT1R), whose role in osteogenic differentiation had been previously suggested.

For both candidates that we analyzed for this project we confirmed the effects of silencing on osteogenic differentiation and we analyzed expression in tissues and in our cell culture system. Furthermore, we characterized function and mechanism of action in osteoblastogenesis for both genes.

INTRODUCTION

1 - Adult stem cells

An adult stem cell is an undifferentiated cell, found in tissues or organs; this cell type has the ability of renew itself and differentiate to yield some or all of the major specialized cell types of the resident tissues and organs. The primary role of adult stem cells in a living organism is to maintain and repair the tissue in which they are found. Adult stem cells have been identified in many organs and tissues, including brain, bone marrow, peripheral blood, blood vessels, skeletal muscle, skin, teeth, heart, gut, liver, ovarian epithelium, and testis. They are thought to reside in specific areas of each tissue, called "stem cell niches" (Li and Xie, 2005). Stem cells may remain quiescent for long periods of time until they are activated by the need for more cells to maintain tissues, or by diseases or tissue injuries (Walker et al., 2009). Generally, adult stem cells are thought to be able to develop into a limited number of cell types related to the resident tissue. Typically, there is a very small number of stem cells in each tissue, and once removed from the body, their capacity to divide is limited, making generation of large quantities of stem cells difficult. Research on adult stem cells has generated a great deal of excitement: if differentiation of adult stem cells can be controlled in the laboratory, these cells may become the basis of transplantation-based therapies.

1.2 - Adult stem cell differentiation

Adult stem cells can differentiate through normal pathways to form the specialized cell types of the tissue in which they reside or they can differentiate to other tissue from the cells predicted lineage (Wagers and Weissman, 2004).

Normal differentiation pathways of adult stem cells. In a living animal, adult stem cells are able to divide, when needed, and can give rise to mature cell types that have characteristic shapes and specialized structures and functions of a particular tissue.

Transdifferentiation. A number of experiments have reported that certain adult stem cell types can differentiate into cell types seen in organs or tissues other than those expected from the cells predicted lineage. This phenomenon is called transdifferentiation.

Although isolated instances of transdifferentiation have been observed in some vertebrate species, whether this phenomenon actually occurs in humans is under debate by the scientific community. Instead of transdifferentiation, the observed instances may involve fusion of a donor cell with a recipient cell. Another possibility is that transplanted stem cells are secreting factors that encourage the recipient's own stem cells to proliferate and begin the repair process. Even when transdifferentiation has been detected, only a very small percentage of cells appears to be able to undergo the process.

In a variation of transdifferentiation experiments, scientists have recently demonstrated that certain adult cell types can be

"reprogrammed" into different cell types using a well-controlled process of genetic modification (Takahashi and Yamanaka, 2006). This strategy may offer a way to reprogram available cells into different cell types that have been lost or damaged due to diseases.

In addition to reprogramming cells to differentiate into a specific cell type, it is now possible to reprogram adult somatic cells to acquire a pluripotent state; these cells have been demonstrated induced pluripotent stem cells, (iPSCs) (Welstead et al., 2008). Thus, a source of pluripotent cells specific to the donor, (thereby avoiding issues of histocompatibility) can be generated and used for tissue regeneration. However, like for embryonic stem cells, methods by which iPSCs can be completely and reproducibly committed to appropriate cell lineages are still under investigation.

2 - The bone marrow system

The bone marrow is a tissue found in the hollow interior of bones. This organ is composed of hematopoietic and the mesenchymal lineages: the first give rise to the blood cells while the second includes non-hematopoietic cells that can support hematopoiesis and mesenchymal stem cells and their progeny, such as osteocytes, chondrocytes and adipocytes (Fig. 1).

2.1 - Bone marrow stromal cells

Bone marrow stromal cells, BMSCs, are a well-characterized population of adult stem cells. These cells can differentiate into a variety of cells, including adipocytes, cartilage, bone, tendon and ligaments, muscle cells, skin cells and even nerve cells.

Unlike most other human adult stem cells, BMSCs can be obtained in quantities that are suitable for clinical applications, making them good candidates for use in tissue engineering. Techniques for isolation and amplification of BMSCs have been established and the cells can be maintained and propagated in culture for long periods of time, maintaining their capacity to form all the above cell types.

The majority of culture techniques still take a colony forming unit-fibroblast, CFU-f, approach: raw unpurified bone marrow or ficoll-purified bone marrow monocytes are plated directly into cell culture plates or flasks and BMSCs, but not red blood cells or haematopoietic progenitors, are adherent to tissue culture plastic within 24 to 48 hours (Friedenstein et al., 1966; Friedenstein et al., 1970; Owen et al., 1988). Other flow cytometry-based methods allow the sorting of bone marrow cells for specific surface markers. They are typically negative for CD34, CD45, CD14, CD11b, CD19, CD79a and HLA-DR and have been shown to be positive for Stro-1, CD29, CD73, CD90, CD105, CD166, CD44 and CD133 (Dominici M et al., 2006).

Furthermore, BMSCs can be transduced, a phenomenon that could be exploited for the delivery of beneficial molecules to targeted locations.

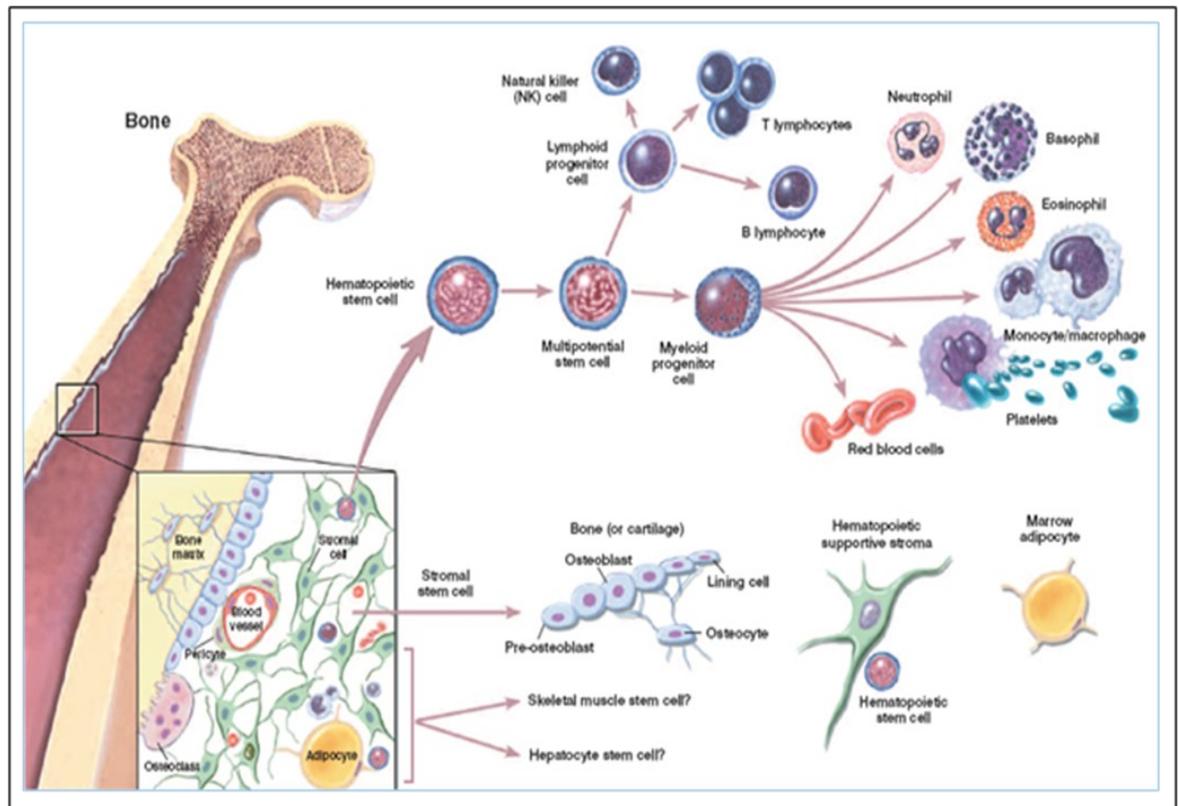


Figure 1: The Bone marrow system

The bone marrow is the source of two distinct lineages, the haematopoietic and the mesenchymal lineage. From the haematopoietic lineage derive blood cells; from the mesenchymal stem cells derive the supporting stromal cells, osteocytes, chondrocytes and adipocytes. Adapted from <http://stemcells.nih.gov>.

Animal *in vivo* experiments aimed at reconstitution of damaged tissues such as cartilage, bone, muscle, heart muscle and tendon using BMSCs cells have shown great promises for clinical applications.

2.1.1 - Differentiation Potential of BMSCs

The differentiation of BMSCs is influenced by convergence of intrinsic cellular signals and extrinsic micro-environmental cues from the surrounding stem-cell niche, but all the specific signals involved remain largely unknown (Watt et al., 2000).

Niche-external signals: the external signals that control stem cell fate collectively make up the stem cell micro-environment, or niche. A complex interplay of short- and long-range signals between stem cells, their differentiating daughters, and neighbouring cells are involved in niche maintenance. Soluble factors, cell-cell interaction and cell-extracellular matrix interaction seem to contribute to the self-renewal of different type of stem cells (Watt et al., 2000; Moore et al., 2006; Wilson et al., 2006). The most important signals implicated in BMSC stemness maintenance are some cytokines such as leukaemia inhibitory factor, LIF (Jiang et al., 2002; Metcalf et al., 2003), fibroblast growth factors, FGFs (Bianchi et al., 2003; Zaragosi et al., 2006), and mammalian homologues of *Drosophila* wingless, Wnts (Boland et al., 2004; Ling et al., 2008).

Niche-intrinsic signals: intrinsic regulators of self-renewal include proteins responsible for setting up cell division, nuclear factors controlling gene expression, chromosomal modification and telomere length (Watt et al., 2000). Embryonic stem cells (ESCs) maintain pluripotency by a transcriptional program that suppresses differentiation; this property is regulated by a small number of ESCs specific transcription factors such as Nanog, Oct-4 Sox-2 and Klf-4, whose expression is down-regulated early during embryogenesis (Boiani et al, 2005). In the last years several groups have shown the expression of Oct-4, Nanog and Sox-2 in the most undifferentiated BMSCs (Jiang et al., 2002; Beltrami et al., 2007).

The identification of specific signalling networks and master regulatory genes that regulate BMSC differentiation in specific lineages remain a challenge. Gene repression and induction may be main mechanism involved in the maintenance of the niche (Quesenberry et al., 2002). Upon stimulation, multipotent uncommitted BMSCs undergo asymmetric division, giving rise to two daughter cells, one being exact replica of the mother cell and maintaining multilineage potential, and other daughter cell becoming a precursor cell, with a restricted developmental program (Quesenberry et al., 2002). To date, the mechanism that governs the transit of uncommitted stem cells to partially committed precursor or progenitor cells, and then to fully differentiated cells is not fully understood.

2.1.1.1 - Adipogenesis

The nuclear hormone receptor peroxisome proliferation-activated receptor gamma, PPAR γ , is a critical adipogenic regulator promoting BMSC 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 PPAR γ , thus promoting osteogenesis while blocking adipogenesis (Hong et al., 2005). A similar role is played by Wnt signalling: the suppression of Wnt signalling is required for adipogenesis (Liu et al., 2004).

2.1.1.2 - Chondrogenesis

Chondrogenic differentiation of BMSCs *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 (Goldring et al., 2006). 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) (Massague et al., 2000), growth and differentiation factor, GDF (Chen et al., 2004), and Wnt ligand (Hartmann et al., 2006).

2.1.1.3 - Osteogenesis

During osteogenesis, multipotent BMSCs undergo asymmetric division and generate osteoprecursors, which then progress to form osteoprogenitors, pre-osteoblasts, functional osteoblasts and osteocytes. This progression from one differentiation stage to the following is accompanied by activation and subsequent inactivation of transcription factors and expression of bone related marker genes.

3 - Osteogenic differentiation of BMSCs

In the osteogenic differentiation of BMSCs are involved a number of growth factors, the relative signaling pathways that they activate and a number of cellular products, especially transcription factors. In this section are described some of the most important growth and transcription factors involved in osteogenesis (Fig. 2).

3.1 - Growth factors involved in osteoblast differentiation

3.1.1 - Bone Morphogenetic Proteins

BMPs are members of the TGF-superfamily of polypeptides, which includes TGF-s, activins, and inhibins (Wozney JM et al, 1988); they were originally identified because of their ability to induce endochondral bone formation. BMP proteins display extensive conservation among species having seven characteristic cysteine knot domains (Wang EA et al., 1990). The conserved cysteine domains participate in the formation of an interchain disulfide bond between two monomers to form a dimeric precursor protein. The precursor dimers are secreted as propeptides, which are activated by proprotein convertases (Cui Y et al., 1998).

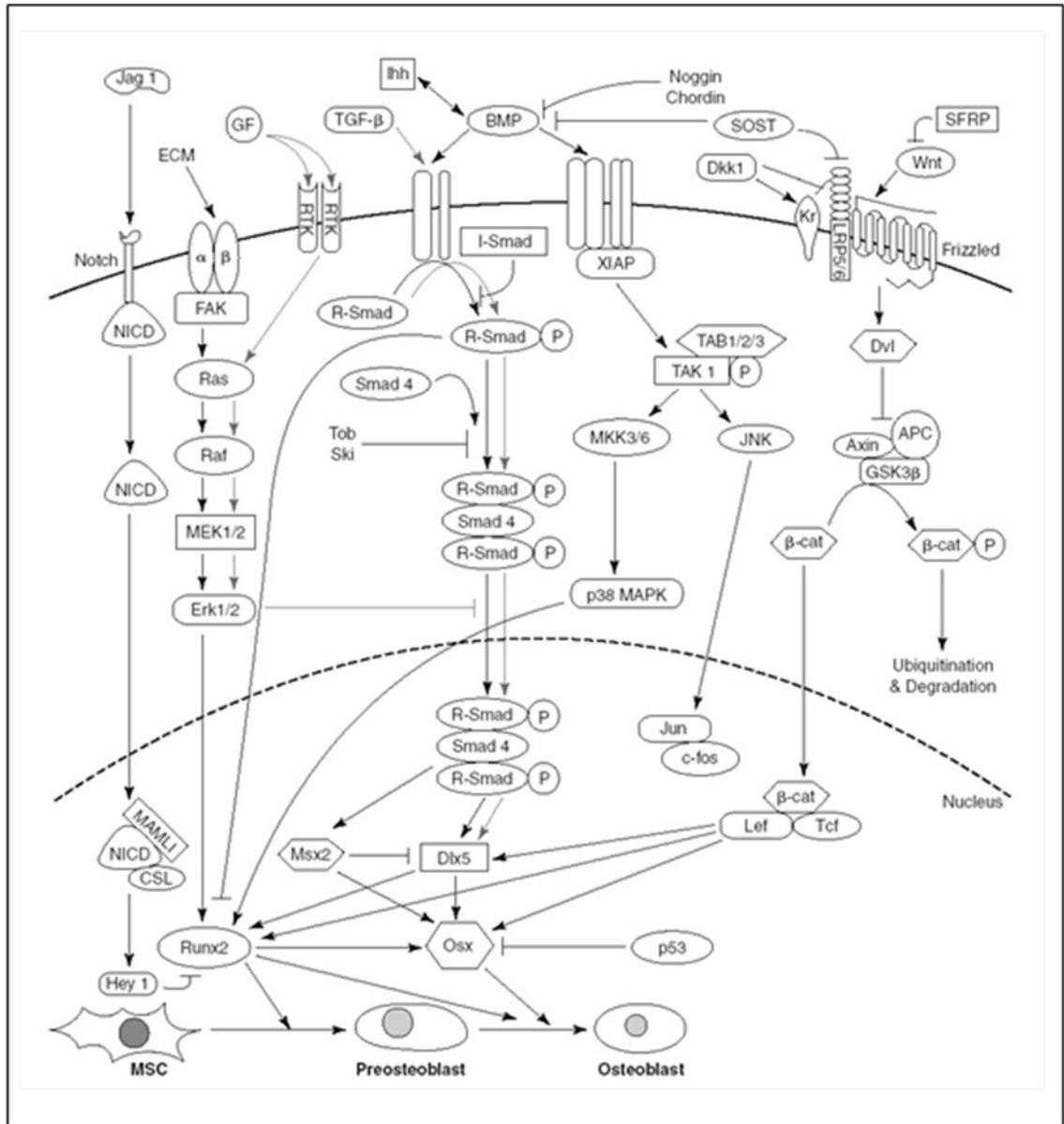


Figure 2: Signalling and transcription factor regulation in osteogenic differentiation

Wnt and BMP pathway induce osteogenic differentiation through the up-regulation of some osteo-specific transcription factors such as Runx2, Osterix, Dlx5 and Msx2.

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 II subtypes. Three type I receptors have been shown to bind BMP ligands, type IA and IB receptors (BMPR-

IA or ALK-3 and BMPR-IB or ALK-6) and type IA activin receptor (ActR-IA or ALK-2) (Koenig et al., 1994; ten Dijke et al., 1994). Three type II receptors for BMPs have also been identified and they are type II BMP receptor (BMPR-II) and type II and IIB activin receptors (ActR-II and ActR-IIB) (Yamashita et al., 1995; Rosenzweig et al., 1995). These receptors are differentially expressed in various tissues. Type I and II BMP receptors are both indispensable for signal transduction. After ligand binding they form a heterotetrameric-activated receptor complex consisting of two pairs of a type I and II receptor complex (Moustakas et al., 2002). The type I BMP receptor substrates include a protein family, the SMAD proteins, that play a central role in relaying the BMP signal from the receptor to target genes in the nucleus. Smad1, 5 and 8 are phosphorylated by BMP receptors in a ligand-dependent manner (Hoodless et al., 1996; Chen et al., 1997). After phosphorylation they form a complex with Smad4 and then are translocated into the nucleus where they interact with other transcription factors. BMPs can activate Smad-independent pathway such as Ras/MAPK pathway (Attisano et al., 2002; Lai et al., 2002). BMP-2 activates ERK and p38 through Ras kinase stimulation and, as result of this activation, ATF-2 and Fos/Jun protein are up-regulated and interact with activating protein-1, AP-1, sequences in the regulation of the expression of different genes. The activation of p38 is essential for the expression of BMP-2, type I collagen, osteocalcin, alkaline phosphatase and the activation of p38 and ERK is essential for the expression of fibronectin and osteopontin

(Lai et al., 2002). MAPKs pathway can activate different pathways independently or in synergistic way with SMAD (Attisano et al., 2002).

3.1.2 - BMP pathway in osteoblast differentiation

Although BMPs are synthesized by skeletal cells, their synthesis is not limited to bone because they are expressed by a variety of extraskeletal tissues in which they play a critical role in development and cell function. BMPs increase commitment of cultured marrow stromal cells to osteoblast phenotype by increasing CBFA1 mRNA expression (Lee et al., 2002; Ito et al., 2003). BMP-4 mRNA levels are BMP-dependent: in fact, BMPs cause an early, short-lived, induction of BMP-4 mRNA in osteoblasts followed by an inhibitory effect, suggesting autocrine regulation (Pereira et al., 2000). The transient increase in BMP-4 expression induced by BMPs may be required to force cell progression toward a differentiated state, whereas the down-regulation suggests a local control mechanism. BMP-2 also can be up- and down-regulated by other BMPs in osteoblasts, and it is of interest that BMP-2 and -4 promoters contain Runx-2/Cbfa-1 binding sequences (Helfering et al., 2000; Ghosh-Choudhury et al., 2001). This opens the possibility for a positive feedback loop regulating BMP-2 and -4 expression involving Runx-2/Cbfa-1 because BMPs induce Runx-2/Cbfa-1 expression (Banerjee et al., 2001). In long-term cultures of osteoblasts, there is an increase in BMP-4 mRNA expression after cell maturation, which may be secondary to a larger pool of cells expressing Runx-2/Cbfa-1 (Chen et

al., 1997).

3.1.3 - Wnt pathway

Wnt proteins act on target cells by binding to Frizzleds (Fzs), seven-span transmembrane receptor proteins, and LRP-5/6, single-span transmembrane co-receptor proteins. The canonical Wnt pathway affects cellular functions by regulating β -catenin levels and subcellular localization (Akiyama, 2000). The canonical Wnt pathway is initiated by the binding of appropriate Wnt ligands to the Fzs and LRP-5/6 co-receptor. In absence of appropriate Wnt ligands, β -catenin is phosphorylated and polyubiquitinated for the following proteasome-mediated degradation (Ikeda et al., 1998; Eastman et al., 1999). In presence of an appropriate Wnt ligand, binding of Wnt to receptor complex leads to the activation of the intracellular protein, Dishevelled (Dvl), that inhibits β -catenin degradation that accumulates and translocates to the nucleus, where in concert with members of the T cell factor/lymphoid enhancer factor (TCF/LEF) family, activates the transcription of a wide range of genes.

The Non-canonical Wnt pathway functions in a β -catenin independent manner (Gordon et al., 2006). Non-canonical Wnt signals are transduced through Fz family receptors and coreceptors, such as ROR2 and RYK, but not LRP-5 or LRP-6 (Kato et al., 2007). This binding mediates the signaling through G-protein, protein kinase C (PKC) or calcium/calmodulin-dependent protein kinase II (CamKII) to induce

nuclear factor of activated T cells (NF-AT) and other transcription factors (Wang et al., 2003; Kohn et al., 2005). Taken together, although the specificity between Wnt molecules and Fz receptors remains unclear, it is known that different Wnt ligands will preferentially activate canonical or non-canonical Wnt pathways (Johnson et al., 2007).

3.1.4 - Wnt pathway in osteoblast differentiation

Members of the Wnt family participate and play a central role in a variety of fundamental processes during embryonic development, such as limb skeletogenesis (Yang et al., 2003). Wnt pathway also represents a mechanism in mesenchymal cells proliferation and differentiation during chondrogenesis or osteogenesis. For example, Wnt-3a has been shown to promote proliferation and suppress osteogenic differentiation of adult MSCs (Boland et al., 2004), suggesting canonical Wnt signaling pathway functions in maintaining an undifferentiated, and proliferating progenitor MSC population. Wnt pathway is also involved in BMPs-mediated osteogenesis (Fischer et al., 2002; Chen et al., 2007) and is able to induce the osteoblastic transcription factors, Cbfa-1, Dlx-5, and Osterix. These data strongly indicate that Wnt pathway is intimately associated with bone regenerative process. Two microarray gene expression studies in a rat closed fracture model revealed that several genes for Wnt signaling components and their target genes are up-regulated (Hadjiargyrou et al., 2002; Zhong et al., 2006). These data indicate that Wnt signaling pathway is also activated during fracture

healing.

3.2 - Transcription factors involved in osteoblast differentiation

3.2.1 - Runx2

Runt-related transcription factor (Runx)-2 or corebinding factor (Cbfa)-1 plays a critical role in the differentiation of cells toward an osteoblastic pathway (Banerjee et al., 2001). Gene-targeted disruption of Runx-2/Cbfa-1 results in disorganized chondrocyte maturation and a complete lack of bone formation due to an arrest of osteoblast development (Ducy et al., 1997; Komori et al., 1997). Runx-2/Cbfa-1 also plays a role in mature osteoblastic function, and transgenic animals overexpressing a dominant negative form of Runx-2/ Cbfa-1, under the control of the osteoblastic specific osteocalcin promoter, display decreased bone formation due to impaired osteoblastic function (Ducy et al., 1999). This indicates a dual role of Runx-2/Cbfa-1 in cells of the osteoblastic lineage, regulating osteoblastogenesis as well as the function of mature osteoblasts. The role of Runx-2/Cbfa-1 in later stages of differentiation is less clear, and its overexpression under the control of the type I collagen promoter results in osteopenia because of the lack of terminal maturation of osteoblastic cells (Liu et al., 2001).

3.2.2 - Osterix

Osterix is a zinc finger transcription factor expressed by osteoblasts and required for endochondral and intramembranous bone formation. Osterix-null mice have normal cartilage development but fail to develop a mineralized skeleton (Nakashima et al., 2002). Osteoblast differentiation is arrested, and histological analysis reveals absence of trabecular bone. Osterix-null mice have reduced or absent expression of a variety of bone matrix proteins, including type I collagen, bone sialoprotein, osteonectin, osteopontin, and osteocalcin, confirming a role in the induction of osteoblast differentiation and function. In contrast to Runx-2/Cbfa-1-null mice that do not form osteoblasts, osterix-null mice form cells of the osteoblastic lineage that express Runx-2/Cbfa-1, but the cells do not mature. This would indicate that osterix has effects on skeletal development that are independent of Runx-2/Cbfa-1 and that acts downstream of Runx-2/Cbfa-1 (Nakashima et al., 2002).

3.2.3 - Msh

The Msh family of homeobox genes includes Msx 1, 2, and 3. Msx 3 is expressed in the central nervous system, whereas Msx 1 and 2 are expressed in skeletal tissue and modulate osteogenesis. Msx 1-null mice display cleft palate and craniofacial and dental developmental abnormalities, and Msx 2-null mice have defects in skull ossification, which are enhanced in double Msx 1/Msx 2 mutants (Satokata et al.,

2000). Msx 2-null mice have defective chondrogenesis and osteogenesis due to a decreased number of osteoprogenitor cells. The skeletal abnormalities are associated with decreased expression of Runx-2/Cbfa-1, indicating that Msx 2 is necessary for osteogenesis and acts upstream of Runx-2/Cbfa-1. However Msx2 represses transcription of osteocalcin directly by binding the promoter. Because osteocalcin is expressed late in osteoblast differentiation and is potentially regulated by Msx2, this transcription factor may have role in both early and late differentiation events (Dodig et al., 1999).

3.2.4 - Dlx 5

The mammalian homolog of *Drosophila* distalless (Dlx) 5 is homeobox genes essential for craniofacial and skeletal development (Robledo et al., 2002). Dlx 5 mRNA is expressed in osteoblasts after differentiation, concomitant with a decline in Msx 2 mRNA and with the appearance of osteocalcin transcripts (Ryoo et al., 1997). BMP induces Dlx 5 expression in osteoblasts and overexpression of Dlx 5 induces an increase of alkaline phosphatase activity, osteocalcin, and mineralization of the extracellular matrix (Miyama et al., 1999). Targeted gene inactivation of Dlx 5 results in severe skeletal abnormalities but Runx2 expression is unaltered (Robledo et al., 2002).

4 - Bone tissue

Bone, although strong, is a constantly changing tissue that has several

functions. Bones serve as rigid structures to the body and as shields to protect delicate internal organs. They provide housing for the bone marrow, where the blood cells are formed and a point of attachment for skeletal muscles. Bones also maintain the body's reservoir of calcium and phosphate.

Bones have two shapes: flat, such as the plates of the skull and the vertebrae, and tubular, such as the thighbones and arm bones, which are called long bones. All bones have essentially the same structure. The hard outer part, cortical bone, consists largely of proteins, such as collagen, and a substance called hydroxyapatite, which is composed mainly of calcium and other minerals. Hydroxyapatite is largely responsible for the strength and density of bones. The inner part of bones, trabecular bone, is softer and less dense than the hard outer part. Bone marrow is the tissue that fills the spaces in the trabecular bone. Bone marrow contains specialized cells (including stem cells) that produce blood cells. Blood vessels supply blood to the bone, and nerves surround the bone.

Bones undergo a continuous process of remodeling. In this process, old bone tissue is gradually replaced by new bone tissue. Every bone in the body is completely reformed about every 10 years. To maintain bone density and strength, the body requires an adequate supply of calcium, other minerals, and vitamin D.

Bones are covered by a thin membrane called the periosteum. Injury to bone transmits pain because of nerves located mostly in the

periosteum. Blood enters bones through blood vessels that enter through the periosteum.

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. 3).

- Osteogenic cells are pre-osteoblast cells derived from mesenchyme, which is the precursor for all forms of connective tissue. When osteogenic cells undergo mitosis, the resulting daughter cells are called osteoblasts
- Osteoblasts are fully differentiated cells that cannot reproduce. They are responsible for bone formation, secreting the organic substances and mineral salts used in ossification processes. Osteoblast possess receptors for hormone regulating bone growth
- Osteocytes are osteoblasts that have become isolated in the intercellular substance that they have deposited around themselves. They are cells that have stopped laying down new bone, but play a role in maintaining the cellular activities of bone tissue in their immediate area
- Osteoclasts are formed by the fusion of monocytes in the endosteum. They are found on bone surfaces and function in bone resorption. They have receptors for various hormones that regulate their activity

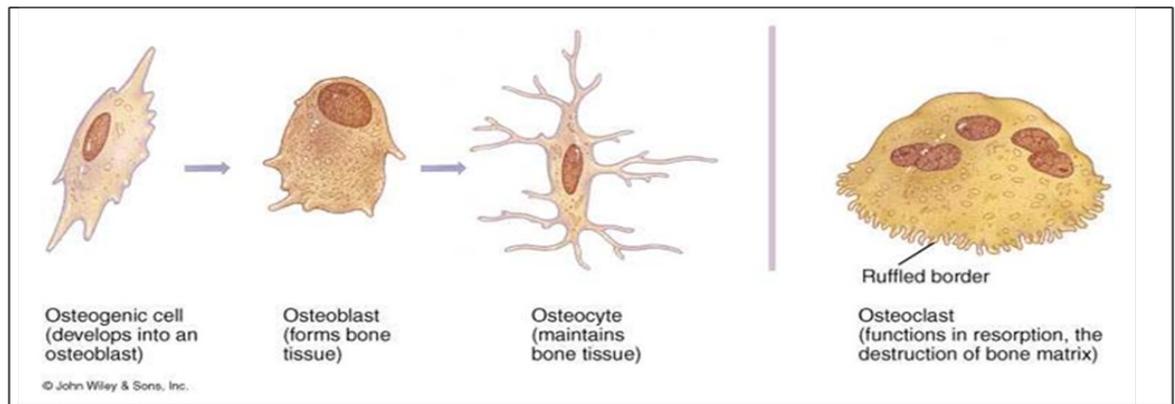


Figure 3: Bone tissue cells

There are four main types of bone cells in bone tissue. Osteogenic cells respond to traumas, such as fractures, by giving rise to bone-forming cells. Osteoblasts (bone-forming cells) synthesize and secrete protein and matrix important for the mineralization and are found in areas of high metabolism within the bone. Osteocytes are mature bone cells made from osteoblasts that have bone tissue around themselves. These cells maintain healthy bone tissue by secreting enzymes and controlling the bone mineral content; they also control the calcium release from the bone tissue to the blood. Osteoclasts are large cells that break down bone tissue. They are very important to bone growth, healing, and remodeling.

4.1 - Ossification

There are two kinds of bone formation: intramembranous ossification and endochondral ossification. In both ossification processes, pre-existing connective tissue is replaced by bone. In intramembranous ossification, some mesenchyme cells are transformed into osteoblasts and start laying down bone.

This is an ossification process that transforms membrane into bone. Additional bone is formed around the edge of this center until the entire membrane is ossified. Intramembraneous ossification is the process used to make flat bones such as the mandible and flat bones of the

skull. In endochondral ossification, a hyaline cartilage model of the bone is replaced and ossified. This is the process associated with fetal bone development, day-to-day bone growth, and fracture repair. This is the type of bone formation found in the development of long bones such as the femur and humerus (Fig. 4).

5 - Bone related pathologies

5.1 - Fractures

A fracture is 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.

5.1.1 - Fracture repair

There are four phases in fracture repair (Fig. 5):

Hematoma formation: when a bone breaks, the blood vessels in the bone, periosteum and surrounding tissues also break. This results in a mass of clotted blood, or hematoma, forming at fracture site

Fibrocartilaginous callus formation: capillaries grow into hematoma and

phagocytic cells consume the cellular debris. Fibroblasts and osteoblasts also migrate into the hematoma from the periosteum and endosteum and starts to produce cartilage. Some fibroblasts produce collagen fibers that link the broken bones together, while some osteoblasts start producing spongy bone. The entire mass of soft tissue produced is called fibrocartilaginous callus and serves to split the broken bone together.

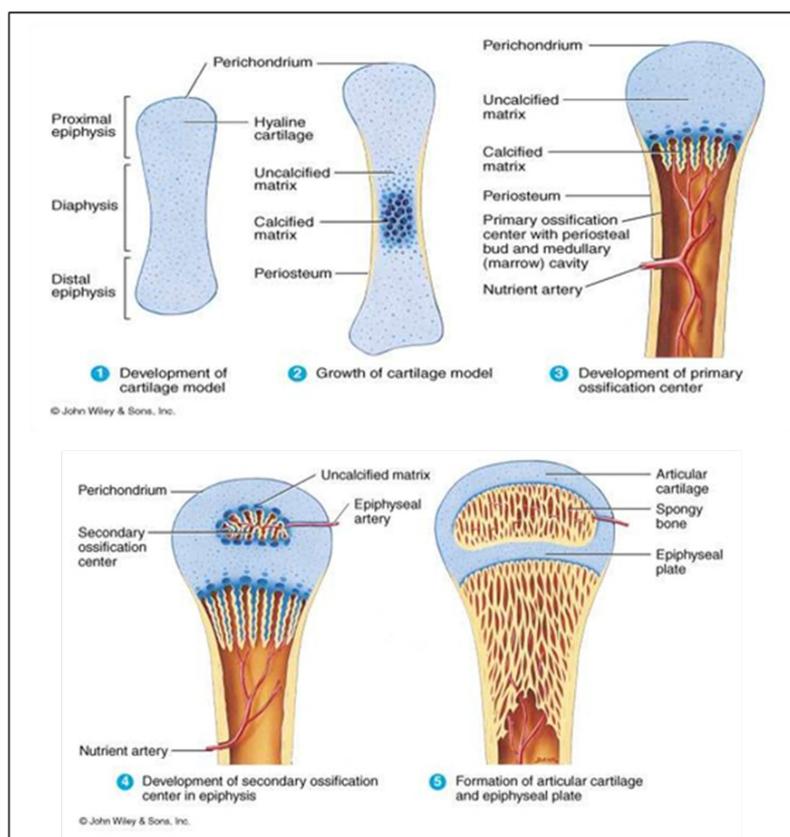


Figure 4: Endochondral ossification

Endochondral ossification begins in points in the cartilage called primary ossification centers. They mostly appear during fetal development, through 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.

Bone callus formation: osteoblasts and osteoclasts continue to migrate

into the fibrocartilaginous callus and convert the existing callus to a bone callus made up of spongy bone.

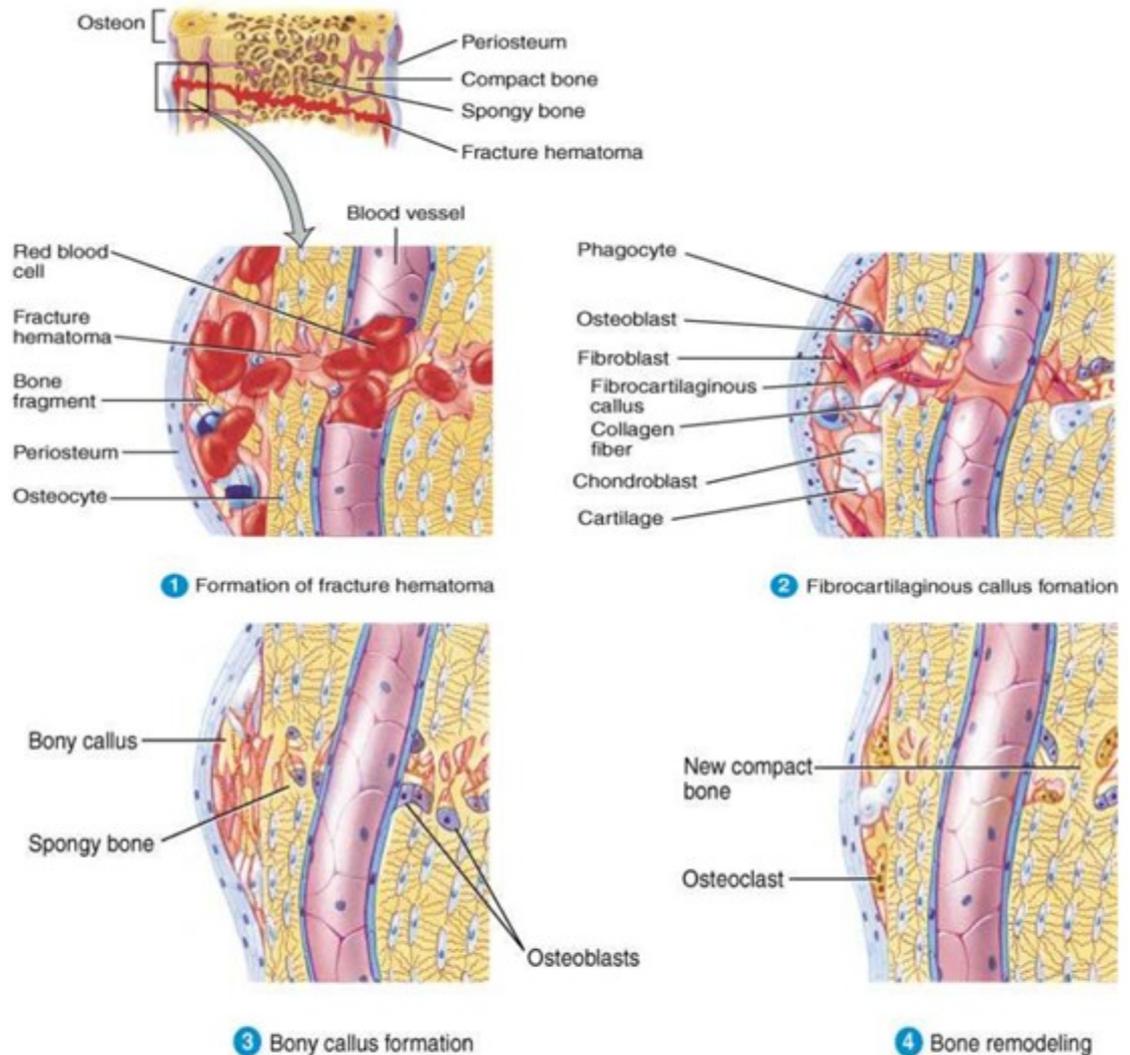
5.2 - Osteoporosis

Osteoporosis refers to a set of diseases in which bone resorption overwhelms bone formation. Normal bone mass becomes reduced, and bones become more porous, weaker, and lighter. Osteoporosis usually affects the spongy bone of the spine, leading to compression fractures between vertebrae. The head of the femur also often fractures in people with osteoporosis.

Estrogen acts to inhibit osteoclasts activity, and when estrogen 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 consequences of other diseases such as hyperthyroidism.

5.3 - 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 years



From Priscilla LeMone and Karen M. Burke, *Medical-Surgical Nursing*, p. 1560 (Menlo Park, CA: Benjamin/Cummings, 1996). ©1996 The Benjamin/Cummings Publishing Company.
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Figure 5: Physiology and process of fracture healing

After the bone fracture, there are four major phases of bone tissue healing: formation of fracture hematoma, fibrocartilaginous callus formation, bony callus formation and bone remodeling.

6 - High-throughput screening

High-Throughput Screening (HTS) allow to quickly conduct large numbers of biochemical, genetic or pharmacological tests. Through these processes it is possible identify active compounds, antibodies or genes which modulate a particular biomolecular pathway. The results of these

experiments provide starting points for understanding the interaction or role of a particular biochemical process in biology and for drug design. Essentially, HTS uses automation to perform screenings of a particular assay against a library of candidate compounds.

An assay is a test for a specific activity such as inhibition or stimulation of a biochemical or biological mechanism. The key labwares for HTS are microplates which generally have 384, 1536 or 3456 wells; these are all multiple of 96 well plates. Each well contains a specific compound to test. After the test, it is necessary manual or automated analysis of results.

Automation is an important element in HTS utility. Typically, an integrated robot system transports assay microplates from station to station for sample and reagent addition, mixing, incubation, and finally read out or detection. An HTS system can usually prepare, incubate and analyze many plates simultaneously; currently HTS robots can test up to 100,000 compounds per day.

Occasionally, HTS cannot be completely automated: in this case an operator need to perform the assay for the candidates compounds. The difference between automated and non automated system depends upon the type and the complexity of the screening.

6.1 - High-throughput screening with RNA interference-based approach

A classic example of activity used by HTS is gene silencing with RNA

interference technique. In fact, many libraries of RNA interference molecules validated for their sequence and acting versus most of the genome sequence of human and mice are available.

Target sequences are selected on the basis of thermodynamic criteria in order to reach a strong silencing efficiency.

Loss of function phenotypes are often the key to understand the function of genes involved in biological processes: in this case RNA interference technique is used to identify the role of a gene towards its silencing.

Recently, a lot of research groups focused their activity on this type of approach: many scientific articles refer about the use of high-throughput screening to investigate about a specific biological process. There are many studies about the investigation of genes functioning in a specific cellular processes in mammalian cells: One example is the research of several genes involved in mammalian cell cycle using a strategy based on RNA-interference (Kittler et al., 2008). Furthermore, HTS had been applied also in other system such as *Drosophila Melanogaster* to find genes involved in development (Perrimon and Mathey-Prevot, 2007).

7 - RNA-Interference

7.1 - Biological functions and effectors

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 block messenger RNA 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

RNA interference 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 non coding transcripts synthesized within the cell (miRNAs, micro RNA): in this case not always the mRNA target is degraded 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 (Lagos-Quintana et al., 2001; Lau et al., 2001; Maurelato et al. 2002).

miRNA are first synthesized as primary miRNA (pri-miRNA) containing the 5' Cap and a polyadenylation 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-miRNA are translocated from nucleus to cytoplasm by a complex of receptors on the nuclear membrane, such as exportin5 (Lund et al., 2004; Bohnsack et al., 2004).

Pre-miRNA 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 drove toward the mRNA target by a RISC complex subunit, Argonata 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 ribosomes 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 bt 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. 7).

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.

7.2 - Off-target effects

Off-target effects are defined as the consequences of not desired interactions between RNA interference molecules and cellular components or no target mRNA.

7.2.1 - Sequence-independent effects

Among the sequence-independent effects there are transfection conditions and inhibition of the endogenous activity of miRNA. miRNA endogenous activity inhibition may be provoked by RNA-interference machinery saturation: an example is the saturation of the exportin5 (Yi et al., 2005).

7.2.2 - Sequence-depended effects

Among the sequence-depended effects there are immune response stimulation and interaction with no mRNA targets. The immune

response can be provoked by type I interferons when siRNA are longer than 30 base pair. Another mechanism is the recognition of single- or double-strand RNA molecules by Toll-like receptors (TLRs) which leads the inflammatory response towards NFkB, IRF and ATF2 transcription factors in the nucleus.

It is possible to reduce the interferon-mediated response avoiding the presence of some nucleotids motifs, such as GU traits, in the small RNA sequences (Judge et al., 2005). The main reason of sequence-depended off target effects is however the interaction between RNA-interference effectors and no target mRNA. To avoid this problem is important verify that a RNA-interference molecule has the minimal omology with other mRNA sequences other than its mRNA target.

Two solution for this problem are redundancy and phenotype rescue experiments (Fig. 8). In the redundancy experiments are used different effectors having the same mRNA target: in this way, probability that two distinct RNA molecules share the same off-target effects is minor. In the phenotype rescue experiments it is used a functional version of the target gene resistant to RNA-interference effector (Echeverri et al., 2006).

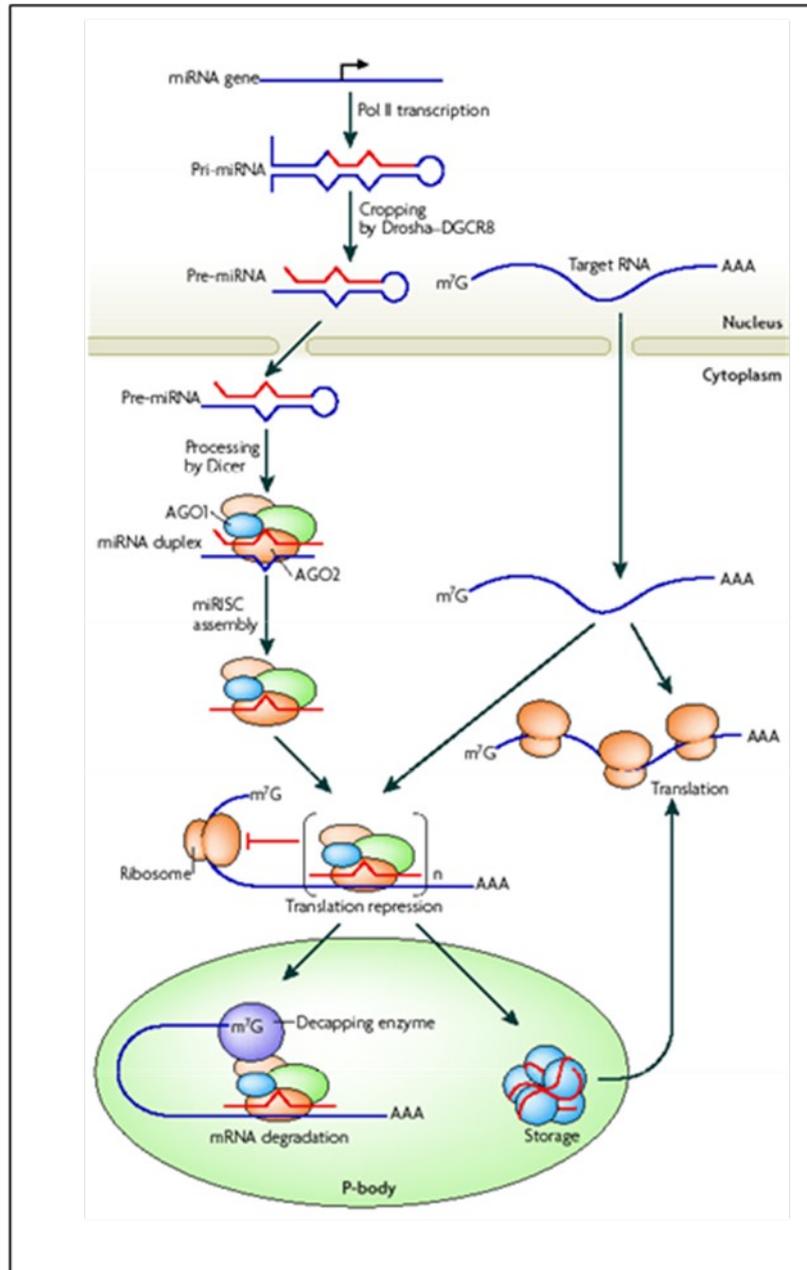


Figure 6: Functional model of the miRISC complex in human

miRNA are transcribed by RNA polymerase II in primary miRNA (pri-miRNA) of 60-100 nucleotids. Pri-miRNAs are cleaved by Drosha to form precursor miRNA (pre-miRNA) of 60-70 nucleotids containing 2 nucleotids 3' protruding. Pre-miRNAs are traslocated into cytplasm 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.

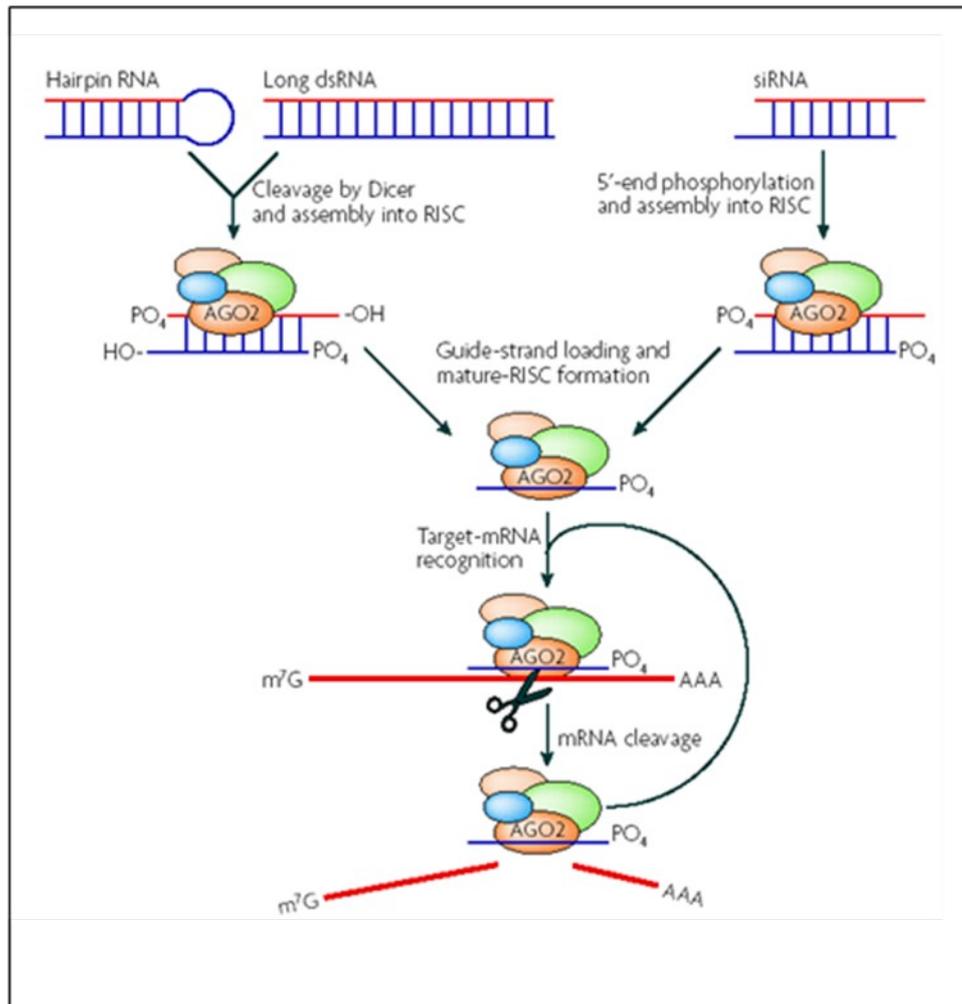


Figure 7: Functional model of the siRISC complex in human

Long double strand RNA or hairpin RNA are cleaved by Dicer into short interfering RNA molecules of 21-nucleotids (siRNAs) with 2 nucleotids 3' protruding and a phosphate group at 5'. Alternatively, siRNAs can be introduced into the cells where are phosphorylated at 5' by cellular kinases. These small RNA assembled into RISC complex that contains AGO2, Dicer and other cellular factors. RISC find mRNA target which is cleaved with the possible recycle of the RISC complex for several cleavage events.

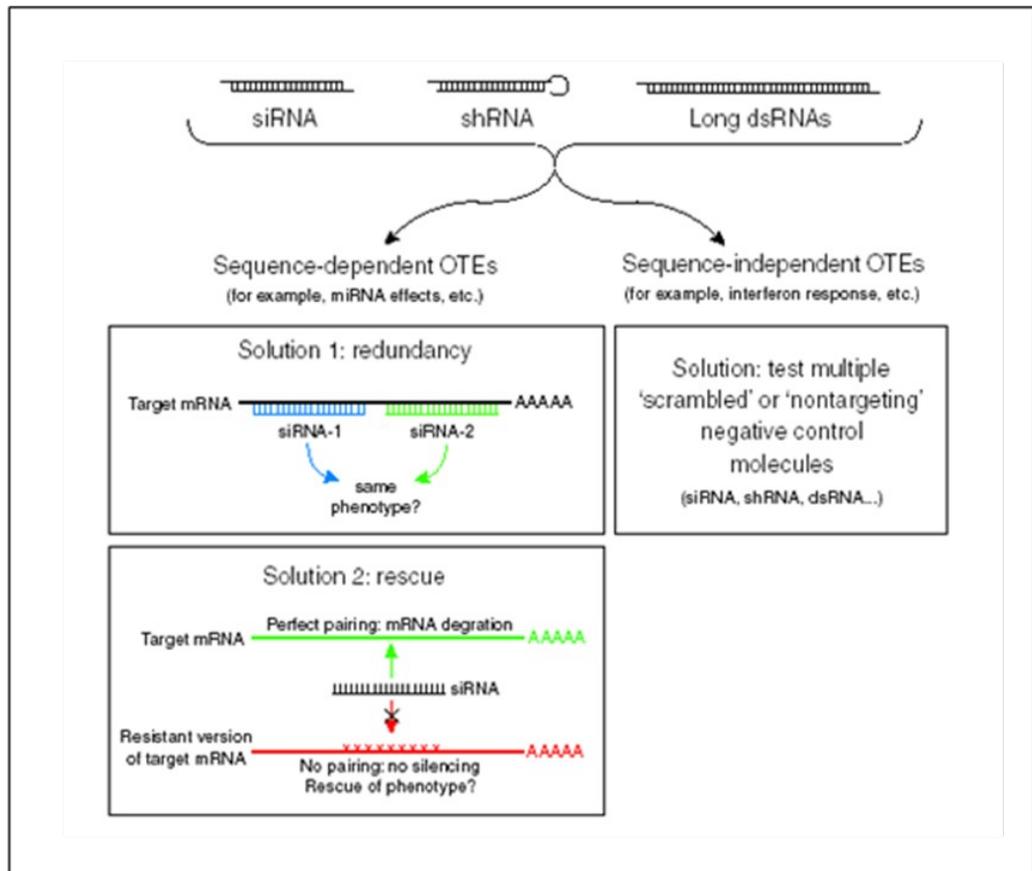


Figure 8: Experimental controls to minimize off-targets effects in i-RNA experiments

In redundancy experiment two RNA interference molecules acting against two different regions of the same target are used. In phenotype rescue experiment a resistant version of the mRNA target is used.

8 - Zinc-finger transcription factors containing the Krüppel-associated box family

The largest family of zinc-finger transcription factors comprises those containing the Krüppel-associated box (KRAB-containing proteins): they were first discovered almost 30 years ago (Bellefroid et al. 1991) and represent the largest single family of transcriptional regulators in mammals. Many genes encoding KRAB-containing proteins are arranged

in clusters, but other occurs individually across the genome. Their structure consist in different domains with a range that can change in the different proteins (Urrutia, 2003) (Fig. 9). The main domains are: KRAB domain located near the N-terminus of the protein which act as a strong transcriptional repressor (Witzgall et al., 1994), and various zinc-finger domains at C-terminus which bind DNA (Looman et al., 2002).

The most remarkable feature of the KRAB-containing proteins is that they are present only in vertebrate genomes (Urrutia, 2003). The KRAB domain is present in the sequences of zinc-finger proteins in the human, mouse, rat, chicken and frog genomes while is absent in fish, *Drosophila*, plants, yeast and other fungi: this distribution suggest that the presence of KRAB domain is relatively recent in the evolution. The functions known for the members of this protein family include transcriptional repression of RNA polymerase I, II and III promoters and binding and splicing of RNA; however, functions of most of the proteins of this family have not been completely studied.

An example of study on one of these proteins was the characterization of a novel zinc-finger transcription factor containing a KRAB domain involved in bone development (Jheon et al., 2001). This study demonstrated the capacity of this factor to modulate Runx2 activity and osteogenic differentiation.

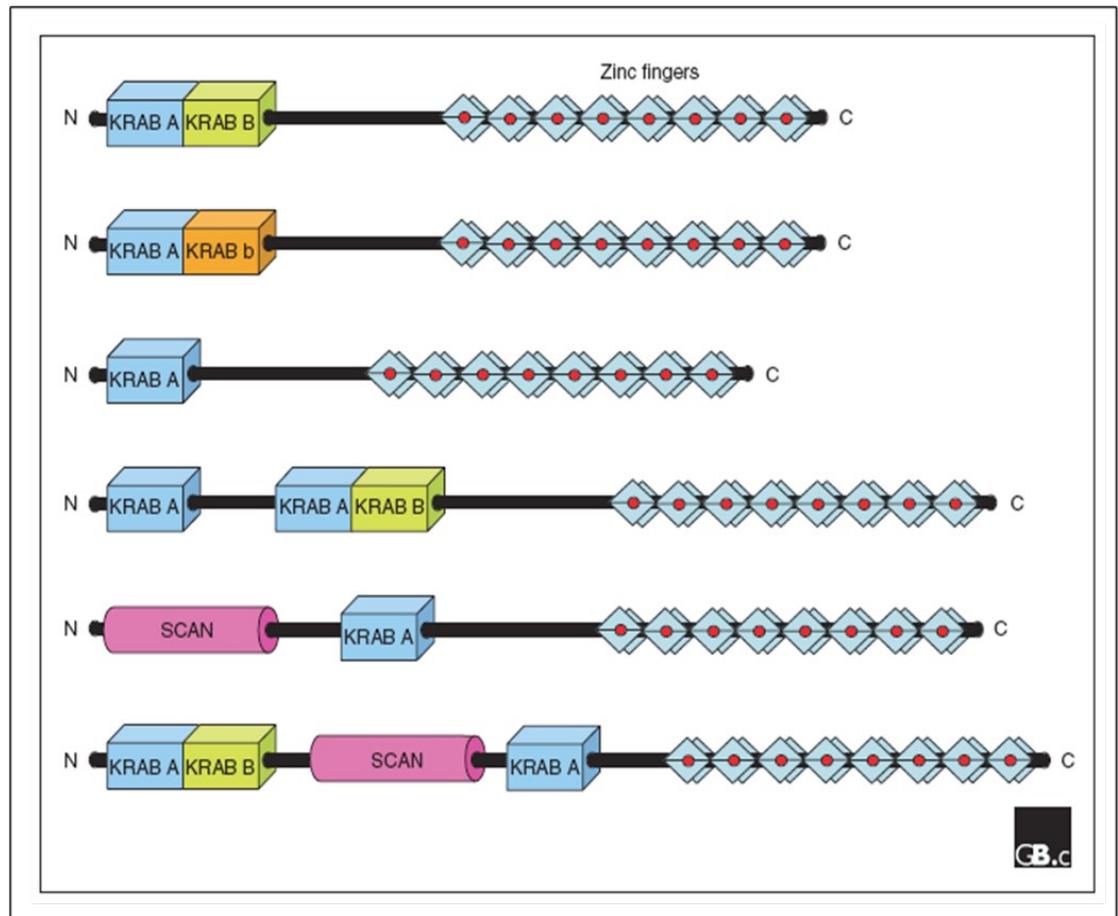


Figure 9: Primary structures of typical KRAB-containing zinc-finger proteins

Different proteins of this family contain different range of functional domains. The number of zinc fingers vary from 4 to over 34 (for simplicity here are shown only 8 for each structure). The KRAB domain consist of the A and B boxes. Some members of the family have a leucine-rich SCAN domain that allows homo- and hetero-dimerization with other SCAN-containing zinc-finger proteins.

9 – Renin-Angiotensin System: Overview

9.1 - The Circulating Renin-Angiotensin System

Angiotensin belongs to renin-angiotensin system (RAS), that is important in regulating blood pressure, sodium homeostasis and fluid balance (Hall JE, 2003). The discovery of a metabolic pathway that contributed a major role to circulatory homeostasis was made in the

1950's (Braun-Mendez E et al., 1940; Page IH et al,1940; Cleland SJ et al., 1997). In the circulating RAS, a decrease in renal perfusion pressure stimulates the production and release of renin, a proteolytic enzyme, from the juxta-glomerular cells of the kidney into the circulation. Renin cleaves angiotensinogen, a circulating α 2-globulin synthesised by the liver, to form the decapeptide angiotensin-I. Angiotensin-converting enzyme (ACE), which is found both as a membrane bound protein at the cell surface and in circulating plasma (soluble ACE), subsequently further cleaves angiotensin-I to form the octapeptide Ang-II (Lavoie JL et al.,2003). Ang-II exerts several effects including salt and water retention via the release of aldosterone from the adrenal cortex, and vasoconstriction (Cleland SJ et al., 1997). Moreover, another important role of ACE is in the degradation and inactivation of BK, which otherwise has potent vasodilatory effects and influences muscle metabolism. Finally, Ang-II can be further degraded to form angiotensin III, angiotensin IV and angiotensin-(1-7). These biologically active peptides may contribute some of the effects of RAS, although their relative contribution is unclear (Campbell DJ, 2003).

9.2 - Angiotensin-II Receptors

Ang-II produces its effects via two main receptors, type I (AT1) and type II (AT2), which were originally classified according to their ability to bind with various ligands antagonists (Murphy et al, 1991; Mukoyama M et al, 1993; De Gaspero M et al,2000). More recently, two other

angiotensin II receptors (AT3 and AT4) have been described, but their role has yet to be clearly defined. AT1 and AT2 receptors are both seven transmembrane domain G-protein coupled receptors, although they appear to have quite different pharmacology.

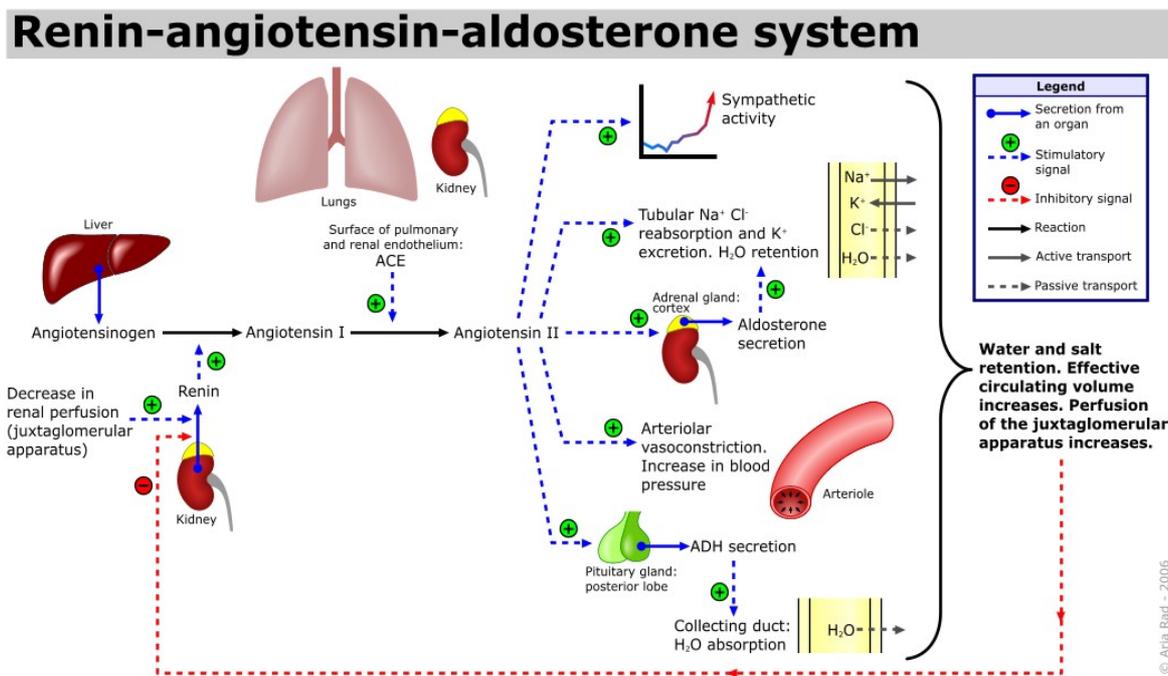


Figure 10: Summary of the Renin-Angiotensin System

Angiotensin is an oligopeptide that causes blood vessels constriction. It is derived from the precursor molecule angiotensinogen, a serum globulin produced in the liver. Angiotensin I is formed by the action of renin on angiotensinogen. Renin is produced in the kidneys in response to decreased intra-renal blood pressure at the juxtaglomerular cells. Angiotensin Converting Enzyme (ACE) catalyses the production of angiotensin II from angiotensin I.

The AT1 receptor is expressed ubiquitously and appears to mediate most of the well known biological effects of Ang-II. In contrast, the role of the AT2 receptor is less well understood. Although it is highly expressed in foetal tissues during development, its expression decreases markedly after birth. However, the AT2 receptor can be

upregulated in pathological conditions, for example after injury, during wound healing and by ischaemia (De Gasparo M et al,2000). It appears to have an opposing role to the AT1 receptor, antagonising its actions. Angiotensin receptor elicits its responses predominantly via G-protein. Ang II stimulates PLC through G_{α_q} coupled to AT1 receptor and subsequently the formation of DAG and IP_3 , that activate PKC and release of Ca^{2+} from the sarcoplasmic reticulum (Hunyady L et al, 2006). AT2 receptor is coupled to G_{α_i} and subsequently to several phosphatases including tyrosine and Ser/Thr phosphatases (Nouet S et al., 2000). Angiotensin receptor can also be stimulated by G protein-independent signal transduction mechanism. Recent studies demonstrated that several scaffold protein can organize signalling complexes for the activation of MAPKs, ERKs and JNKs pathway (Prossnitz ER, 2004; Thomas WG et al., 2003). In cardiac fibroblast and the kidney Ang II-induced AT1r activation also stimulates the Jak/STAT pathway (Booz GW et al., 2002).

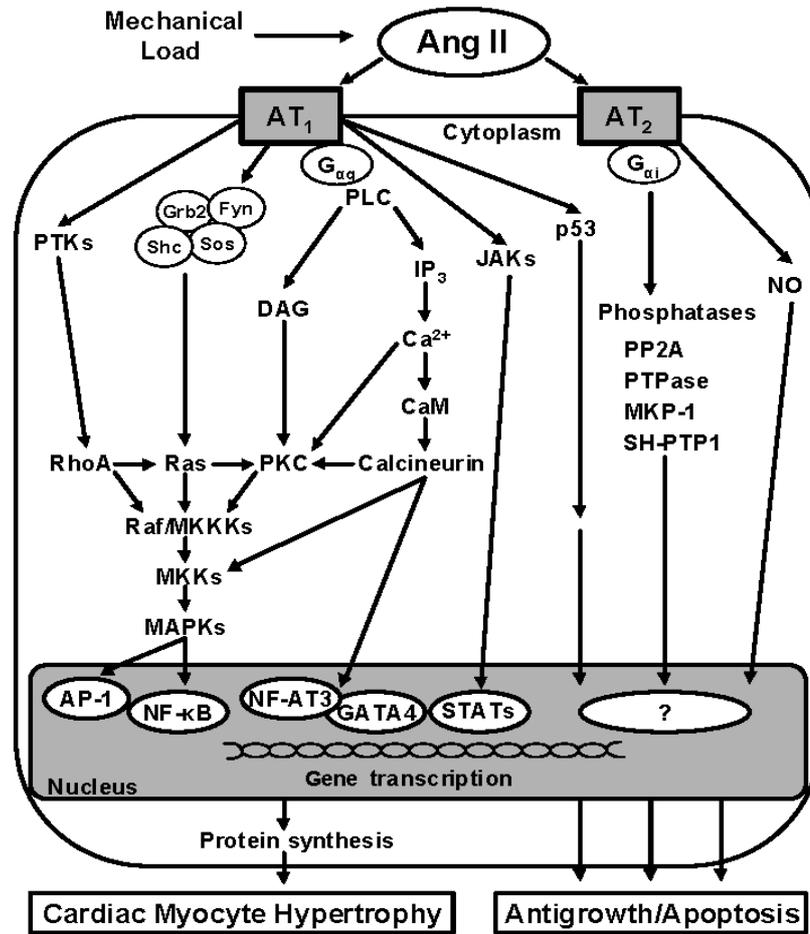


Figure 11: Putative model of signal mechanisms induced by angiotensin II (Ang II)

AP-1, activator protein-1; AT_x, angiotensin II receptor subtype; CaM, calmodulin; DAG, diacylglycerol; IP₃, inositol-1,4,5-triphosphate; JAK, janus kinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MKKK, MAPK kinase kinase; MKP-1, MAPK phosphatase-1; NF-AT3, nuclear factor of activated T-cells 3; NF-κ B, nuclear factor κ B; NO, nitric oxide; PKC, protein kinase C; PLC, phospholipase C; PP2A, protein phosphatase 2A; PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; SH-PTP1, Src homology domain-containing protein tyrosine phosphatase 1; STAT, signal transducer and activator of transcription.

9.3 - The Local Renin-Angiotensin Systems

In addition to the circulating RAS, local RAS have also been described in various tissues including the heart, brain, lung, pancreas and adipose

tissue (Jones A et al,2003; Izu Y et al, 2009). A local RAS has now been discovered in skeletal muscle (Dragovic T et al.,1996; Schaufelberger M et al, 1998). This local skeletal muscle RAS depends on a combination of in-situ synthesis of all the RAS components and the uptake of renin or pro-renin from the circulation (Danser AH et al, 2003). ACE has been identified on both skeletal muscle membranes and on endothelial cells of the capillary bed (Dragovic T et al.,1996; Schaufelberger M et al, 1998). Osteoblasts and osteoclasts have been demonstrated to express angiotensin receptors 1 and 2 (AT1R and AT2R, respectively) and, recently, involvement of the RAS in osteoporosis development has been demonstrated (Shimizu H et al, 2008; Asaba Y et al, 2009); in addition, AT2R blockade has been shown to increase bone mass (Izu Y et al, 2009). Angiotensin II (Ang II) treatment of osteoblast seems to indirectly stimulate osteoclasts proliferation through expression of the receptor activator of NF-kb ligand (RANKL) (Shimizu H et al, 2008); however, results regarding the use of AT1R inhibitor are controversial (Izu Y et al, 2009; Shimizu H et al, 2009, Broulik PD et al, 2001). On the other hand, inhibition of RAS blocks primary calvaria osteoblasts differentiation and mineral deposition in vitro (Hagiwara H et al, 1998). The role of this local RAS is still debated but may include growth stimulation, neovascularisation, and the regulation of inflammation (Igic R et al, 2003). The importance of tissue RAS as a potential therapeutic target comes from evidence that the anti-hypertensive properties of ACE inhibitors correlate better with the inhibition of tissue ACE, rather

than plasma ACE. Furthermore, the additional benefits of ACE inhibitors such as improved endothelial function and reduction in inflammation, appear to be independent of changes in blood pressure, and are thought to relate to effects on local RAS systems (Dzau VJ et al, 2001). In addition, AT1R inhibitors, such as losartan, have been effective in the treatment of Marfan syndrome, a disease due to mutation in the fibrillin I gene that leads to an excess of TGF-beta signaling (Hagiwara H et al, 1998; Brooke BS et al, 2008; Cohn RD et al, 2007; -25); TGF-beta had been previously identified as one of the genes involved in mesenchymal cell proliferation and in mesenchymal progenitors commitment (Roelen BA et al, 2003).

MATERIALS AND METHODS

10 - MATERIALS

10.1 - Mouse shRNA library

shRNA library consist in 64,000 bacterial stocks containing plasmid DNA expressing shRNA constructs that cover the entire mouse transcriptome. Bacterial stock are stored in single wells in 96 well plates in glycerol stock at -80°C (Fig 11).

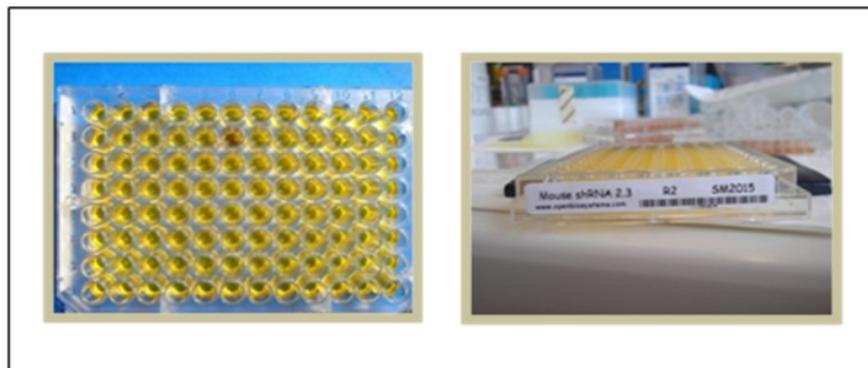


Figure 11: Images of a mouse shRNA library plate

Bacterial stock of the shRNA library are stored in single wells in 96 well plate (left side). Library plates have an ID number and a bar-code to identify the specific plates for each shRNA construct (right side).

10.1.1 - Bacterial cells

PirPlus (Pir-1)-competent bacteria (Open Biosystems). It's a *Escherichia Coli* stock expressing the pir1 gene.

10.1.2 - Plasmid DNA

pSM2 (pShag Magic Version 2.0; Open Biosystems) retroviral vector. This plasmid contains the following features: 1) Murine Stem Cell Virus (MSCV) backbone with Retroviral Signaling Sequences; 2) U6 promoter; 3) RK6γ conditional origin of replication. Requires the expression of pir1 gene within the bacterial host to propagate; 4) Kanamycine and Chloramphenicol resistance as bacterial selection markers; 5) Puromycine resistance for selection after transfection in mammalian cells . 6) Sequence that express the shRNA construct (only sequence difference between all the plasmids (Fig 12).

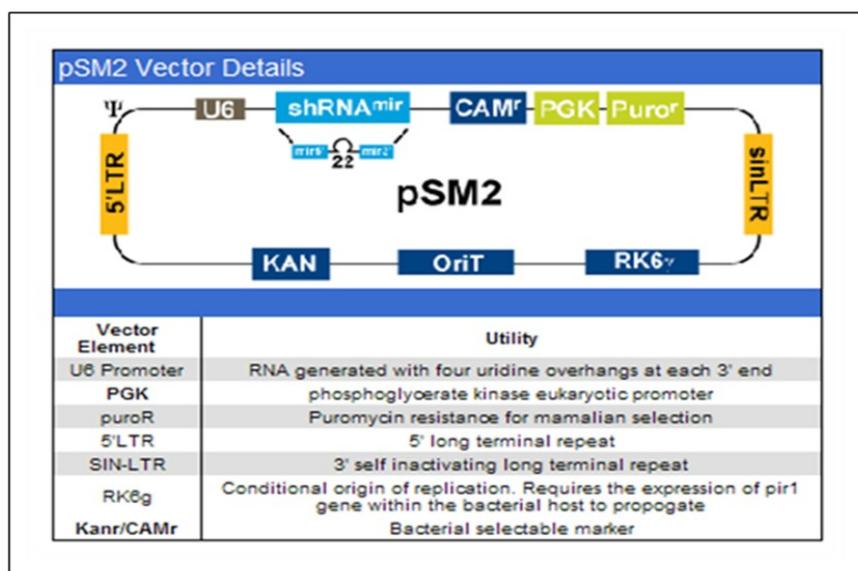


Figure 12: pShag Magic Version 2.0 features

Structure of the DNA plasmids contained within bacterial stocks of the mouse shRNA library. Each plasmid contain a DNA segment for the expression of a specific DNA construct. Furthermore, plasmids contain several common features.

10.2 - Cell cultures

10.2.1 - Culture of cell lines

W20-17 cells were obtained from ATCC (American Type Culture Collection). Cells were cultured in Dulbecco's Modified Eagle's Medium, High Glucose (D-MEM) (Euro Clone) supplemented with 10% selected Fetal Bovine Serum (FBS) (HyClone) and 2mM L-Glutamine (Gibco, Invitrogen).

10.2.2 - Culture of murine bone marrow stromal cells

Murine Bone Marrow Stromal Cells (mBMSC) were obtained by isolation from mouse tibias in our laboratory (Esposito et al., 2009). Cells were cultured in alpha minimum essential medium (α -MEM) (Cambrex Bio Science) supplemented with 10% selected Fetal Bovine Serum (FBS) (HyClone) and 2mM L-Glutamine (Gibco, Invitrogen).

10.3 - Differentiation media

10.3.1 - Osteogenic medium

D-MEM supplemented with 10% selected FBS, 2mM L-Glutamine, 0.05 mM ascorbic acid 2-phosphate (Sigma Aldrich), 10 mM β -glycerolphosphate (Sigma Aldrich), 5×10^{-3} M dexametasone (Sigma Aldrich). Angiotensin II (Sigma, St. Louis, MO US), PD123319 (Sigma, St. Louis, MO US) and losartan potassium (Fluka, St. Louis, MO US) was

added to the medium every day accordingly with the differentiation protocol.

10.3.2 - Adipogenic medium

D-MEM supplemented with 10% selected FBS, 2mM L-Glutamine, 5µg/mL insulin (Sigma Aldrich), 0,5 µM isobutylmethylxanthine (IBMX) (Sigma Aldrich), 50 µM indometacin (Sigma Aldrich).

10.4 - Solutions

10.4.1 - Trypsin/EDTA

0.2 % Trypsin (Invitrogen), 1% chicken serum (Euro Clone), 10 mM EDTA (pH=8).

10.4.2 - Formalin 10%

Cells fixing solution. 10% Formaldehyde (Sigma Aldrich), 90% Water.

10.4.3 - Alizarin Red S

2% Solution (2 g Alizarin Red S powder per 100 mL water). pH = 4.1-4.3.

10.4.4 - Solutions for ALP staining

Fixing solution:

Neutral Formalin Buffer (NFB) 10% (+4°C).

Staining solution:

Naphtol AS MX-PO₄ powder (0.005 g) in DMF (N,N-Dimethylformamide) (200 µL).

Tris-HCl (MW = 157.6) 0.2 M, pH 8.3 (25 mL) + distilled water (25 mL).

Red Violet LB salt (0.03 g)

Note: Prepare (a) and (b) separately; add (a) + (b); add (c).

10.4.5 - Oil Red

0.5% solution (0.5 g Oil red-O per 100 mL water).

10.4.6 - Guanidine HCl

Used for Alizarin Red S staining quantitation; it was prepared at 4M.

11 - METHODS

11.1 - Transfection with Lipofectamine™ 2000

Transfection with Lipofectamine™ 2000 (Invitrogen) was performed following the procedure's guidelines. Initially, cells were plated in the plastic support and allowed to grow overnight to reach the confluence of 70%-80% optimal for the transfection. The day after, cells were transfected with different DNA: DNA and Lipofectamine 2000 were separately diluted in D-MEM without serum and incubated for 5 minutes at room temperature (the amount of the dilution medium and of DNA and lipofectamine depends upon the plastic support used). After the 5

minutes incubation the diluted Lipofectamine and diluted DNA were combined, mixed gently and incubated for 20 minutes at room temperature. Finally the reagent/DNA mixture was added to the plated cells after the change of the medium in which they were. After 6 hours, medium with the mixture was removed and replaced with standard medium.

In the table 1 are reported volumes of medium and quantities of DNA and reagent used in our experiment in 96 and 6 well plates (Tab. 1).

CULTURE VESSEL	SURFACE AREA PER WELL	VOLUME OF PLATING MEDIUM	VOLUME OF DILUTION MEDIUM	DNA	LIPOFECTAMINE™ 2000
96 well	0,3 cm ²	100 µL	2 x 25 µL	0,2 µg	0,5 µL
6 well	10 cm ²	2 mL	2 x 250 µL	4,0 µg	10 µL

Table 1: Quantity of components for DNA transfection in 96 and 6 well plates

Note: For DNA transfection in 96 well plates during the screening a gene reporter plasmid was co-transfected at concentration of 70-100 ng per well in order to evaluate the efficiency of the transfection after 24-48 hours.

11.2 - Puromycin selection

Cells transfected with shRNA plasmids of the library were cultured for 3 days in medium supplemented with puromycin in order to enrich cell population that incorporated the plasmid DNA. Puromycin amount was established on the basis of puromycin kill-curve experiments on cells for 3 days: for W20-17 the concentration of puromycin used is 2.0-2.5

µg/mL.

11.3 - Differentiation assay

11.3.1 - Osteogenic differentiation

Cells are cultured for 21 days with osteogenic medium (section 10.3.1) at 37°C in humidified atmosphere at 5% CO₂. Osteogenic medium is replaced every 3-4 days.

11.3.2 - Adipogenic differentiation

Cells are cultured for 14 days with adipogenic medium (section 10.3.2) at 37°C in humidified atmosphere at 5% CO₂. Adipogenic medium is replaced every 3-4 days.

11.4 - Staining assay

11.4.1 - Alizarin Red staining

Cells were washed with PBS and fixed in 10% formaldehyde for 1h; after rinsing with distilled water they were incubated with Alizarin Red S 2% solution (section 10.4.3) with gentle agitation for 10 min. Excess staining was removed using PBS.

11.4.2 - ALP staining

Cells were washed with cold PBS and fixed with 10% cold NFB (section

10.4.4) for 15 min. Cells were incubated with the staining solution (section 10.4.4) for 15 min and then were washed 3-4 times using distilled water.

11.4.3 - Oil Red staining

Cells were washed with PBS and fixed in 10% formaldehyde for 15 min; after rinsing with distilled water, they were incubated with Oil Red-O working solution (section 10.4.5) for 10 min. Excess staining was removed using PBS and cells were then counter-stained hematoxylin (Bio-Optica) for 3 min and washed with distilled water.

11.5 – Staining Quantitation assay

11.5.1 - Alizarin Red staining quantitation

Alizarin Red S solution bound to the calcium deposits was extracted using over-night incubation with Guanidine HCl 4M (10.4.6) at room temperature. Absorbance at 490 nm of the resulting supernatant was used for quantitative calcium determination.

11.5.2 - Alkaline phosphatase assay

The cells were washed twice with Tyrode's balanced salt solution (50mM Tris-HCl pH 7.4, 0.15M sodium chloride) and then incubated with 5mM p-nitrophenylphosphate in 50mM glycine, 1mM magnesium chloride pH 10.5 at 37°C for 5 to 20min. The reaction was stopped by adding 3M

sodium hydroxide to each well. Optical density (OD) was measured at 405nm.

11.5.3 - Oil Red staining quantitation

The lipid bound dye was extracted using isopropanol incubation for 15 min at room temperature. Quantitative assessment was obtained by measuring adsorbance of the extracted dye at 550nm.

11.6 - Proliferation assays

W20-17 cells were seeded in 96 microwell plates and cultured for 4 days in osteogenic differentiation media. Then cells were washed with PBS and cultured with regular media for 48 h. The proliferation was assessed with CellTiter 96 AQueous Non-Radioactive assay (Promega, Madison, WI US) in according to manufacturer's instructions and the absorbance measured at 490nm.

11.7 - RNA extraction

TriReagent (Sigma Aldrich) was used in order to extract RNA from the cells. 1 mL of TriReagent was added to the cells (1 mL per each well of a six well plate in our experiments) after aspirating culture medium and washing with PBS. After addition of the reagent the cell lysate was passed several times through pipette to form an homogenous lysate. After homogenization, the homogenate was centrifuged at 12,000 g for

10 minutes at 4°C. Following the centrifuge the clean supernatant was transferred to a fresh tube and left for 5 minutes at room temperature. After, 0.2 ml of chloroform was added per 1 ml of TriReagent used initially. The sample was shook vigorously for 15 seconds and allowed to stand for 2-15 minutes at room temperature, resulting mixture was centrifuged at 12,000 g for 15 minutes at 4°C.

After centrifugation the upper aqueous phase containing RNA was transferred in a fresh tube, mixed with 1/10 of isopropanol, left for 5 minutes at room temperature and centrifuged at 12,000 g for 10 minutes. Subsequently, the supernatant was transferred to a fresh tube and the RNA was precipitated by adding 0.5 ml of isopropanol per 1 ml of TriReagent used in sample preparation. The sample was allowed to stand for 5-10 minutes at room temperature and then it was centrifuged again at 12,000 g for 10 minutes at 4°C. After this centrifugation the supernatant was removed and the RNA pellet washed by adding 1 ml of 75% ethanol per 1 ml of TriReagent used initially; the sample was centrifuged at 7,500 g for 5 minutes at 4°C. Finally, the supernatant was removed, the RNA pellet dried for 5-10 minutes by air-drying and resuspended in an appropriate volume of water.

11.8 - RT PCR

2 µg of RNA extracted was added to random nanomers, dNTP mix and water (until 16 µL of final volume). Mix was incubated at 70° for 10 min and then in ice for 1 min. After that, were added Reverse transcriptase

buffer (1X) RNAsi inhibitor and M-MuLV Reverse Transcriptase enzyme (New England BioLabs) (final volume: 20 μ L). Program for reaction was: 42° for 1 h and 90° for 10 min.

11.9 - Real-Time PCR

Real-Time PCR was performed with LightCycler machine using Sybr Green as probe. PCR program for each cycle as: 50° for 2 min, 95° for 10 min, 95° for 15 sec, 60° for 1 min, 95° for 15 sec, 60° for 15 sec. Control is represented by GAPDH determination.

11.10 - Western Blot

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), proteinase inhibitors (Complete Protease Inhibitor Cocktail, Roche) and phosphatase inhibitors (sodium fluoride 5mM and sodium orthovanadate 1mM). Protein extracts were run on 10% SDS-PAGE gels, transferred on Immuno-Blot PVDF membranes (Bio-Rad) and analyzed using the following antibodies: Phospho-Smad2/3 (Cell Signaling, Danvers, MA US), Smad2/3 (Cell Signaling, Danvers, MA US), Phospho-Smad1,5,8 (Cell Signaling, Danvers, MA US), Smad1,5,8 (Cell Signaling, Danvers, MA US), Anti-Flag (Cell Signaling, Danvers, MA US), Anti-GAPDH (Cell Signaling, Danvers, MA US) and secondary HRP-conjugated antibodies (Amersham Bioscience, Uppsala, Sweden). The membrane was detected by chemiluminescence (ECL plus, GE Healthcare)

11.11 - Immunofluorescence

Cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% TX-100, 10% normal fetal bovine serum/1% bovine serum albumin in PBS for 30 min at room temperature. The samples were incubated with primary murine antibody anti-Flag (1:250) and then with an anti-mouse immunoglobulin G conjugated with Alexa Fluor 488 (Molecular Probes, Eugene). Images were captured using an inverted microscope (DMI4000, Leica Microsystem).

RESULTS

12 - Components and experimental conditions set up

12.1 - Mouse shRNA library amplification

Bacterial stock containing shRNA plasmids have been inoculated to amplify the mouse shRNA library. The entire procedure of amplification has been automated using Biomek FX robot from Beckman (Fig. 13). Bacterial stock were stored in 96 well plates, and our plasmid preparations were performed in the same support. In this way we have been able to directly transfect all the plasmids in W20-17 cells seeded in 96-well plate.

To date 15,000 construct have been amplified, corresponding to 150 plates of the library. All the plates were catalogued in order to know the exact plate and well position for each construct.



Figure 13: Biomek FX Robot from Beckman

Biomek FX Robot used to perform automated mini-prep procedure of the bacterial stock of the library.

12.2 - *In-vitro* osteogenic differentiation

In order to differentiate W20-17 cells into osteoblasts, we treated them for 21 days with osteogenic medium or D.A.G. medium (Dexametasone, Ascorbic Acid-2P and β -Glicerophosphate). Treatment of mesenchymal precursors with this medium allows to obtain a good differentiation since these reagents act as osteogenic differentiation inducers.

In order to evaluate completion of osteogenic differentiation we performed an Alizarin Red S staining: cells differentiated into osteoblasts form calcium deposits which are stained in red and are well visible at microscope analysis. We also included in our experiment cells treated with normal medium as negative control (Fig. 14).

We also quantify Alizarin Red S staining using 4M Guanidine-HCl extraction and absorbance reading at 490nm.

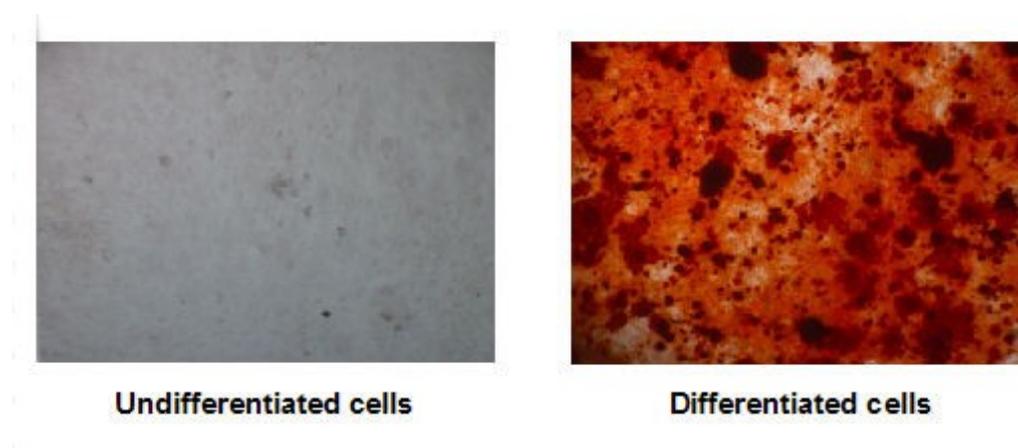


Figure 14: Osteogenic differentiation of W20-17 cell line

Alizarin Red S staining of W20-17 cells cultured in either the presence or absence of osteogenic medium. Cells not cultured with osteogenic medium were grown with normal medium.

In order to further analyze the osteogenic differentiation process in our cells, we performed a time course with staining and quantitation of Alizarin Red adsorbance cells at different time points from day 0 until

day 21. We choose day 5, 7, 9, 12, 14, 16, 18 and 21 as different time points and we grew cells in osteogenic and normal medium. For the cells grown in osteogenic medium, Alizarin Red S staining positivity is evident starting from day 12 with a gradual increase until day 21. On the contrary, cells grown in normal medium do not show positivity to Alizarin Red S staining for any of the time points considered (Fig. 15A). Microscope analysis of stained cells was confirmed by quantitation of staining: in fact, there is an increase of absorbance at 490 nm in the wells containing cells treated with osteogenic medium, while the absorbance remains constant in the wells containing cells treated with normal medium (Fig. 15B).

12.3 - W20-17 cell line characterization

In order to characterize W20-17 cells differentiation into osteoblasts we evaluated the expression of several osteogenic marker genes along the entire process; this analysis was necessary to demonstrate the effective changes at molecular levels during differentiation. At this purpose, we evaluated the expression of RunX2 and Osterix, as early markers, and Osteocalcin and Osteopontin, as late markers, at different time points: T0, T4, T14 and T21.

RunX2 expression has a bimodal curve: it increases from day 0 to day 4, then it decreases at day 14 to increase again at the last time point (Fig. 16A). On the other hand, Osteopontin levels increase steadily from day 0 to day 21 (Fig. 16B). Osterix and Osteocalcin present an

expression pattern similar to Osteopontin, with a very high pick at day 21 (Fig. 16C and 16D).

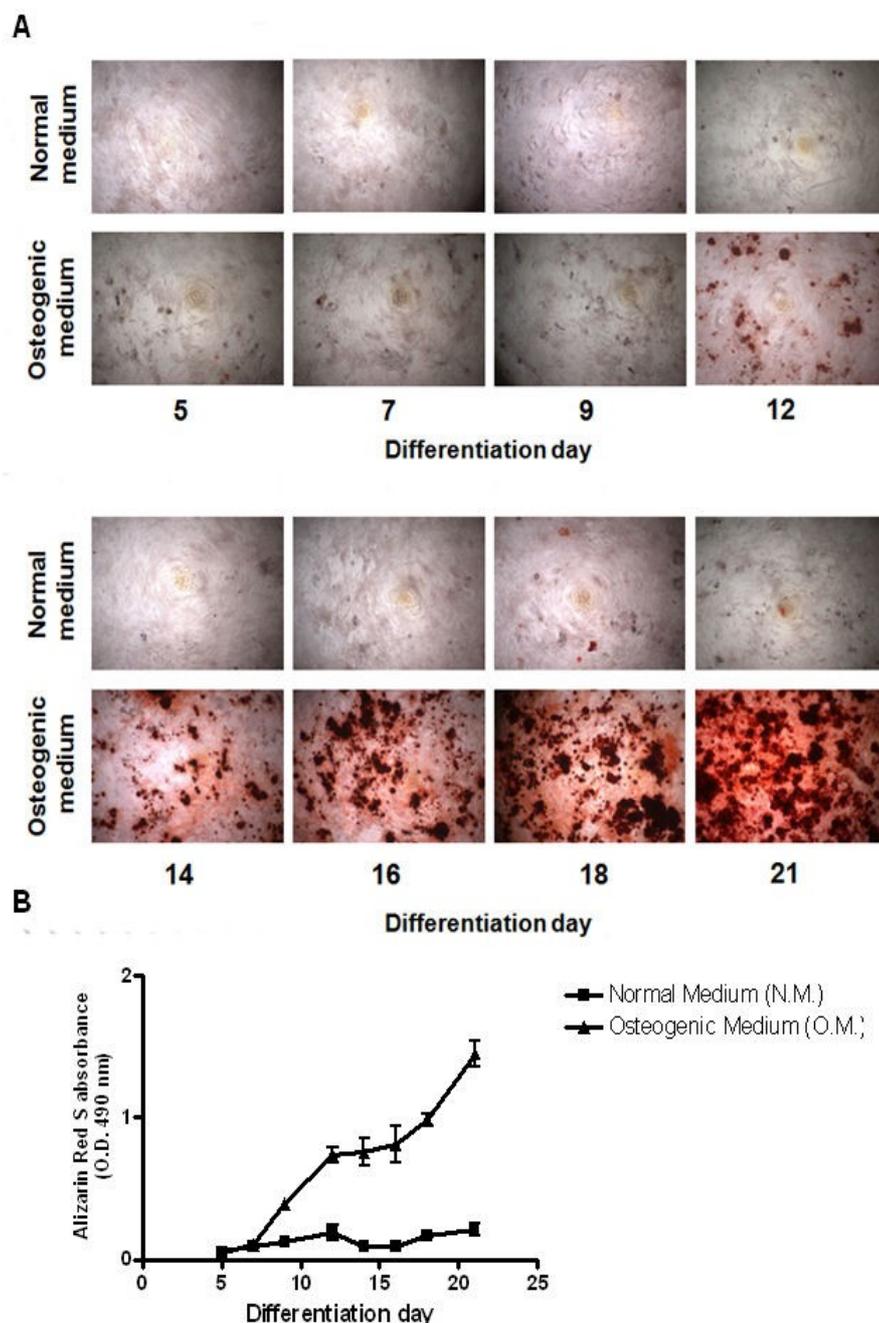


Figure 15: Osteogenic differentiation time course of W20-17 cell line

Alizarin Red S staining of W20-17 cells cultured in normal and osteogenic medium at days 5, 7, 9, 12, 14, 16, 18 and 21 (A). Calcium deposition quantitation using Guanidine HCl 4M and adsorbance reading at 490 nm (B).

12.4 - Experimental approach validation

Prior to the screening we verified our experimental approach and tested the functionality of shRNA constructs of the library in the inhibition of the differentiation process. We prepared a 96-well plate with several plasmids expressing shRNA constructs against genes previously shown to be involved in the osteogenic differentiation process. We chose about 40 genes with 64 corresponding constructs. Among these genes we included transcription factors, such as Runx2 and Osterix, growth factors, such as BMPs and FGFs, cellular regulators, such as SMAD protein and also differentiation markers such as osteocalcin and genes codifying for matrix proteins such as collagen.

We transfected W20-17 cells with the 64 constructs in a 96-well plate and proceeded with puromycin enrichment for three days in order to obtain a larger number of cells containing the library plasmids.

After puromycin treatment we continued with osteogenic differentiation for 21 days and, finally, we performed Alizarin Red S staining and its quantitation with Guanidine HCl 4M (Fig. 17).

In this experiment we chose several controls: positive and negative controls of differentiation, respectively represented by untransfected cells grown in osteogenic and normal medium, and a scrambled control for the RNA-interference technique, achieved using a library construct codifying for an shRNA that does not interfere with any known gene, shRNA Non Silencing (shRNA-NS). For this experiment we also chose several controls to use during the screening, obtained using shRNA

constructs that interfere with genes whose role in osteogenic

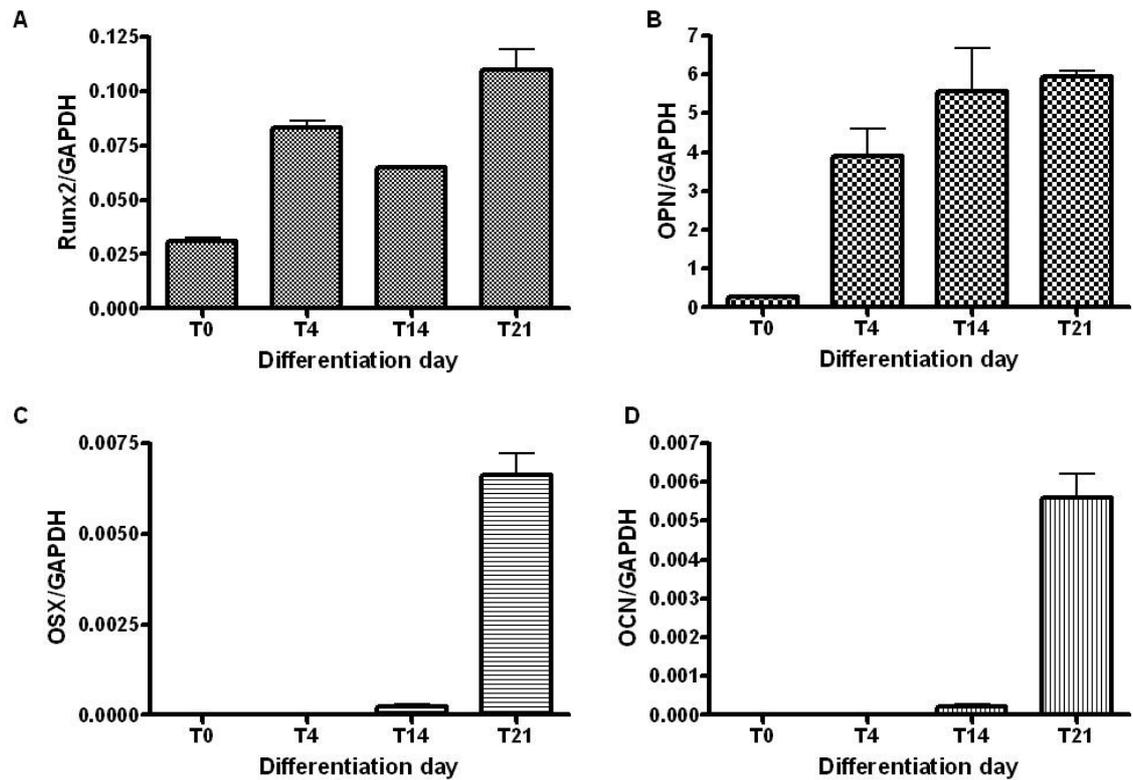


Figure 16: Expression analyses of osteogenic differentiation markers during osteogenic differentiation of W20-17 cells.

Expression profiles of Runx2 (A), Osteopontin (B), Osterix (C) and Osteocalcin (D) at days 0, 4, 14 and 21 of differentiation of W20-17 cells. Data are means \pm SD of 3 experiments.

differentiation is well demonstrated. Following Alizarin Red S staining after 21 days of osteogenic differentiation, results were variable:

for some genes we did not obtain any osteogenic differentiation impairment, while for other genes we obtained different levels of silencing, in some cases similar to the negative control of differentiation.

Cells transfected with the shRNA-NS showed staining result similar to those obtained for the positive control of differentiation. Finally, for constructs interfering with genes with a demonstrated role in osteogenic

differentiation we obtained different levels of Alizarin Red S staining impairment. We reported staining and relative quantitation for the negative and positive control of osteogenic differentiation; furthermore, we reported the staining for cells transfected with the shRNA-NS and cells transfected with shRNA interfering with Smad5 and Bmp1 genes as best examples of genes involved in the osteogenic differentiation which silencing produced effects in our experiment (Fig. 18A and 18B). Library constructs that interfere with these two genes were chosen as control for the screening.

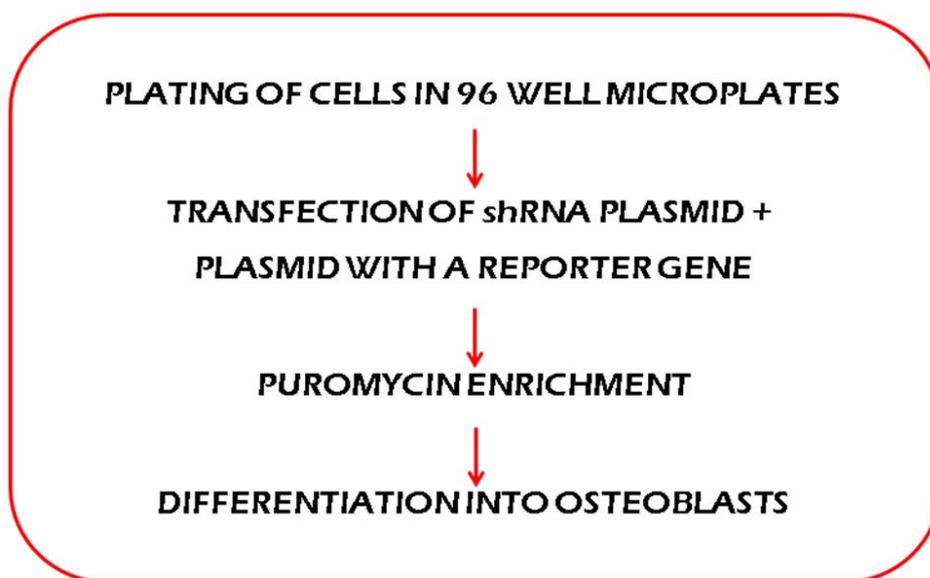


Figure 17: Experimental approach to perform the screening

Cells are plated in 96 well-plates and transfected after one day with shRNA plasmids. Co-transfection with a plasmid for the expression of a reporter gene is performed to supervise transfection efficiency. Then, cells are cultured for 2-3 days in presence of puromycin in order to enrich cellular population that incorporated the shRNA plasmids. Finally, cells are cultured in osteogenic medium to differentiate into osteoblasts.

13 - Screening

13.1 - Transfection of shRNA mouse library

To date we have transfected about 10,000 constructs of the library in single wells and we collected the relative results. We acquired pictures of plates after staining and of the specific wells with the most interesting results. Collection of the pictures, together with the

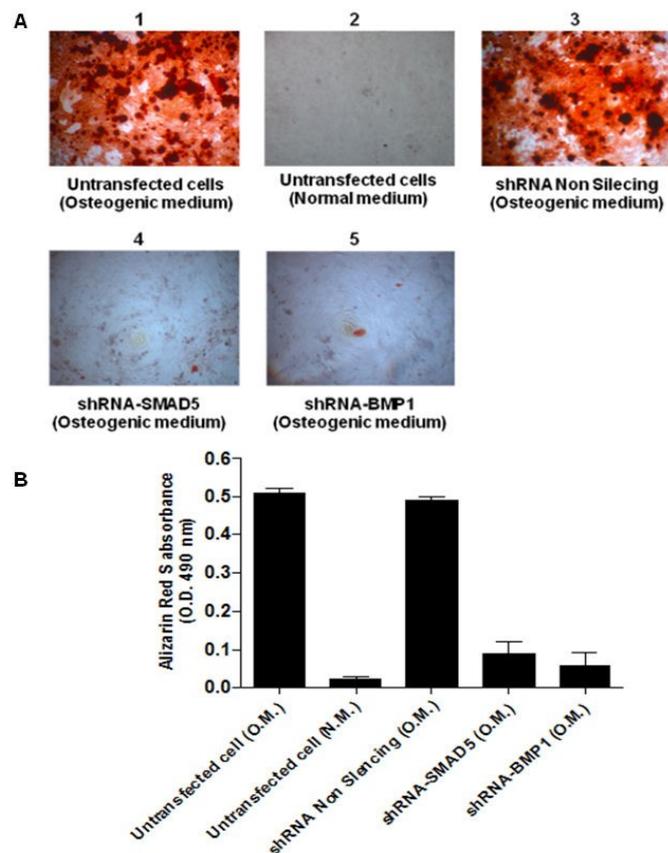


Figure 18: Experimental approach validation

Alizarin Red S staining after 21 days of: 1) untransfected W20-17 cells cultured in osteogenic medium; 2) untransfected W20-17 cells cultured in normal medium; 3) W20-17 cells transfected with shRNA Non Silencing and cultured in osteogenic medium; 4) W20-17 cells transfected with shRNA-SMAD5 and cultured in osteogenic medium; 5) W20-17 cells transfected with shRNA-BMP1 and cultured in osteogenic medium (A). Calcium deposition was quantified using Guanidine HCl 4M and reading the adsorbance at 490 nm (B). Data are means \pm SD of 3 experiments. collection of the data corresponding to the quantitation of the staining,

was crucial to catalogue and record all the results of the screening (Fig.19).

Quantitation of the staining was very important to select a pool of most relevant candidate genes: in fact, we established a threshold of the OD sample/OD positive control of differentiation value and on the basis of this value we were able to further consolidate our results.

Up to now we have identified 650 candidate genes that correspond to 6.5% on the total number of the screened genes.

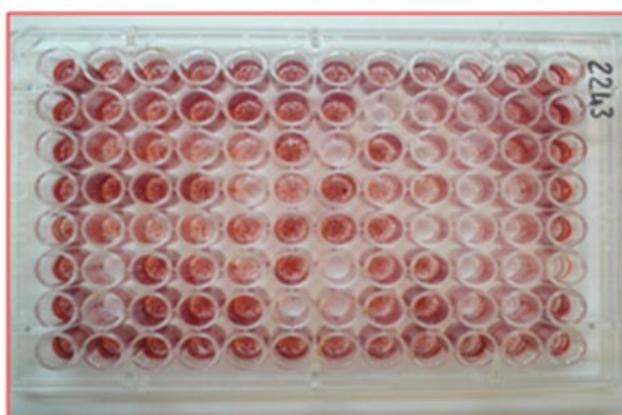


Figure 19: Representative image of a plate after Alizarin Red S staining

This is a representative image of a plate as example of our screening results. Several wells don't show red staining: genes silenced in these wells are putative candidates of our screening.

13.2 - Analysis of candidate genes

We started with an in-silico analysis of our results using bioinformatic tools to obtain a Gene Ontology classification of the candidate genes (Carmona-Saez et al., 2007). We found candidate genes involved in signal transduction, transcription, transport within and between the cells and in developmental processes (Fig. 20).

It was interesting to find candidate genes belonging to the same pathway, such as apoptosis: this result further validate our screening

system (Fig. 21). A remarkable result of Gene Ontology analysis was to find a consistent fraction of genes with unknown function corresponding to the 30% of all the candidates identified in the screening. These genes belong to the Riken collection. In particular we focused our attention on a short number of these genes and we named them ObI- that stands for Osteoblasts inducers-. These genes were chosen on the basis of their putative function: in fact, bioinformatic analysis of the chosen genes predicted that they were transcription factors.

We also considered several genes identified during the screening with a known function but an unknown role during the osteogenic differentiation process. Among these candidates we mainly focused on angiotensin receptor gene.

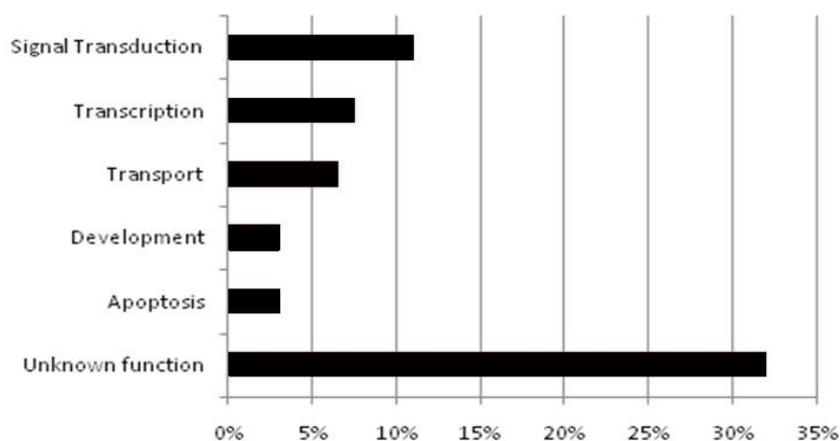


Figure 20: Gene Ontology classification of candidate genes

Results of Gene Ontology classification of the candidate genes arose from the first part of our screening. Biological processes represented by more than 3% of our candidates are shown in the graphic.

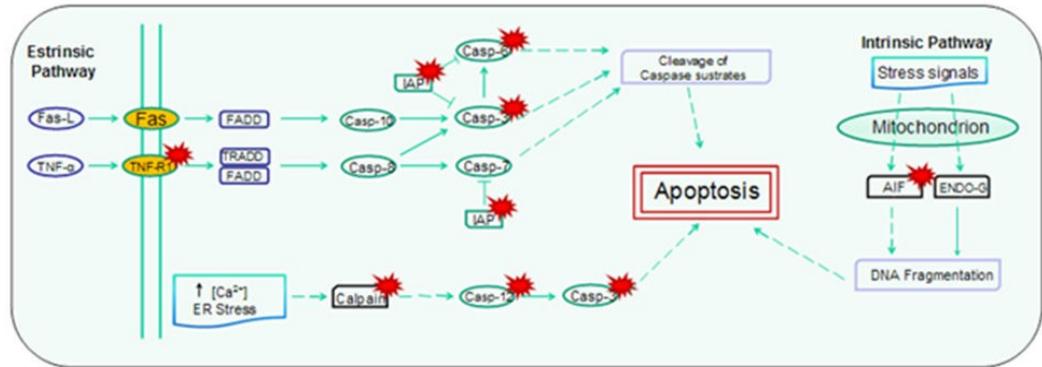


Figure 21: Identification of a pathway involved in osteoblast differentiation (Apoptosis).

Several gene of the apoptosis pathway were identified as candidate genes of our screening. Genes that are necessary for osteoblast development (i.e., absence of mineral deposit after silencing of these specific genes) are indicated with a star.

14 - ObI-1 candidate gene

14.1 - Interference with osteogenic differentiation

Among the candidate genes with unknown function, interference with ObI-1 gene with corresponding shRNA construct of the library produced an almost total absence of Alizarin Red S staining in comparison to the positive control of differentiation. Furthermore, the effect seems to be specific of the action of the shRNA construct since transfection with the shRNA-NS produced the same effect of positive control of differentiation (Fig. 22).

After the result obtained from the screening, we repeated the interference experiments to confirm our data. We found two additional shRNA constructs interfering with ObI-1 and tested them in our system. Results obtained with all three constructs showed a significant reduction of osteogenic differentiation (Fig. 23A and 23B). To validate the

specificity of the result achieved by the silencing, transcript levels of ObI-1 gene was evaluated by Real Time PCR analysis. In ObI-1 silenced cells, with all three constructs, the levels of transcript were extremely reduced compared to untransfected cells and Non-Silencing transfected cells. These results confirmed that the shRNAs were specifically silencing the W20-17.

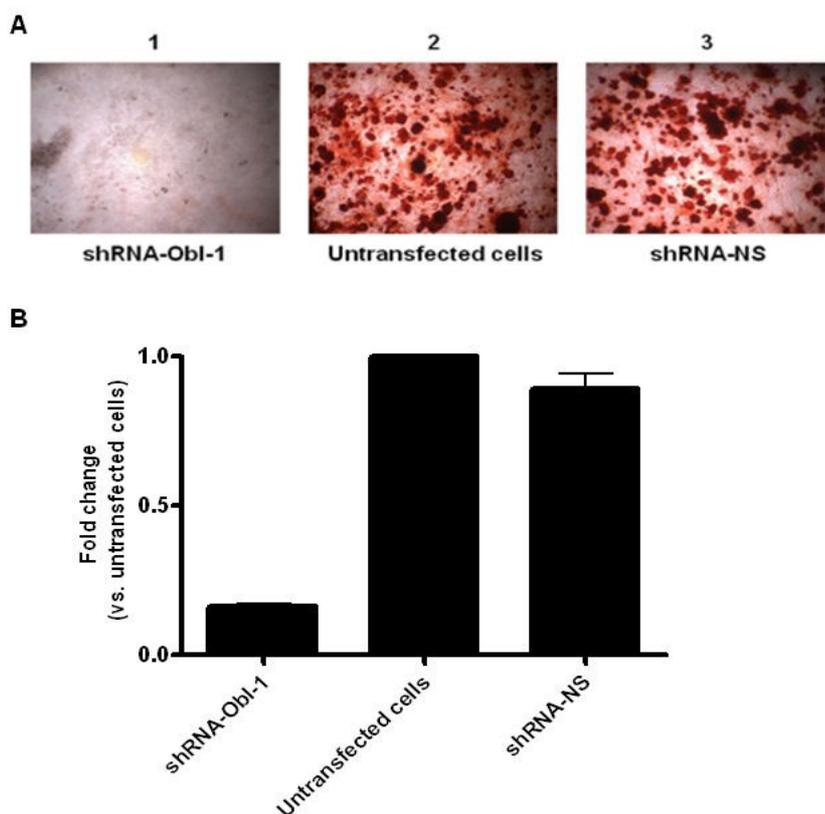


Figure 22: ObI-1 results from the screening

Alizarin Red S staining after culture in osteogenic medium for 21 days of: 1) W20-17 cells transfected with shRNA-ObI-1; 2) untransfected W20-17 cells; 3) W20-17 cells transfected with shRNA Non Silencing (A). Calcium deposition quantitation using Guanidine HCl 4M and adsorbance reading at 490 nm (B). Data are means \pm SD of 3 experiments.

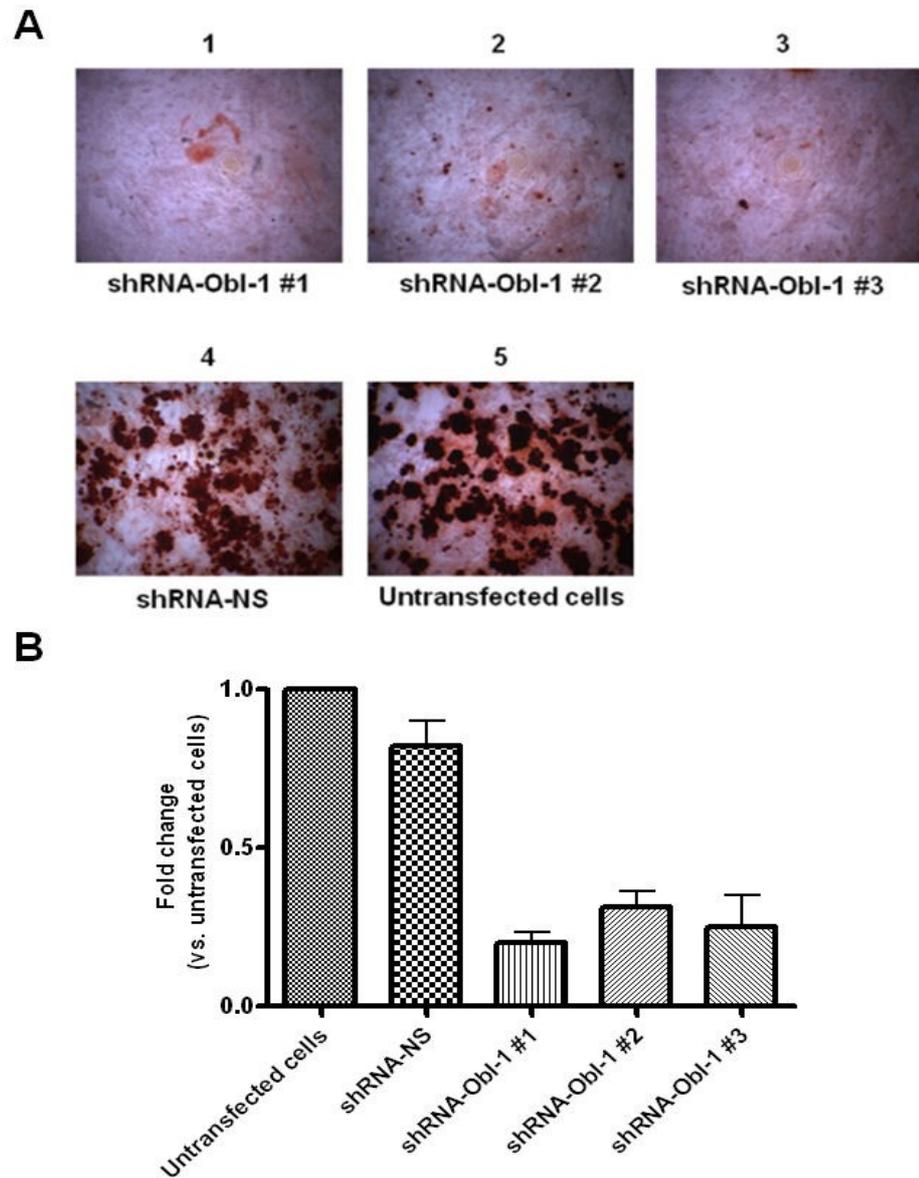


Figure 23: Confirmation of screening results on Obl-1

Alizarin Red S staining after culture in osteogenic medium for 21 days of: 1) W20-17 cells transfected with shRNA-Obl-1#1 (shRNA construct used during the screening); 2-3) W20-17 cells transfected with shRNA-Obl-1#2 and shRNA-Obl-1#3 (other two library constructs acting against Obl-1 gene); 4) W20-17 cells transfected with shRNA-Non Silencing; 5) Untransfected cells (A). Calcium deposition quantitation using Guanidine HCl 4M and adsorbance reading at 490 nm (B). Data are means \pm SD of 3 experiments.

14.2 - Protein domains and phylogenetic analyses

Prior to an experimental analysis of the role of ObI-1, we proceeded with an in-silico analysis in order to understand its structure and function. Analysis on Ensembl database showed two transcripts for ObI-1: a long and a short form (Fig 24A). Analysis of the protein revealed the presence of a KRAB and several zinc-finger domains for the long form; the short form contained only the KRAB domain (Fig. 24B). A phylogenetic analysis of ObI-1 gene was also performed on the Ensembl database. We found orthologue genes in many species: among these, we found the correspondent Human (*Homo sapiens*), Macaque (*Macaca mulatta*), Gorilla (*Gorilla gorilla*), Rat (*Rattus norvegicus*) and Cow (*Bos Taurus*) orthologues.

In total, 14 orthologue genes were present but only the Human and Rat genes correspond to demonstrated proteins while in other species their presence is only predicted.

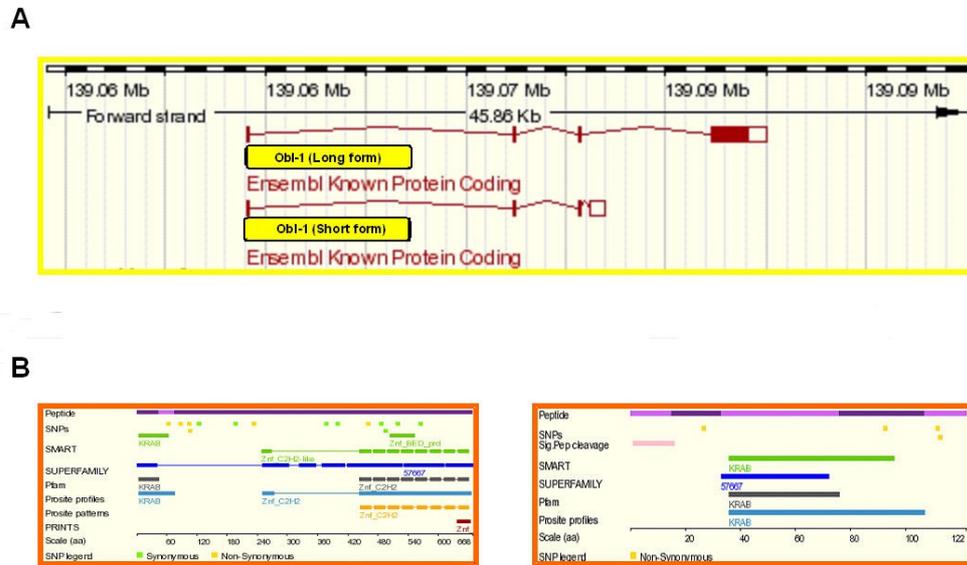


Figure 24: Transcripts and proteins domains of Obi-1 gene

Bioinformatic analysis to search for transcripts of Obi-1 gene: two different transcripts are evident, a long and a short form (A). Bioinformatic analysis to search for functional domains in the Obi-1 protein: long form present a KRAB domain and several Zinc finger domains, short form present only a KRAB domain (B).

14.3 - Expression analyses

14.3.1 - Expression analysis in tissues

We first performed an in-silico analysis of the expression of ObI-1 in different tissues. Consulting UniGene database of NCBI (National Center of Biotechnology Information) we looked for EST profiles and we found the presence of ObI-1 in different tissues at different levels (Fig 25).

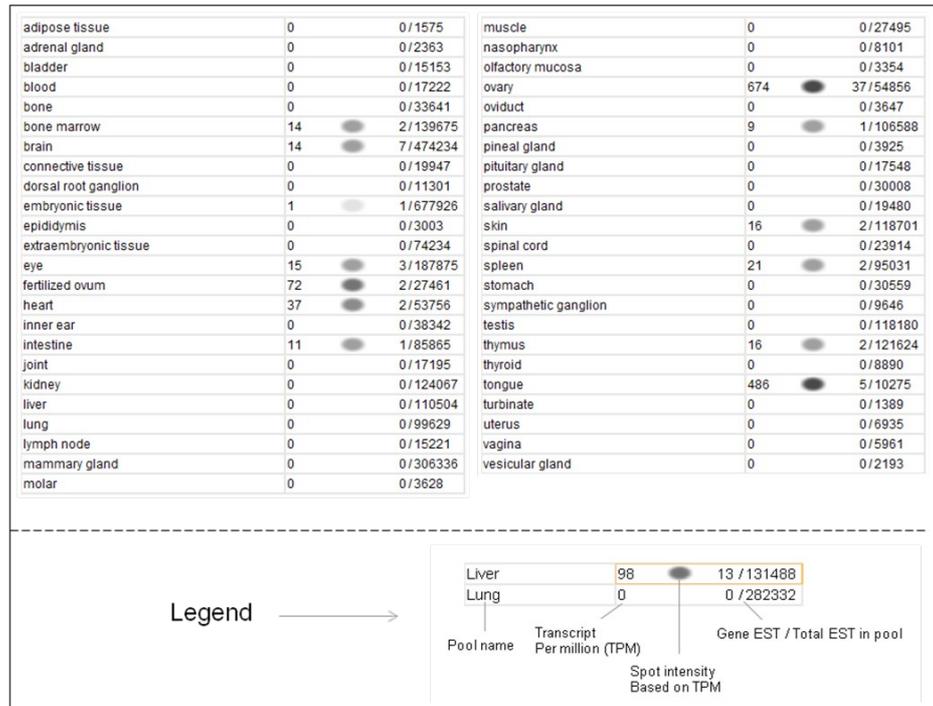


Figure 25: In silico analysis of Obi-1 expression in tissues

EST profile of Obi-1 transcript in mouse tissues

(<http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Mm.36612>).

After bioinformatic analysis, we also performed Real-Time PCR experiments to directly evaluate the expression of Obi-1 in different mouse tissues (Fig. 26).

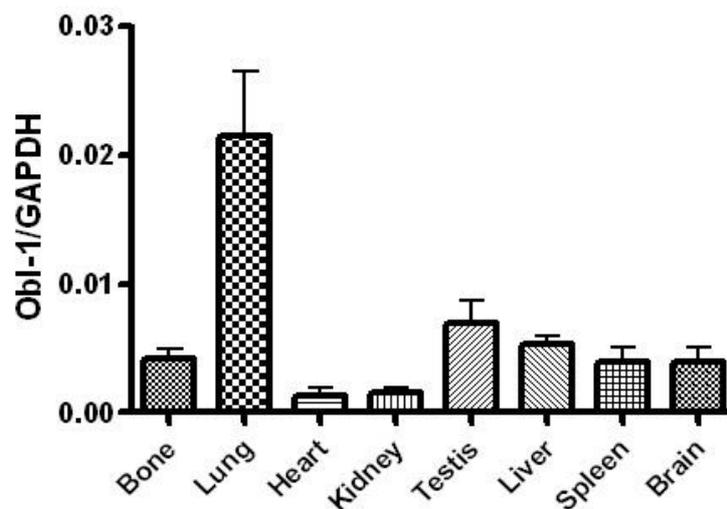


Figure 26: Obi-1 expression in tissues

Expression profile of Obi-1 transcript in different tissues isolated from mouse.

14.3.2 - Expression analysis in W20-17

Expression analysis of ObI-1 gene in W20-17 cell line was performed to evaluate the presence of its transcripts during osteogenic differentiation. We analyzed the presence of both Obi-1 transcripts, with Real-Time PCR experiments at different time-points (T0, corresponding to the undifferentiated cells, and T4, T14 and T21). The levels of the long form of ObI-1 increase at day 4 of differentiation and they remain high during all the process. The levels of the short form are very low, therefore they might not have a significant role (Fig. 27).

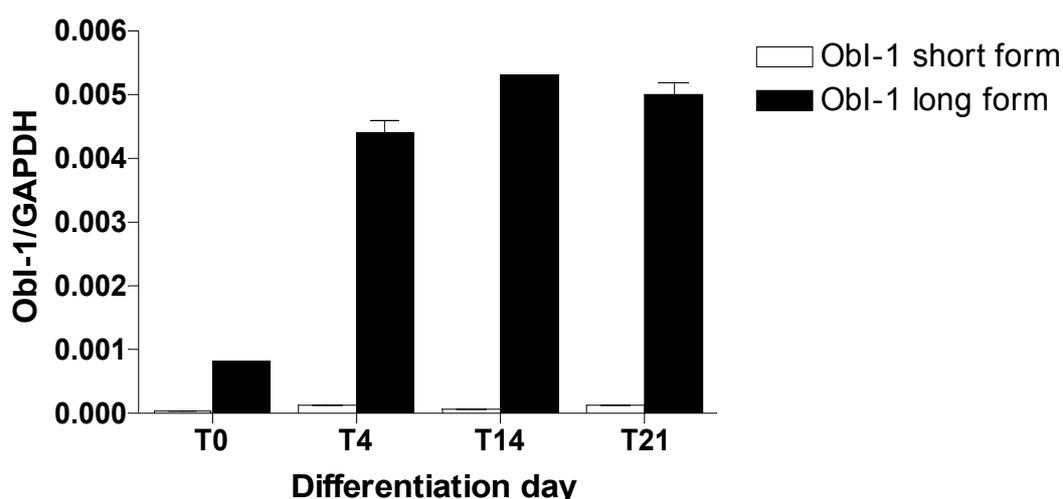


Figure 27: Expression analysis of ObI-1 during osteogenic differentiation of W20-17

Expression profile of ObI-1 transcripts during osteogenic differentiation of W20-17 cells at day 0, 4, 14 and 21. Data are means \pm SD of 3 experiments. * $p < 0.05$ (respect to T0).

14.3.3 - Expression analysis in primary cells

After analysis in W20-17 cell line, we also performed the expression analysis of ObI-1 gene in primary murine mesenchymal stem cells (mMSC). Also in this case we analyzed the presence of ObI-1 transcript

by Real-Time PCR during osteogenic differentiation.

We considered three different time points: T0, corresponding to undifferentiated cells, and T3 and T21. As previously observed during osteogenic differentiation of W20-17 cells, we detected an increase of ObI-1 transcript levels in osteogenic differentiation of primary mMSC: ObI-1 expression rose at day 4 and further increased at day 21 (Fig. 28).

In addition to these data we also performed expression analysis of ObI-1 during adipogenic differentiation of MSC. We considered three different time points: undifferentiated cells (T0) and other two time points corresponding to day 3 (T3) and day 14 (T14). In this case we did not detect any increase of ObI-1 transcript levels during differentiation, but rather a decrease of the expression at day 14 (Fig. 29).

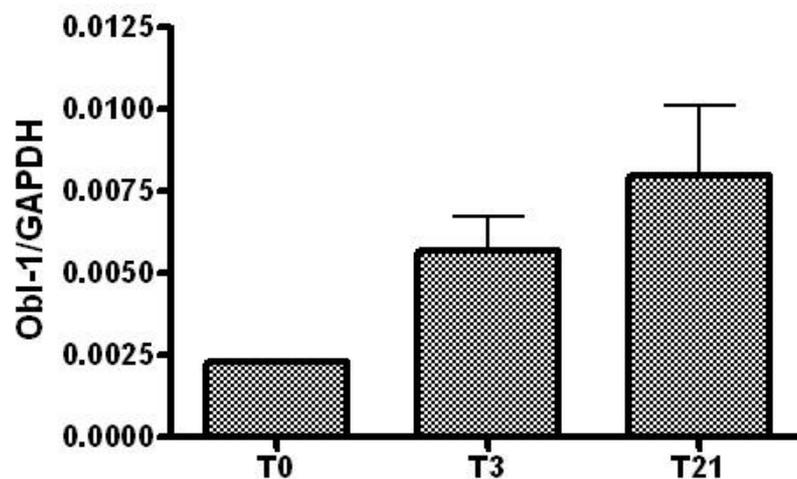


Figure 28: Expression analysis of ObI-1 during osteogenic differentiation of mMSC

Expression profile of ObI-1 transcript during osteogenic differentiation of mMSC cells at day 0, 3 and 21. Data are means \pm SD of 3 experiments.

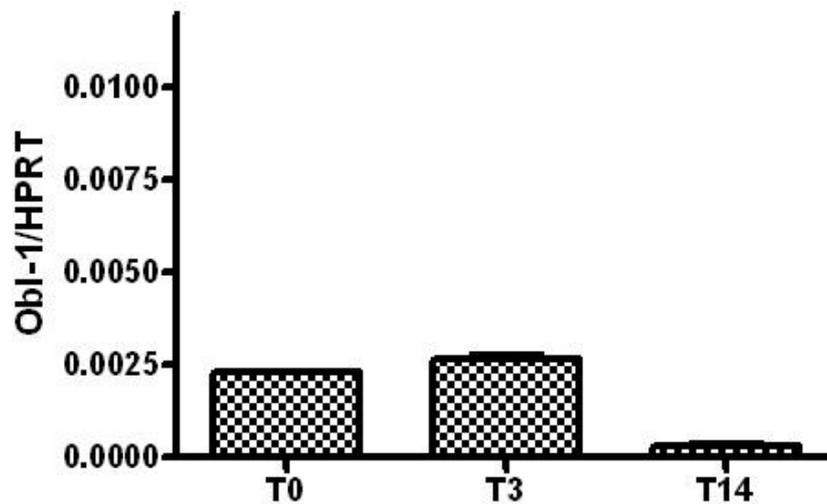


Figure 29: Expression analysis of ObI-1 during adipogenic differentiation of mMSC

Expression profile of ObI-1 transcript during adipogenic differentiation of mMSC cells at day 0, 3 and 14.

14.4 - Sub cellular localization analysis

In order to check for the sub-cellular localization of the protein product of the ObI-1 gene we cloned the cDNA of ObI-1 in pcDNA3 (Invitrogen) with a Flag. We first evaluated the expression of ObI-1 gene by Western Blot analysis comparing protein lysates obtained from W20-17 cells transfected with the pcDNA3 plasmid with or without the ObI-1 cDNA insert (Fig. 30).

After this experiment we performed Immuno-fluorescence analysis to find the sub-cellular localization of ObI-1. Merge between the cells treated with DAPI and Anti-Flag antibody indicate the nuclear localization of ObI-1 gene (Fig. 31).

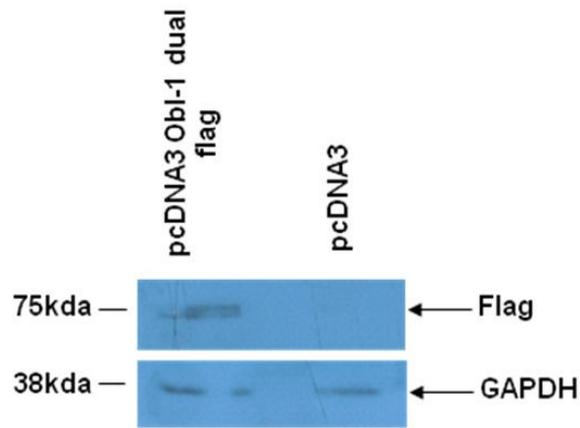


Figure 30: Detection of pCDNA Obl-1-Flag by Western Blot analysis

Protein lysate of cells transfected with pcDNA3 Obl-1 dual-Flag and not of cells transfected only with pcDNA3 was positive to anti-Flag antibody. GAPDH was used as control for each protein loading.

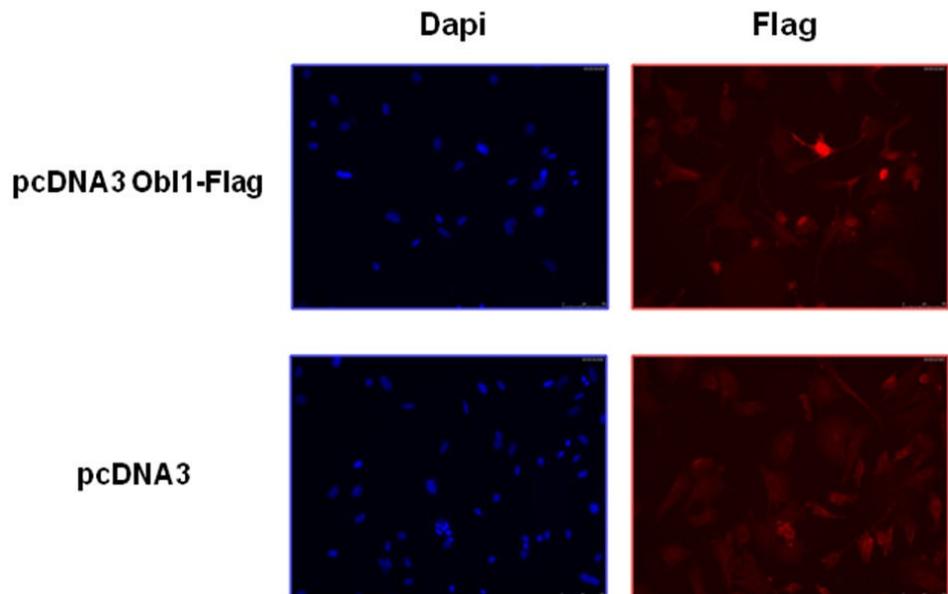


Figure 31: Detection of Obl-1 sub-cellular localization by immunofluorescence analysis

Expression of pcDNA3 Obl-1-confirm the nuclear localization of Obl-1. pcDNA3 expression was used as control.

14.5 - Expression profile of osteoblast differentiation markers in ObI-1 silenced cells

To investigate the role of ObI-1 during osteogenic differentiation we evaluated, by Real Time, the expression profile of some of the early

differentiation markers in untransfected cells and in cells transfected with either ShObI-1 or ShNS. The transcript level of Runx2 and osteopontin (OPN) were significantly reduced in ObI-1 silenced cells while, in cells transfected with ShNS, the transcript level was comparable to untransfected cells (Fig. 31).

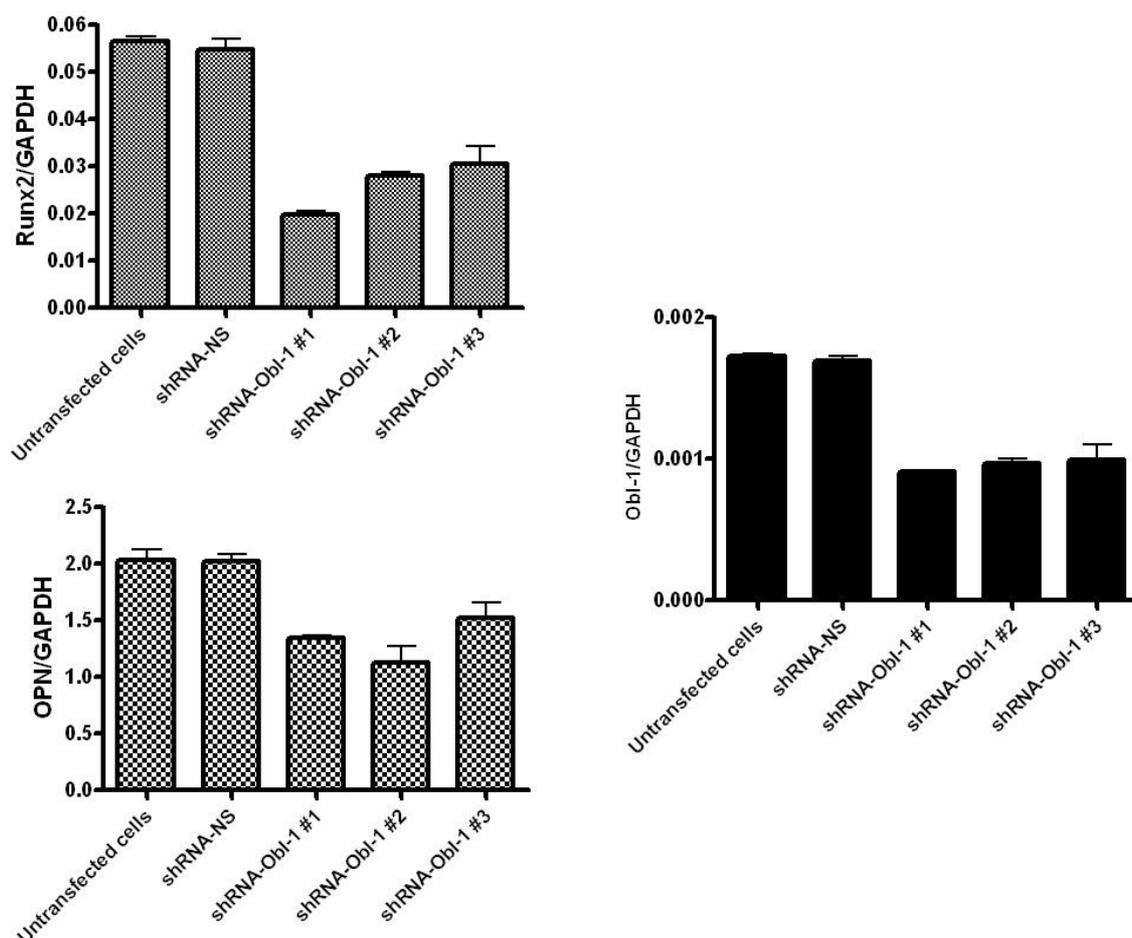


Figure 32: Effect of ObI-1 silencing on bone differentiation marker

Expression profile of early differentiation markers in ObI-1 silenced cells. Effect of ObI-1 silencing on bone differentiation marker. Runx2 and osteopontin results decreased in ObI-1 silenced cells.

15 – Angiotensin receptor 1 candidate gene

15.1 - Interference with osteogenic differentiation

Silencing of Angiotensin receptor 1 gene (AT1R) produced a strong impairment of the osteogenic differentiation of W20-17 cells. Alizarin Red S staining of cells transfected with an shRNA interfering with AT1R expression showed a reduced mineralization at day 21 of differentiation. Cells transfected with the shRNA-NS show a phenotype similar to untransfected cells.

We also analyzed a second shRNA-AT1R construct from our library that reproduced the same effect. We were able to demonstrate and confirm the same inhibition effect on osteogenic differentiation for both the shRNA-AT1R constructs. In order to validate the specificity of the result of silencing, the level of AT1R protein was evaluated by Western blot analysis. In AT1R silenced cells the level of protein is extremely reduced compared to untransfected cells and Non-Silencing transfected cells. Taken together this results were suggesting that AT1R gene may have a role in differentiation process.

15.1.1 - Effects of angiotensin receptor antagonist on osteoblast differentiation

In order to confirm the results obtained with shRNA-mediated silencing, we differentiated the cells in presence of losartan, a specific AT1R inhibitor, and PD123,319, a specific AT2R inhibitor. Losartan was able to

inhibit W20-17 differentiation at a very low concentration, thereby confirming a specific effect. Same concentrations of PD123,319 were not able to inhibit this process, confirming the involvement of type-1 receptor.

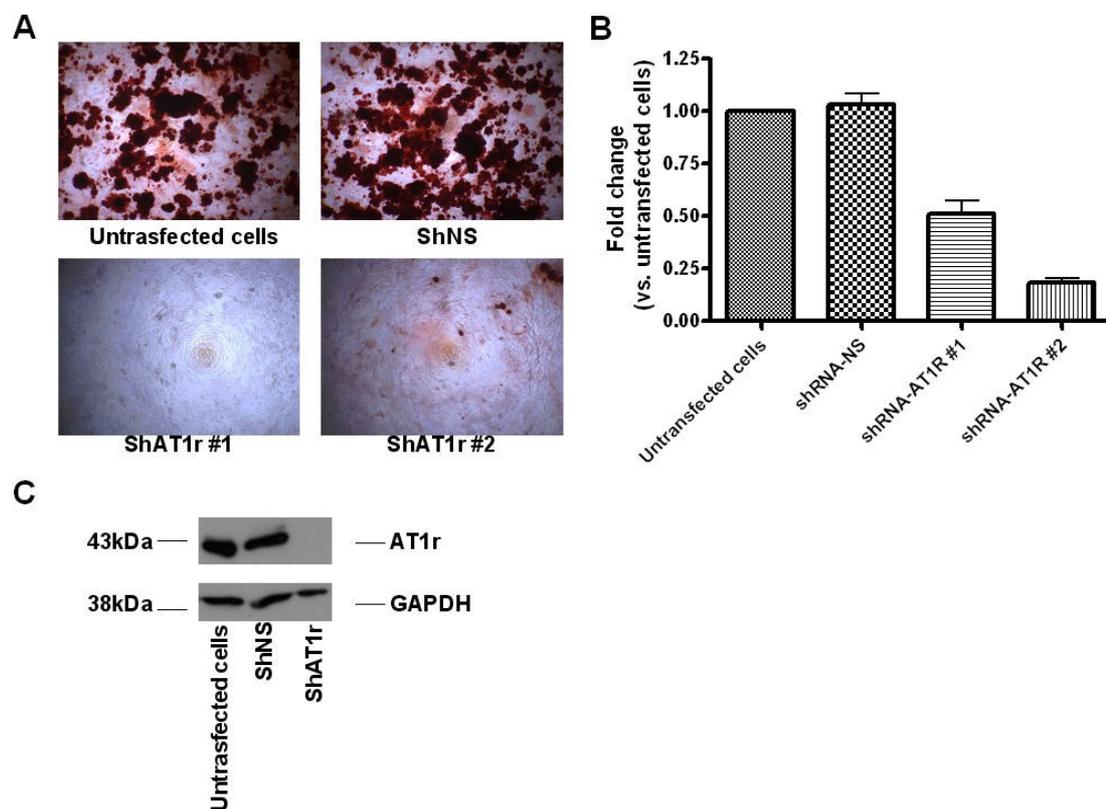


Figure 33: AT1R results from the screening

Alizarin Red S staining of cells transfected with the constructs against AT1r gene, with a Non-Silencing construct (ShNS) and non transfected cells (A). Quantification of Alizarin Red S staining of silenced and non silenced cells using Guanidine HCl 4M and adsorbance reading at 490 nm (B). Protein expression of AT1R in silenced and not silenced cells was evaluated by western blot (C).

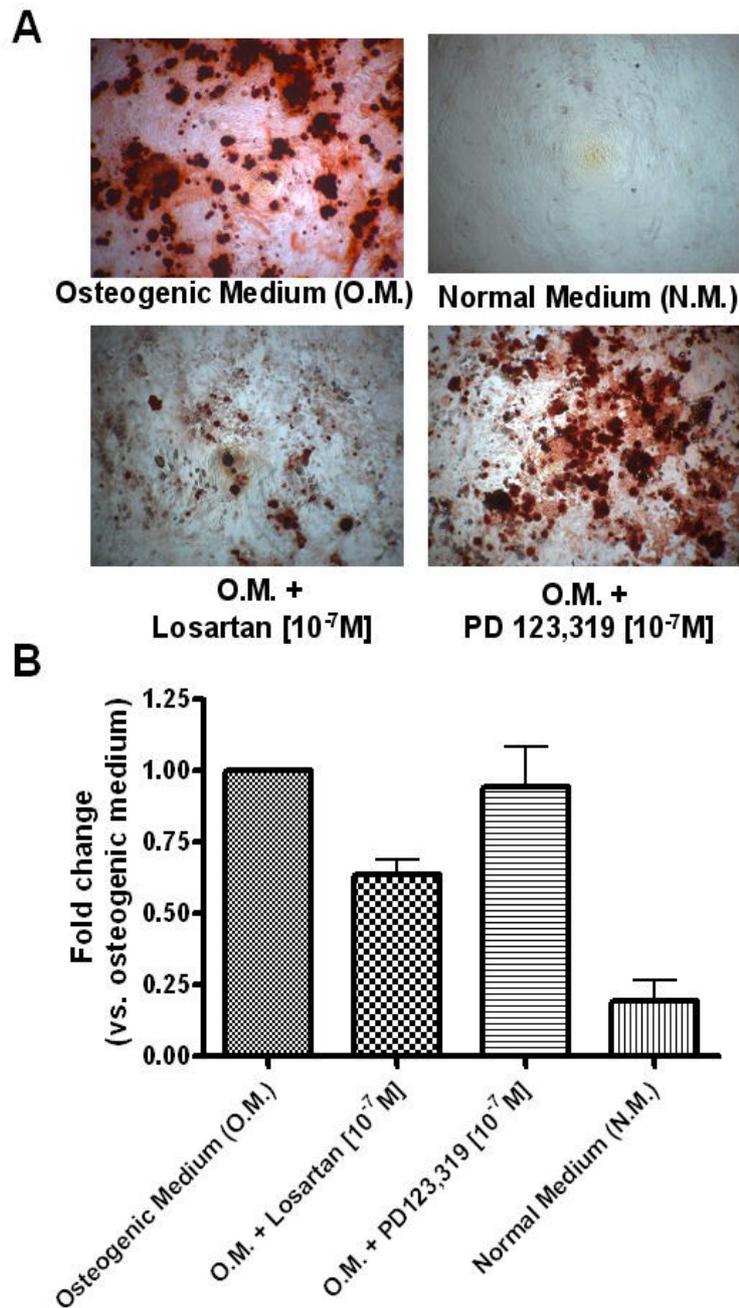


Figure 34: Effects of angiotensin receptor antagonist on osteoblast differentiation

Inhibition of mineralization of osteoblastic cells in response to angiotensin receptor antagonists. Alizarin Red S staining of cell treated with osteogenic medium and selective AT1r antagonist (Losartan [10^{-7} M]). A selective AT2r antagonist (PD123,319 [10^{-7} M]) was used as control to test the specificity of the involvement of AT1r during osteogenic differentiation (A). Quantification of Alizarin Red S staining of treated cells (B).

15.2 - Expression analysis in W20-17 cell line

We confirmed the presence of the angiotensin receptors transcript in our *in-vitro* differentiation system. At this purpose we performed a Real-Time PCR experiment to evaluate presence and possible variations of AT1R transcript levels during osteogenic differentiation of W20-17 cells. We considered four different time points during the process: T0, corresponding to the undifferentiated cells, T4, T14 and T21. AT1R is expressed at low levels in undifferentiated cells but increases immediately during the first days and remaining at similar levels throughout the process; conversely, AT2R expression increased during differentiation peaking only at the very end of the process.

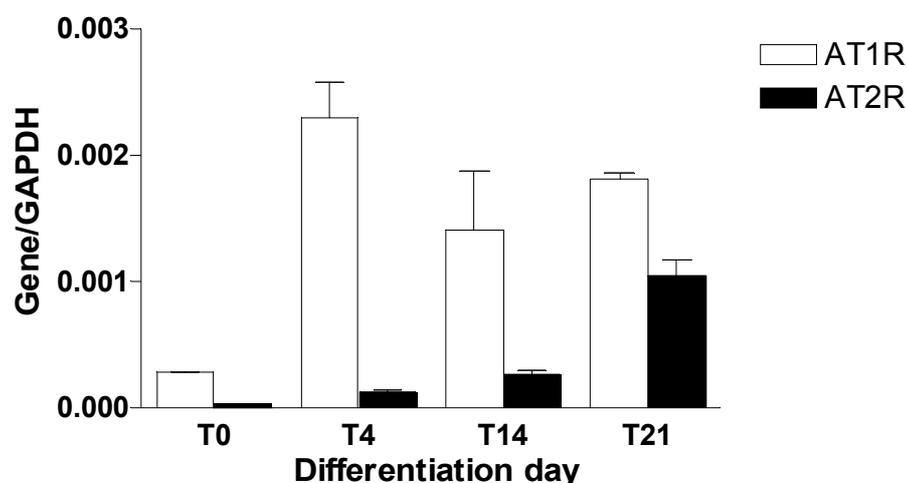


Figure 34: Expression profile of angiotensin receptor during osteogenic differentiation
Expression analysis of distinct AT1R and AT2R subtypes during the differentiation.

15.3 - Study of the role of AT1R in osteogenic differentiation

15.3.1 - Effect of AT1R inhibition on alkaline phosphatase

To identify whether AT1R was involved only in the latter mineralization phase, we analyzed ALP expression and activity in W20-17 cells after 14 days of treatment with osteogenic media. W20-17 cells treated with losartan showed a reduced ALP staining compared to cells treated only with osteogenic medium. Analysis of ALP activity confirmed the observation, showing that cells treated with losartan had values similar to W20-17 kept in absence of osteogenic stimuli. These data therefore confirmed that AT1R was not involved only in the mineralization phase, but differentiation and/or proliferation were dependent by the expression of this gene.

15.3.2 - Time course analysis of the AT1R stimulation during the differentiation

In order to further characterize the phase of the differentiation process where AT1R stimulation was more relevant, a time course experiment was performed starting to add losartan and PD123,319 at different time point (0, 7, 14 days) from the beginning of the differentiation. At any time of administration, PD123,319 treated cells showed an osteogenic

differentiation comparable with untreated W20-17. On the other end,

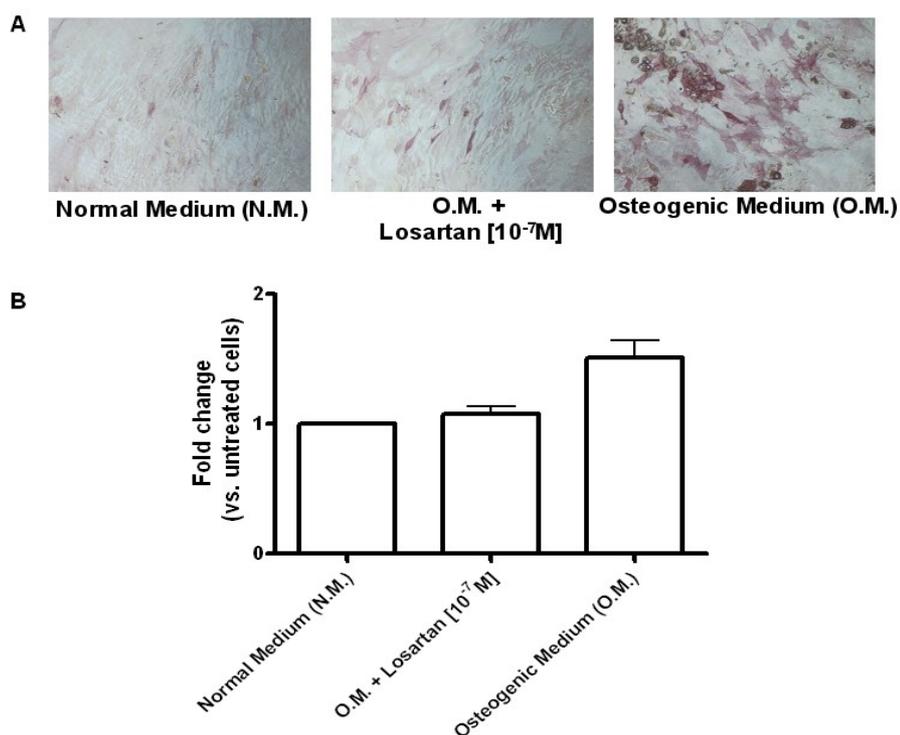


Figure 35: Effects of angiotensin receptor antagonist on ALP expression

Inhibition of ALP expression in response to angiotensin receptor antagonists. ALP staining (A) and activity (B) of cell treated with osteogenic medium and selective AT1r antagonist (Losartan [10⁻⁷M]).

Days of treatment:

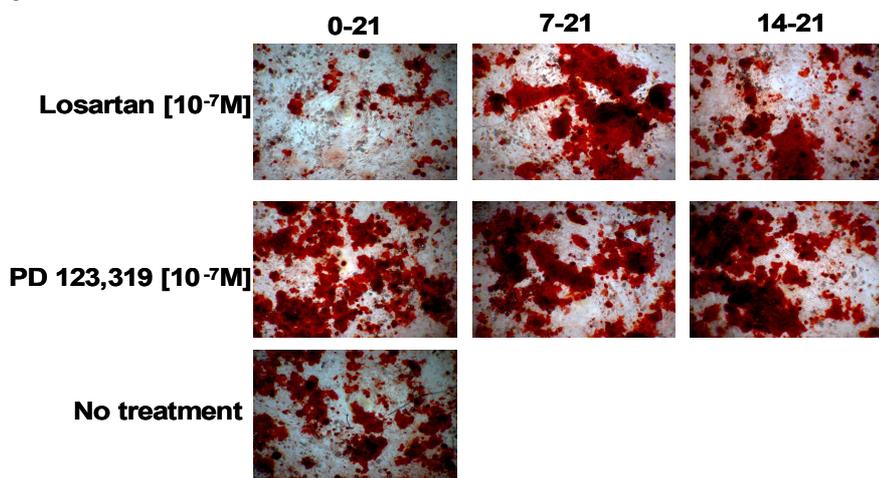


Figure 36: The effects of angiotensin receptor I inhibitor are time-dependent

The activation of angiotensin receptor I is essential for osteoblastic differentiation only in the first part of the process. The administration of losartan the osteoblastic mineralization only when its administration starts in the first week of the differentiation.

losartan-treated cells showed a significant reduction of alizarin red staining only when losartan was added at timepoint 0; on the other hand, W20-17 produced an almost completed mineralized matrix when losartan was added later. These results are compatible with an involvement of AT1R in the very early phases of differentiation when we observed the most abundant increase of the transcript.

15.3.3 - Evaluation of AT1R stimulation on cell proliferation

We therefore decided to analyze Ang II effect on stimulation of pre-osteoblast, administering different doses of recombinant protein to W20-17 cells differentiating in osteogenic medium. Addition of Ang II to the cells, after 3 days of treatment with differentiation media, significantly increased proliferation in a dose-dependent manner; this effect could be reversed by the addition of losartan, whereas addition of PD123,319 did not affect proliferation at this stage. A proliferative effect of Ang II was also observed in undifferentiated W20-17 and primary mesenchymal stem cells (MSCs) although was not comparable to what observed in differentiating cells. It's probably due to the low expression of AT1R in completely undifferentiated cells

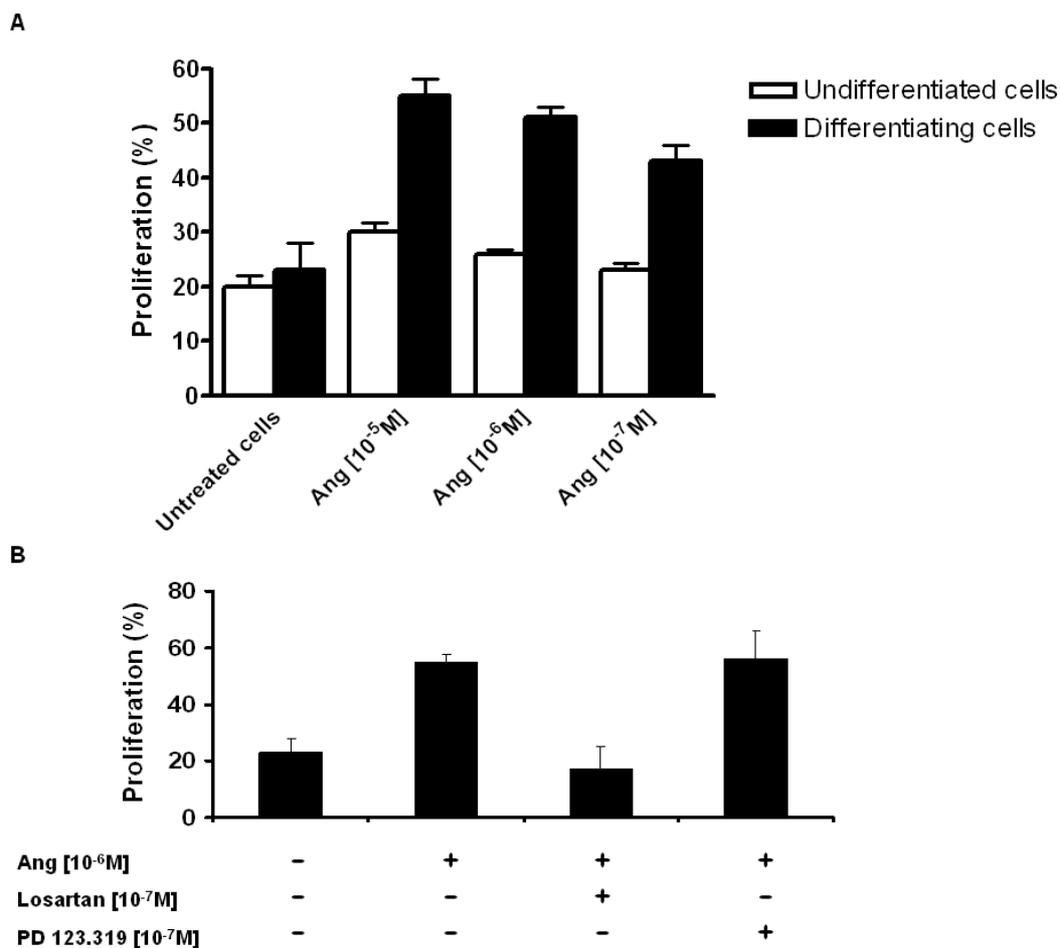


Figure 37: Angiotensin II has a proliferative effect at the pre-osteoblast stage of differentiation acting on AT1R

Addition of angiotensin II to the differentiation media increase proliferation of undifferentiated (white bars) and partially differentiated (black bars) cells (A). This effect can be reversed by the addition of losartan [10⁻⁷M], whereas addition of PD123,319 [10⁻⁷M] does not affect proliferation (B)

15.4 - AT1R inhibition blocks SMAD-mediated induction of proliferation

In order to identify what is the molecular mechanism activated by AT1R stimulation, we evaluated if the reduction of MSCs and pre-osteoblast proliferation was due to an effect of AT1R inhibition on the TGF- β pathway. In fact, recent evidences linked AT1R blockade with inhibition of TGF- β pathways in different cell types. We analyzed the level of phosphorylated SMAD2 and 3 (p-SMAD2/3) as downstream

effectors of TGF- β and phosphorylated SMAD1, 5 and 8 (pSMAD1/5/8) as downstream effectors of BMPs, during the differentiation in presence or absence of losartan. In presence of losartan we observe a significant decrease of p-SMAD2/3 compared to control cells, indicating a reduction in TGF- β signaling. The reduction is specific for this pathway since the complex SMAD1/5/8 is not affected by the losartan treatment. This data confirm that AT1R inhibition induces an over-stimulation of TGF- β pathway effectors during osteogenic differentiation.

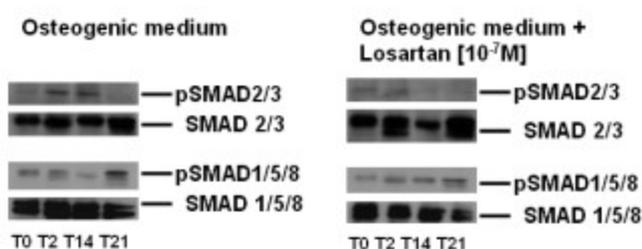


Figure 38: AT1R inhibitors decrease the phosphorylation of the protein SMAD2/3

Addition of losartan [10⁻⁷M] significantly decreased p-SMAD2/3. The reduction is specific for this pathway since the complex SMAD1/5/8 is not affected by the treatment.

15.5 - Effect of angiotensin receptor 1 inhibitors on TGF- β expression

Recent evidences suggest that the AT1R-mediated induction of TGF- β pathway may be transcriptionally mediated. At this purpose we performed a Real-Time PCR experiment to evaluate the presence and the possible variations of TGF- β transcript levels. We observed a reduction of TGF- β transcription in W20-17 cells treated with osteogenic

medium in presence of losartan. Even in absence of osteogenic conditions, TGF- β transcription is induced by Ang-II treatment; this induction is specifically reversed by losartan. TGF- β receptors levels were identical in any of the previously mentioned conditions.

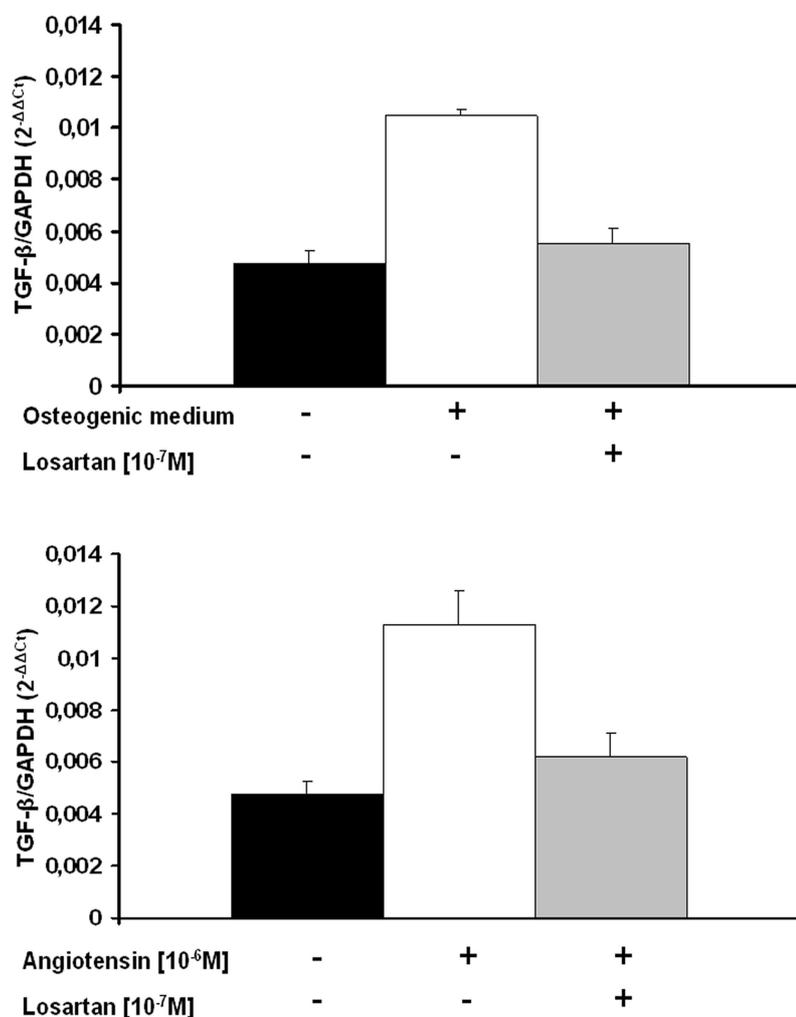


Figure 39: Expression analysis of TGF- β in angiotensin and losartan treated cells

Expression profiles of TGF- β in cells treated with osteogenic differentiation medium (A) and angiotensin [10⁻⁶M] (B). In both case the addition of losartan [10⁻⁷M] is able to reduce the expression of TGF- β .

15.6 - Effect of angiotensin receptor inhibitor on mesenchymal differentiation

In order to identify the cell fate of the AT1R inhibited cells, we evaluated the adipogenic differentiation of cells in presence or absence of losartan in the osteogenic medium. This medium contains beta-glycerophosphate and ascorbic acid that are specific for osteoblast differentiation protocols but also dexamethasone that is present also in adipogenic medium. Cells treated with losartan in osteogenic medium showed a higher amount of adipocytes compared to cells treated with only osteogenic medium when stained with oil red-O. On the other hand, addition of PD123,319 did not produce any significant change compared to cells treated with only osteogenic medium.

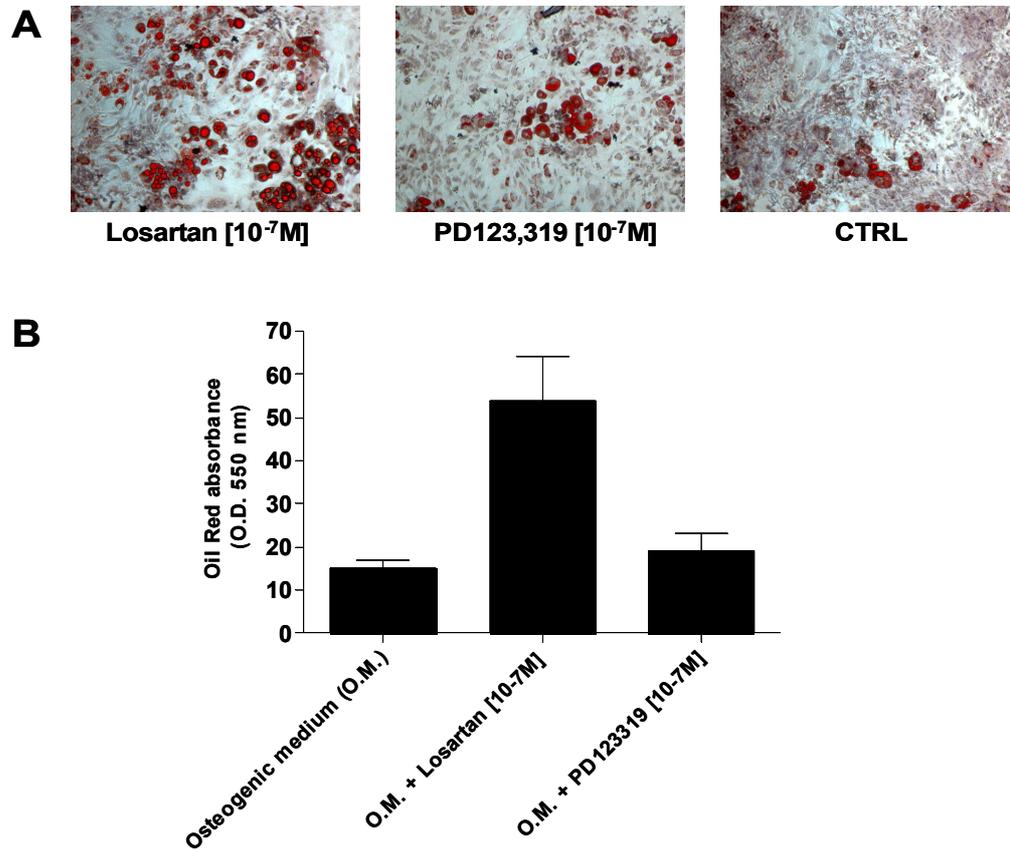


Figure 40: Inhibition of angiotensin receptor I during the osteoblast differentiation increase the presence of adipocyte

Oil Red staining of cell treated with osteogenic medium in presence of angiotensin receptor inhibitors Losartan [10^{-7} M] and PD123,319 [10^{-7} M] (A). Quantification of Oil Red staining of treated cells (B).

DISCUSSION

16 - shRNA-mediated screening for the identification of genes relevant for osteoblast differentiation

16.1 - Set up

The first challenging of this study was to identify the components and the methods to perform the screening. We have decided to apply RNA-interference technique in a screening approach to investigate on new possible genes involved in osteogenic differentiation for two main reasons: the first consisted in a long-term interest of our laboratory in osteoblast differentiation and bone formation and the second was the availability of a mouse library of short interfering RNA.

An important boost to begin with our study came from the success of RNA approach in another line of research in our research group, with the same mouse shRNA library. In the above-mentioned a gene essential for the maintenance of stemness embryonic stem cells (Esc) was identified evaluating the effects of the shRNAs on the neural differentiation of ESc (Parisi et al., 2009).

We decided to evaluate osteogenic differentiation of murine Mesenchymal Stem Cells (mMSC); therefore we chose a specific cell line, a method to differentiate these cells into osteoblasts and a system

to detect osteogenic differentiation. Finally, we have adapted all these components to perform experiments as high-throughput screening.

We decided to use W20-17 among different types of MSC-derived cell lines since they are able to differentiate into osteoblasts; furthermore, this cell line was previously used by other authors to perform osteogenic differentiation experiments (Blum et al., 2001; Kempen et al., 2008). In order to differentiate W20-17 cells we used osteogenic medium (or D.A.G. medium): this medium consist in DMEM with 10% fetal bovine serum supplemented with dexametasone, ascorbic acid 2P and β -glicerophospate.

Dexametasone is a glucocorticoid agonist capable to act on responsive promoters of transcription factors necessary for MSCs commitment for osteogenic lineage. Ascorbic acid-2P promotes the extracellular matrix formation and the maturation of all the types of collagens; furthermore, it induces the activity of plasma membrane-associated ALP in osteoprogenitors. β -glicerophospate promotes mineralization since phosphate groups are incorporated into idroxiapatite crystals of the matrix.

Finally, we chose Alizarin Red S staining for osteogenic differentiation detection because it was largely described and used in literature and gave solid and reproducible results in our hands.

16.2 - Experimental approach validation

After the choice of all the components and methods, we had to establish and test the experimental conditions to adopt. This point was crucial to execute a reliable process to carry out the high-throughput screening of our assay with the library of shRNA constructs.

We described experimental approach in the results: essentially it implicates plating of the cells in 96-well microplates, transfection of the shRNA plasmids of the library, antibiotic treatment for 3 days in order to enrich for cell population that incorporated the plasmids and, finally, osteogenic differentiation protocol. After the above-mentioned optimization, we had to test the functionality of the shRNA plasmids for our purpose and, above all, the ability of our system to detect the osteogenic differentiation impairment by shRNA plasmids acting against candidate genes. We therefore performed the test that we described in the section 12.4 of the results, in which we chose shRNA plasmids interfering with genes whose role in osteogenic differentiation had been largely described in literature.

We obtained different grades of osteogenic differentiation impairment with these shRNA plasmids and this probably depended upon two main reasons: the first reason was that not all the constructs in the library are able to silence genes with the same efficiency; the second consisted in the different grade of involvement in the osteogenic differentiation process of the genes selected.

The most important outcome from these preliminary experiments was

the demonstration that using our experimental approach was possible to identify genes involved in osteogenic differentiation process and therefore we were able to proceed with the screening.

16.3 - Implementation of the screening

Performing of the screening was probably the most relevant commitment of this study. We chose the mouse shRNA library because of the availability of murine mesenchymal stem cells-derived cell lines and the prospective to work with a mouse model for the best candidates.

The mouse shRNA library is composed of 66,313 shRNA constructs corresponding to 699 plates; we were able to screen 1/7 of the library corresponding to 100 plates and about 10,000 constructs. A number of library plates contains genes whose function is unknown, we have tried to reduce the presence of these plates, in order to screen a higher percentage of known murine genes. So, even though we screened 1/7 of the total number of genes, we were able to screen about 1/3 of all the known genes in mouse. Nonetheless, we equally screened genes whose function is still unknown and, in fact, we selected some of these genes among our candidates.

Once performed part of the screening described in this study, we collected all the data and we chose a number of candidate genes establishing our choice on the basis of results from Alizarin Red S staining and its quantitation.

As we mentioned in the section 13.1 of the results, we found 650 candidate genes corresponding to 6.5% of the screened genes. This number of candidate genes may seem excessive. A possible explanation is that among the candidate genes are included genes whose function is essential for cell survival, independently from their role in osteoblast differentiation: therefore, the final effect is impairment of osteoblast differentiation even though they are not implicated specifically in this process.

After individuation of candidate genes we performed a Gene Ontology classification: our candidates were subdivided in different groups on the basis of their specific function or the process in which they are involved (Carmona-Saez et al., 2007). Most of our candidates are represented by genes with unknown function; this was a very interesting results for two main reasons: they were about the 30% on the total number of the candidates and genes whose function is still unknown are a challenging subject to investigate. As regards the rest of the candidates with known functions, it was interesting to find a consistent number of genes belonging to the apoptosis pathway (Glynn Denis et al., 2003). This result validated our experimental approach: in fact, genes belonging to the same pathway, when induced in a process, should be almost all positive in a screening. Furthermore, involvement of genes belonging to the apoptosis pathway in our study once more validated our screening system since some genes of apoptosis pathway, such as several caspases, had previously been demonstrated to be involved in

osteoblast differentiation (Mogi and Togari, 2003).

17 - ObI-1 candidate gene

The effect of ObI-1 gene silencing in osteogenic differentiation was clear and therefore we focused our attention on this candidate. Furthermore, we found other constructs of the library that interfere with ObI-1 gene and, therefore, we could perform silencing confirmation experiments with three different constructs: this represents a specificity control for the RNA interference technique. In fact, in RNA-interference experiments a molecule can cause off-target effects, independently by its activity on the putative mRNA target; obtaining the same effects with other RNA molecules interfering with the same gene, we could exclude off-target effects.

We, therefore, proceeded with an in-silico analysis, particularly required for genes with unknown function. Using Ensembl database we found two different transcripts for ObI-1 gene and, above all, we identified KRAB and zinc-finger functional domains in the corresponding protein. This results led us to hypothesize that this gene belongs to the family of the transcription factors with a KRAB domain (Urrutia, 2003). To confirm this hypothesis we also performed immunofluorescence experiments to search for the sub-cellular localization of ObI-1. With these experiments we demonstrated the nuclear localization of ObI-1, reinforcing the idea that this gene may be a transcription factor.

Proceeding with the bioinformatic analysis, we demonstrated a possible

conservation of this gene: in fact we found 14 orthologues in several species. It was interesting to find orthologues in Human with different levels of homology with the mouse gene. The better value of homology was about 46% for one of these orthologues: it was relevant to note that homology between the sequences occurs in the functional domains of the relative proteins.

Expression analysis of ObI-1 in tissues was investigated with both in-silico analysis and Real-Time PCR experiments after RNA extraction from different tissues in mouse. In-silico analysis was performed by EST profile research on UniGene database of NCBI. An EST profiles can give an approximate gene expression pattern of a gene in different tissues since ESTs (Expressed Sequence Tag) are short fragments of transcribed DNA sequenced by cDNA obtained after retrotranscription of longer mRNA. This analysis showed the presence of ObI-1 in several tissues including bone marrow. Our analysis performed by Real-time PCR revealed a different distribution of ObI-1 gene in the tissues considered. We looked for the expression in bone, lung, heart, kidney, testis, liver, spleen and brain. We found a large amount of ObI-1 gene in lung, while no expression of ObI-1 was found in this organ by EST profile analysis. Data regarding other organs were different. Most relevant result consisted in the expression of ObI-1 in bone. Concerning the inconsistencies between the two analyses is important to note that EST profile evaluation can be largely inconsistent: EST sequences are, in fact, not always correct since their sequencing is automated in only

one direction and in a non-overlapping manner.

After analysis in tissues, we also performed expression analysis in W20-17 cells to search for the presence of ObI-1 gene in our system. We analyzed the expression of ObI-1 transcripts during the osteogenic differentiation and we detected an increase for the long form during the process respect to time 0 and very low levels of the short form during all the differentiation. Considering this analysis, we hypothesized a possible role of ObI-1 gene in osteogenic differentiation of W20-17 since its transcript increase since the day 4 of differentiation and remain high during all the process. Furthermore, after this experiments, we did not consider anymore the short form since its levels were extremely low.

In order to complete expression analysis in cells, we also performed experiments in primary mMSCs isolated from mouse in our laboratory (Esposito et al., 2009). Analysis in primary cells was very important because a cell line, such as W20-17, may not exactly reproduce a physiological cellular environment. On the other hand, primary cells better reproduce the biochemical activities observed *in-vivo*: therefore, we decided to proceed with the analysis in these cells.

Expression of ObI-1 gene during osteogenic differentiation of mMSCs was comparable to the data obtained in W20-17 cells. This further confirmed a role of our candidate in osteogenic differentiation. Furthermore, we also performed the expression analysis during MSCs adipogenic differentiation: in this case levels of the transcript did not increase during the process, but rather they appear even lower at the

end of the differentiation compared to day 0. This latter result led us to consider a possible specific role of ObI-1 gene in the osteogenic differentiation of MSC considering the multipotency of these cells.

18 – Angiotensin Receptor 1 candidate gene

Regarding AT1R, we demonstrated the involvement of angiotensin in pre-osteoblast proliferation and how this process is necessary for a correct osteoblast development. Moreover, we were able to demonstrate that angiotensin stimulates TGF- β signaling while has no effect on BMPs signaling. Recent studies confirmed the presence of RAS system locally in bone: in fact, it has been shown that activation of the RAS induces osteoporosis and this effect is independent from hypertension (Asaba Y et al., 2009). The osteoporosis is probably caused by the Ang-II mediated increase of expression of receptor activator of NF-kB ligand (RANKL) in osteoblasts; RANKL secretion induces osteoclasts proliferation with a pro-osteoporotic effect (Shimizu H et al., 2008). However, the relative role of AT1R and AT2R in the induction of osteoporosis appears to be controversial since Izu et al. found that AT2R blockade prevents osteoporosis (Izu Y et al, 2009), whereas Shimizu et al. found that RANKL increase was mediated by AT1R (Shimizu H et al., 2008). In our studies, the most relevant result in our screening was found the same effects with two independent constructs of the library. This result was relevant to demonstrate the specificity of the action of AT1R gene on the

osteogenic differentiation process. Following the first results from the screening, we repeated AT1R silencing experiments and confirmed osteogenic differentiation impairment. The use of specific angiotensin receptors inhibitors was once more an important result to demonstrate the specificity of RNA interference experiment. The expression profile of AT1R suggested that could have a role from the first phase of differentiation. This is, also, confirmed by analyzing the role of AT1R in each phase of differentiation. This preliminary data led us to investigate a possible role of angiotensin receptor 1 in proliferation that is confirmed by adding angiotensin II recombinant protein to the medium. We had the most significant results when the angiotensin II is added to cell that are differentiating. This is probably due to the increase of AT1R transcript in early stage of differentiation when the cells start to differentiate. Several studies correlates the TGF- β signaling to osteoblast proliferation. TGF- β has been reported to stimulate proliferation and early differentiation, while inhibits terminal differentiation. We found that using specific angiotensin receptor 1 inhibitors, such as losartan, is possible to modify the level of phosphorylation of smad2/3 that is effectors of TGF- β signaling. In order to indicate the mechanism of Ang II-mediated increase of TGF- β signaling we evaluated transcription of TGF-beta and its receptors. We found that increased levels of Ang II appears to induce transcription of TGF-beta.

In our study, we did not address any role to AT2R during the

osteogenic differentiation but we can not exclude that this receptor could be involved. In fact, in our in vitro model of osteoblastogenesis, we did not observe a reduction of mineralization after pharmacological inhibition of AT2R even if we found an increase of AT2R transcript in the late phase of differentiation. Moreover, recent studies show how a treatment with AT2 receptor blocker significantly enhanced the levels of bone mass (Izu Y et al, 2009). This is probably due to the fact that our experimental design is optimized to individuate gene whose silencing is able to impair, and not to improve, the osteoblast differentiation.

We can therefore conclude from this study that AT1R stimulation increase pre-osteoblast proliferation through TGF-beta canonical SMAD-signaling pathway. In addition, AT1R inhibition may be proved an interesting approach to diseases due MSCs or pre-osteoblasts hyperproliferation.

CONCLUSIONS

In this work we have described set up and implementation of a shRNA-mediated screening aimed at the identification of genes involved in osteoblast differentiation. Prior this study, this mouse shRNA library present in our institute was previously used to perform a screening to find genes involved in neural differentiation of ESCs. With our work we demonstrated versatility of this library and therefore the possibility to adapt this tool for different types of assay. In fact, the system that we have developed, allowed us to perform a screening of mouse genes to identify those involved in osteoblast differentiation. The high-throughput system can be adapted to pharmacological as well as other screenings to identify modulator of osteoblastic differentiation.

A future perspective of this study consists in completing the characterization of the identified genes by morphological analysis in cells and *in vivo* data: in fact, their identification with the screening and the following analysis in the cellular system represents an interesting starting points to further investigate on their role in osteoblast differentiation and eventually to individuate new target for bone related pathologies *in vivo*.

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