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Role of the Transcription Factor Sox2 in the Osteogenic Lineage

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ABSTRACT

The Sox2 transcription factor is modified by sumoylation at the K247 position although the addition of SUMO1 and Pias1 promotes the sumoylation of Sox2 at the additional K123 site. The role of sumoylation on Sox2 biological functions was analyzed by comparing the activity of WT and sumoylation mutants on the transcription of the FGF4 gene in HeLa cells and on the downregulation of the Wnt pathwayvin 293T cells. When SUMO1 and PIAS1 promote the sumoylation of WT Sox2, the transcriptional activity of the FGF4 promoter is inhibited showing that Sox2 sumoylation is necessary for the repression function. However, there is no effect of Sox2 sumoylation on β-Catenin activity.

Since we were interested in osteoblast differentiation we set up an inducible system for Sox2 in primary osteoblasts. Following Sox2 doxycycline induction, 158 genes were differentially expressed: 120 up-regulated and 38 down-regulated. We annotated as direct Sox2 targets a number of genes involved in osteoblast biology and we further analyzed 3 of them involved in the BMP pathway. The results show that Sox2 regulates the BMP pathway–without affecting SMAD phosphorylation, and that Sox2 sumoylation is not necessary for this function.

We also found that genes involved in the Hippo pathway were direct Sox2 targets. As the Hippo pathway is activated by Sox2 and Sox2 interacts with the NF2 promoter, we checked the effect of Sox2 on the expression of NF2. We showed that Sox2 down-regulates the transcriptional activity of the NF2 promoter, allowing the transcription of the YAP/TEAD genes in osteoblasts, thus acting as an upstream regulator of the Hippo pathway. We conclude that Sox2 induction in osteoblasts triggers FGF dependent inhibition of the BMP, Wnt and Hippo pathways.

INTRODUCTION

BONE TISSUE AND SKELETON.

Bone is composed of dense connective tissue that forms the structural framework of vertebrates. There are 206 bones in an adult human body and 270 in an infant, very heterogeneous for sizes, shapes and locations. The functions of skeletal apparatus can be organized into three categories: mechanical, synthetic and metabolic (Lee and Karsenty, 2008).

Mechanical functions of bone include protection of internal organs, body shape and movement. For instance, skull protects brain and ribcage encloses heart and lungs. Bone, muscles, tendons, and ligaments cooperate to aid in movement of a part of or the whole body. A specialized mechanical function of bone also involves sound transduction in the middle ear.

The synthetic activity of bone tissue primarily comprises of haematopoiesis. The site for the reaction is the medullar cavity of long bones, where blood cells are produced from their progenitors. Moreover bones function as storage for minerals, growth factors and fat, as an energy reservoir: bone tissue plays an essential role in metabolism of entire organism.

Based on structural categorization, compact bone is distinguished by spongy bone.

Compact or cortical bone makes up the shaft of long bones and surfaces of other bones (Galante et al., 1970), providing the typical white and smooth appearance to the 80% of total bone mass of the skeleton. The fundamental functional unit of compact bone is the Osteon or Haversian system. Osteon appears as a long narrow cylinder, that is approximately 10 mm in length and 0.2 μm in width. Each osteon consists of a central or Haversian canal, which contains blood and nerve supplies. Surrounding the canal are compact bone tissue concentric layers, called lamellae. Each lamella consists of osteocytes, lodged in lacunae, spaces of mineralized matrix. The osteocytes within an osteon are connected to each other and to the central canal by fine cellular extensions called canaliculi, even useful for the exchange of nutrients and metabolite waste between osteocytes and blood vessels. Osteons are connected to each other and to periosteum by oblique channels called Volkmann's canals (Fig. 1 A and B).

Over the past years a lot of academic and industrial research laboratories throughout the world have focused on a better understanding of the molecular mechanisms regulating bone formation and growth. A major cause is explained by a steady increase in bone related diseases in humans. For instance, osteoporosis is a disease on a steady rise in the U.S.A. and all over the world.

Statistical estimates reveal that about 52 million people over the population are affected by osteoporosis in the United States and the number is expected to increase of other 10 million by 2020 (from National Osteoporosis Foundation, 2010). In addition, osteoporosis related fracture expenses are expected to rise from 19 billion dollar in 2005 to 25 billion dollars by 2025 (National Osteoporosis Foundation, 2010). Among all the other genetic or metabolic skeletal pathologies our research lab focused on premature skull suture pathology named craniosynostosis (see sections below).

Cellular and non-cellular bone elements.

Bone tissue represents a specialized connective tissue, built by metabolically active cells within a mineralized extracellular matrix (Milat and Ng, 2009). The major cells involved in bone growth and maintenance are osteoblasts, osteoclasts and osteocytes (Fig. 1C).

Osteoblasts produce the major constituents of bone matrix, so they are commonly referred to as bone forming cells. They originate from multipotent mesenchymal stem cells (MSCs), that later differentiate to preosteoblasts and terminally differentiate into functional mature osteoblasts (Aronow et al., 1990; Aubin et al., 1995). A mature osteoblast synthesizes a collagen rich matrix which later mineralizes to form the mature bone. During mineralization, some osteoblasts remain on the bone surface to form flat lining cells. Other osteoblasts get entrapped within the bony matrix and differentiate into osteocytes. These cells undergo a change in morphology and develop the net of canaliculi. Osteocytes have more limited capacity of protein synthesis compared to osteoblasts (Nefussi et al., 1991).

Another major cell involved during bone remodeling is the osteoclast, originated from hematopoetic monocyte-macrophage cell lineage and specific for bone resorption. Osteoclasts are enclosed within a resorption lacuna, the so called Howship's lacuna, at the bone surface. They each harbor 10 to 20 nuclei and are characterized by giant size (up to 100 mm in diameter), ruffled border, sealing zone to the bone matrix and a foamy appearance due to a greater concentration of vesicles and vacuoles (Gothlin and Ericsson, 1976).

Bone formation: Intramembraneous and Endochondral origin.

Bone is formed through a complex process named osteogenesis or skeletogenesis. The formative process depends on the lifespan and proliferation potential of osteoblasts (Manolagas, 2000) and is an integral part of bone remodeling. It starts from MSCs, derived from the embryonic mesoderm and resident in both the vascular stroma of adipose tissue and bone marrow (Tang and Lane, 2012). The determination phase of MSC differentiation involves pluripotent cells committing to lineage specific progenitors, before further differentiating into mature bone, fat, cartilage, or muscle tissues through finely tuned cell proliferation and cell differentiation events (Fig. 1), (Laudes, 2011).

Most of skeletal element development occurs through endochondral ossification, a process in which MSCs condensate and differentiate into chondrocytes that form a cartilage template for future bone. The vascular invasion of compact chondrocytes triggers the template replacement by the three specific bone cell types in a sequential manner, from osteobalsts to osteocytes to osteoclasts.

In a minority of skeletal elements, such as part of the clavicle and part of the skull, cells of the mesenchymal condensation differentiate directly into bone-forming osteoblasts, a process called intramembranous ossification.

A careful coordination of signals within cells, to drive proliferation, migration and differentiation in a chronologically and spatially organized fashion allows a proper osteogenesis. During the last decades, new details on bone mass remodeling and regeneration has been achieved, mainly thank to the growing body of knowledge about the wide number of hormonal molecules, cross-talking pathways and transcription factors involved at both systemic and local levels.

Bone modulation by hormones.

The major hormones that regulate bone remodeling include polypeptide hormones, such as growth hormone (GH), parathyroid hormone (PTH), calcitonin, steroid hormones like 1,25-dihydroxyvitamin D3 (Vit D3), glucocorticoids and sex steroids.

The synthesis and release of polypeptide hormone GH is regulated by promoting growth hormone releasing hormone (GHRH) and inhibiting somatostatin on the anterior pituitary gland (Giustina and Veldhuis, 1998). GH direct and indirect effects are essential for normal bone development, since receptors are on both osteoblasts and chondrocytes. Furthermore, in vitro

studies have demonstrated that GH stimulates the proliferation rate of osteoblastic lineage cells (Kassem et al., 1993) and expression of pro-differentiative bone morphogenetic proteins (BMPs), as described later (Canalis et al., 2003). The indirect effects of GH are mediated by insulin- like growth factor (IGF). GH stimulates the release of IGF-I from the liver, to the bone. Additionally, IGF-I is also produced locally from bone and exerts autocrine or paracrine effect on bone itself. The IGF binding proteins (IGFBPs) are often bound to the circulating IGF-I or complexed with IGF-I produced locally in tissues: IGFBP-3 and -5 stimulate whereas IGFBP-2, -4 and -6 primarily inhibit the actions of IGF-I on bone (Clemmons et al., 1995). In vivo studies in mice further confirm the direct and indirect roles of GH and IGF on longitudinal bone growth (Giustina et al., 2008).

Besides its role on calcium homeostasis in kidney and intestine PTH, along with PTH-related peptide (PTHrP), interacts with receptors on osteoblasts to produce osteoclastogenic factor RANKL that initiates osteoclast differentiation and subsequently bone resorption (Potts et al., 1997; Swarthout et al., 2002). On the other hand, PTH is an anabolic factor in vitro and in vivo: it stimulates the expression of IGF-I, IGF-II, and IGFBPs (Canalis et al., 1988; Kurland et al., 1997).

Sex steroids play an important role in bone and mineral metabolism. For instance, reduced estrogen concentrations lead to a marked reduction in bone mineral density (BMD), thus describing the major rationale for post-menopausal osteoporosis (Syed and Khosla, 2005). Normal concentrations of estrogen in the body reduce the osteoclast formation by decreasing the receptiveness of osteoclast progenitor cells to RANKL (Srivastava et al., 2001) or by induction of osteoclast apoptosis (Kameda et al., 1997). Similar to estrogens, androgens regulate bone growth and have suppressive effects on osteoclastogenesis (Kawano et al., 2003), explaining the reduced risk of osteoporosis, greater mineral density and stronger bones in males.

Besides IGF-I and -II, there are many other growth factors including FGF, Wnt, BMP and Hippo family of proteins that regulate bone formation and remodeling.

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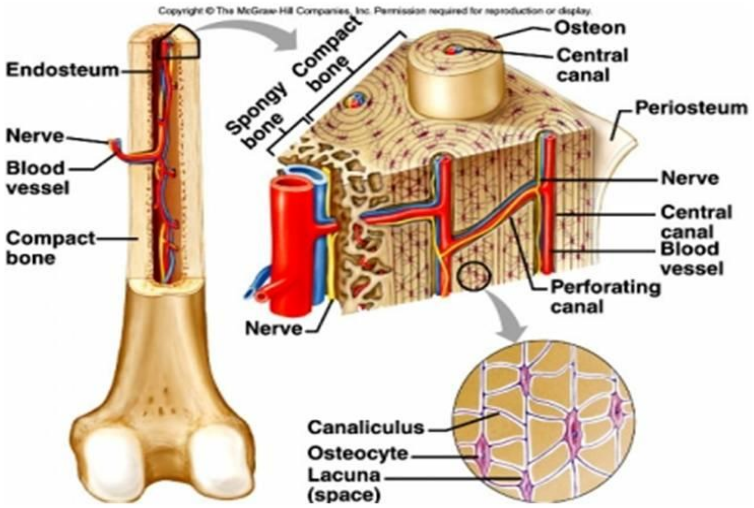

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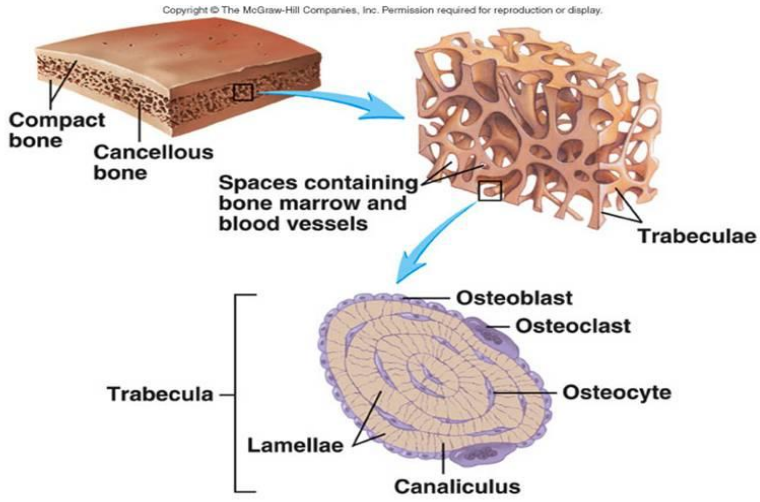

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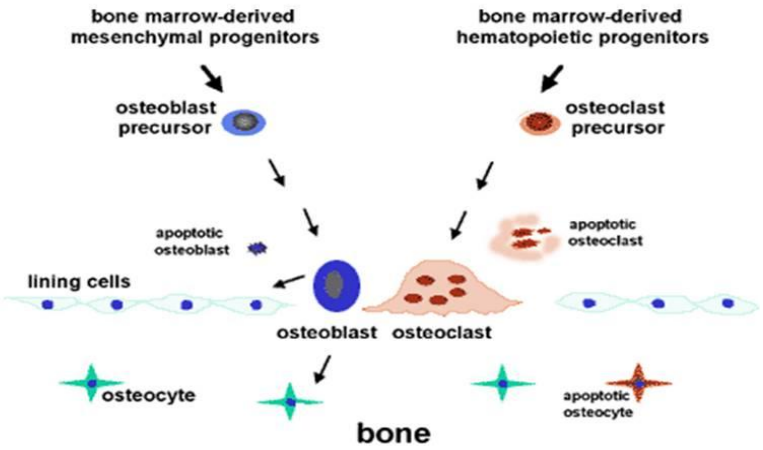

Fig. 1: Bone tissue anatomy and cell line derivation.

A. Long bone components, from skeletal element to cellular organization. **B.** Flat bone structure, from macro- to microscopic organization. **C.** Bone marrow derived mesenchymal progenitor and bone marrow derived hematopoietic progenitor lineages contributes to bone formation and homeostasis.

FIBROBLAST GROWTH FACTOR (FGF) SIGNALING.

FGF pathway components.

The FGF family currently comprises 23 structurally related proteins between 20 and 35 kDa each. FGF members have very different expression pattern, spanning from ubiquitous molecule, like FGF2, to other limited to specific cell populations or development stages, such as FGF4 (Ambrosetti et al., 1997; Ambrosetti et al., 2000). FGFs bind to one of two tissue specific splicing variants among 4 different gene products of tyrosine kinase transmembrane receptors. Upon binding FGFR on its extracellular ligand-binding immunoglobulin- like (Ig) domain, FGF causes partially promiscuous dimerization of receptor monomers, leading to autophosphorylation of tyrosine residues on the intracellular signal transduction domain (Su et al., 2008). Alternative downstream signal transduction pathways have been described, following activated FGFR kinase phosphorylation of FGFR substrate 2α (FRS2α) (Gotoh, 2008), which is constitutively associated with the receptor kinase, and phospholipase Cγ1 (PLCγ1)(Carpenter and Ji, 1999). Activated FRS2α binds the adaptor protein growth factor receptor-bound 2 (GRB2), that then recruits to the signalling complex either the guanine nucleotide exchange factor son of sevenless (SOS) or the adaptor protein GRB2- associated binding protein 1 (GAB1). Recruited SOS stimulates RAS GTPase, which initiates mitogen activated protein kinase (MAPK) cascade (Kouhara et al., 1997): phosphorylation means translocation from the cytoplasm to the nucleus, where it phosphorylates and hence activates immediate early gene transcription factors, such as FOS, to induce transcription of specific genes, primarily stimulating cell proliferation (Fig. 2).

Alternatively, recruited GAB1 leads to Inositol tri-phosphate kinase (PI3K)-mediated activation of AKT kinase, also known as protein kinase B (PKB) (Lamothe et al., 2004). As a result, AKT inactivates pro-apoptotic effectors, such as the BCL-2 antagonist of cell death (BAD) and forkhead box class O (FOXO) transcription factors, thereby promoting cell survival (Brunet et al., 1999) (Datta et al., 1997).

Recruitment and phosphorylation of phopholipase C gamma (PLCγ1) by FGFR kinase initiates a distinct pathway, that is thought to have roles in cell migration and differentiation and that can in turn influence the mentioned RAS–MAPK and PI3K–AKT pathways. Activated PLCγ1 catalyses the hydrolysis of the membrane phospholipid phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5) (P_2)] into diacylglycerol (DAG) and IP_3. DAG phosphorylates and activates protein kinase C (PKC), that in turn activates substrates like the myristoylated Ala-rich C kinase

substrate (MARCKS), a regulator of cell motility (Hartwig et al., 1992); IP3 contribute to release of Ca^{2+} ions from intracellular stores (Li et al., 2011).

FGF signaling in Bone tissue.

Fibroblast growth factors (FGFs) and corresponding receptors (FGFRs) are essential for bone homeostasis and during fracture healing. Functionally, FGFs are osteoblast mitogens. One among several types of FGF, FGF-2 stimulates DNA synthesis and proliferation of both precursors and mature osteoblasts (Canalis et al., 1988).

FGF signaling is crucial in both endochondral and intramembranous ossification. Different FGF ligands are expressed in mesenchymal limb bud soon at the stage of condensation and later in chondrocytes and osteoblasts on developing long bones (Colvin et al., 1999). During the cyclic proliferation and hypertrophy stages of endochondral bone formation, at least ten different FGF molecules are expressed in a perfectly spatio-temporal coordinated manner (Lazarus et al., 2007). Particularly, FGF2 is indicated as the earliest marker gene to be expressed in pre-chondrocyte condensation stages, while FGF1 and FGF3 appear later in differentiated chondrocytes (Yu et al., 2003).

Lessons from both genetically modified mouse models and *in vitro* studies indicated that excessive Fgf2 gene expression inhibits chondrogenesis, resulting in decreased bone elongation, hypertrophic abnormal chondrocyte proliferation (Sobue et al., 2005). Conversely, mice lacking Fgf2 display reduced bone formation and abnormal bone structure (Montero et al., 2000). Overall, the correct dosage of Fgf2 is essential for the bone growth and homeostasis (Su et al., 2008). Other FGF ligands play similar roles from the stage of chondrocyte condensation till osteoblastic differentiation, indicating redundancy among FGF signaling (Fiore et al., 1997; Haub and Goldfarb, 1991).

With regard to intramembranous ossification, only Fgf3 and Fgf4 are not expressed in coronal suture in the mouse embryo and in other mesenchymal sutures during craniofacial development (Su et al., 2008). FGF signaling actually cross-talks with the other osteogenic pathways, including Msx2, Twist, Bmp and other TGF-β superfamily members, during calvarial suture morphogenesis (Opperman et al., 2000; Rice et al., 2005).

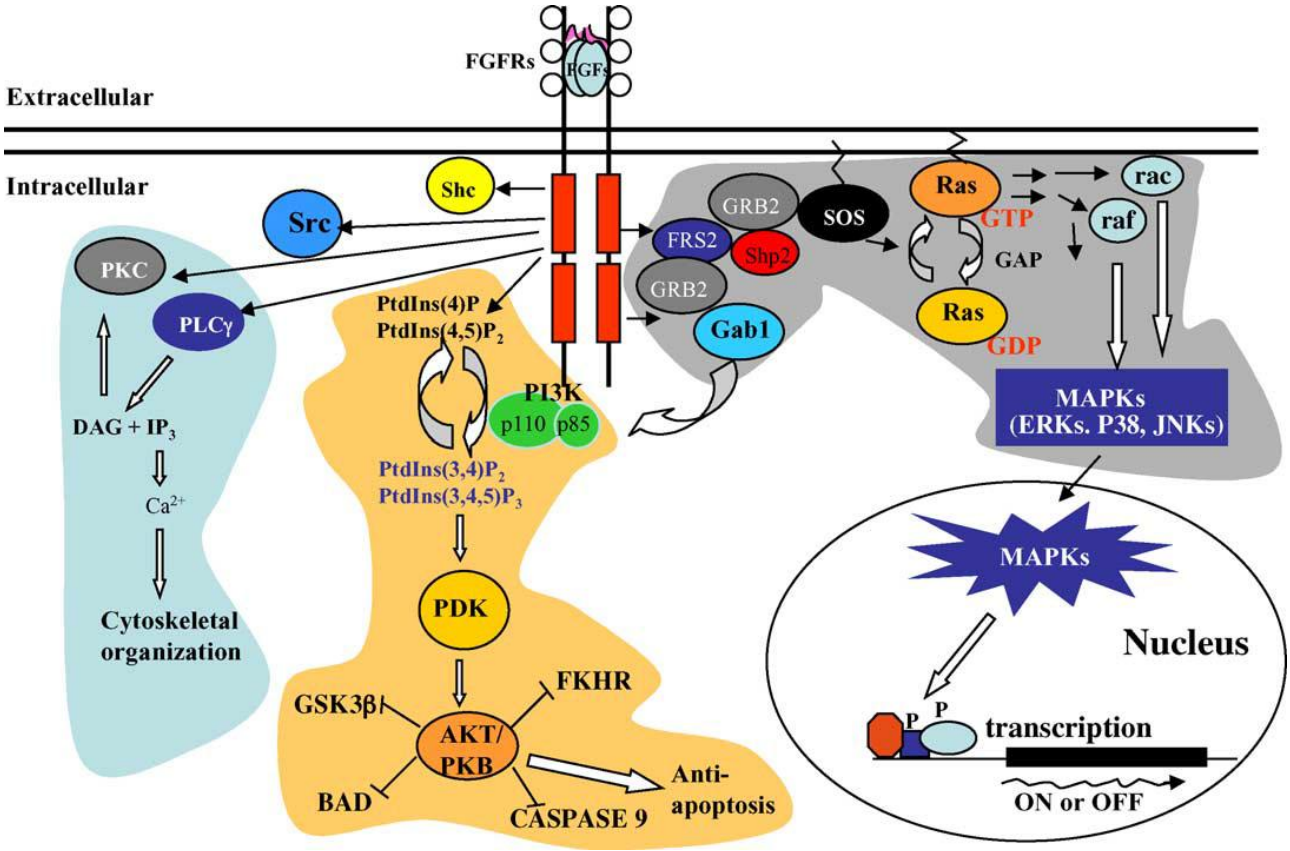

Fig. 2: FGF signaling pathway.

Representation of FGF intracellular signaling pathway from (Dailey et al., 2005): FGFRs activation (red rectangles) stimulate PLCg pathway (blue area), the P-I-3 Kinase-AKT/PKB pathway (yellow highlight) and the FRS2-Ras-MAP kinase pathway (grey area). The activated MAP kinases (ERKs, p38, or JNKs) are translocated to the nucleus, where they phosphorylate (P) transcription factors, thereby regulating target genes. In some cell types FGF signaling also phosphorylates the Shc and Src proteins.

FGF- related syndromes in bone tissue: craniosynostosis.

The role of FGF/FGFR signaling in promoting intramembranous ossification is indeed strongly supported by the association of FGFR1-3 genes in the three main human craniosynostosis syndromes, inside the wide spectrum of congenital skeletal disorders, including also dwarfism, hypo- and achondroplasia among other syndromes. In particular, craniosynostosis syndromes are autosomal dominant skeletal disorders associated to gain-of-function mutations in either of the FGFR-1, -2 and -3 genes, which imply the constitutive activation of the kinase receptor activity regardless of ligand binding on the extracellular domain, mainly due to creation or destruction of Cysteine residues, alterating intramolecular sulfide bonding pattern, and of functional auto-phosphorylation amino acids. The major genetic inheritance of affected children comes from paternal transmission lineage as a result of clonal expansion of selected spermatogonia carrying the pathogenic FGFR2 common mutations for rising Apert (S252W), Crouzon (C342Y) and Pfeiffer (C278F) syndromes (Ornitz and Marie, 2002; Wilkie, 2005).

Phenotypic consequences in 1 of 2000 live born children are malformed skull shape, proptosis, increased intracranial pressure with headache and vomiting, mental retardation and learning disabilities, strabismus and ambyopia (Magge et al., 2002; Shimoji and Tomiyama, 2004; Shipster et al., 2003; Thomson and Rood, 1995); Fig. 3A and B.

The functional relevance of the most common FGFR gene mutations has been confirmed by different comprehensive screenings of genes variably expressed in craniosynostosis syndromes compared to healthy controls reveals heterogeneous set of genes, even explaining the wide spectrum of the pathology (Brugmann et al., 2010; Coussens et al., 2008). There are expected variations in genes already associated with craniofacial syndromes, like Fgfr2, Jagged2, Msx2, Twist, Stab2, Tgfb3. Together with variations in Wnt signaling components Dkk2, Fzd1, Wnt1, Lef1, it also emerges disruption of other intracellular pathways, such as the same FGF, TGFβ/BMP, Ephrin/Eph signaling, and expression changes in early osteoblast differentiation genes, like Rbp4, Gpc3, C1qtnf3, Ili1ra, Ptn, Postn, Wif1, Anxa3, Cyfip2.

At the molecular level these pathologic gene variations consist of increased proliferation rates in suture-derived calvarial cells leading to FGF dependent premature suture closure (i.e. synostosis); (Kim et al., 1998) calvarial cell differentiation misregulation. Therefore, FGF signaling exerts a dual effect on osteoblast (OB) biology, inducing proliferation of immature osteoblasts and apoptosis in differentiated osteoblasts (Mansukhani et al., 2000). The pro-differentiation effects should be the result of Runx2- induced expression of osteocalcin, enhanced by Fgf2 (Kim et al., 2003). Further expression profile analysis in our lab highlights,

instead, the abnormal up-regulation of Sry- related HMG-box 2 factor (Sox2) in Apert and Crouzon osteoblast OB1 cell lines, compared to wild-type OB1 control (Mansukhani et al., 2005). Other significance positive and negative expression program changes cover different functional groups: OB1 up-regulated genes coding for extracellular matrix elements, such as fibromodulin, osteocalcin and cadherin11 (Beck et al., 2001; Garcia et al., 2002; Qi et al., 2003) and transcription factors modulating craniofacial development, like Msx2, Hmlx, Dlx2and Pitx2, are down-regulated or not modified in Apert and Crouzon OB1 cells; Gas6 and Wisp1 apoptotic marker genes (Bellosta et al., 1997; Su et al., 2002) are up-regulated in Apert or Crouzon OB1 rather than in normal OB1 cells; IGF signaling- related genes are more expressed in differentiated OB1 than undifferentiated cells, but there is no difference in craniosynostosis mutated OB1 cells; BMP pathway targets and components, like BMP4, Noggin, Decorin, Osf2 and Fmo1, result in lower or same basal expression levels in FGFR2 carrying mutation cells, compared to OB1 cells, as expected according to papers illustrating FGF- dependent antagonism to BMP triggered differentiation (Bellosta et al., 2003; Vaes et al., 2002). In parallel constitutive active FGF signaling inhibits the expression of 40% of established Wnt target genes on used Affymetrix microarray chip.

Not all genes in each category behave coherently as expected: pro-differentiation IGFBP3 gene and some of indicated WNT target genes have higher basal level in Apert and Crouzon OB1 cells, so expression data upon differentiation have not easy interpretation. However, clear Sox2 involvement has an in vivo confirmation: an Apert mouse model connects early abnormal suture closure and imbalanced osteoblast proliferation, differentiation and apoptosis because of the failure of mutated FGFR2 osteoprogenitor cells to respond to signals that would block their recruitment and advancement along normal suture formation sites (Holmes et al., 2009). The signals can refer to role of other cellular pathways, such as WNT, BMP and Hippo mediated signaling, in bone tissue biology.

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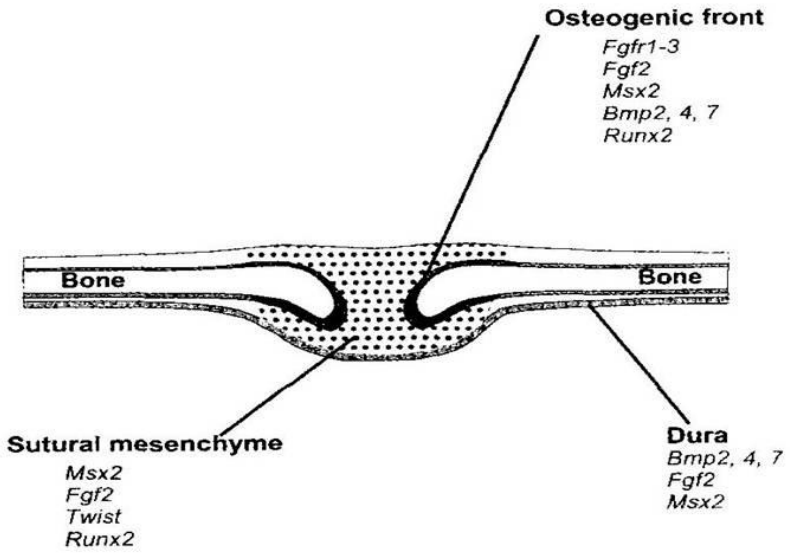

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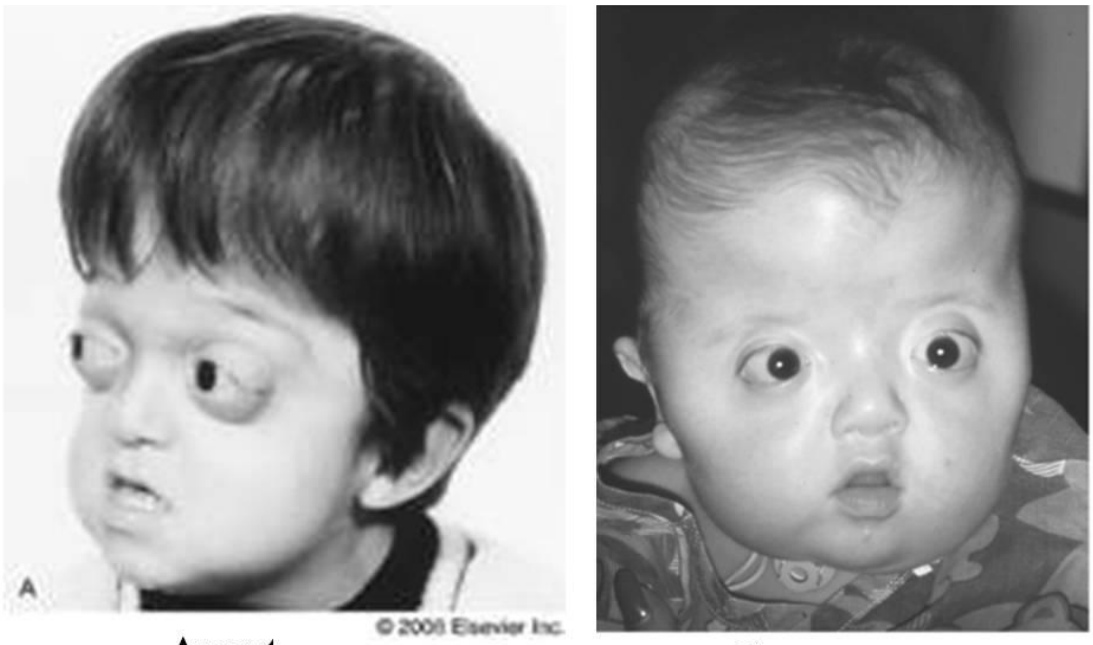


Apert Crouzon

Fig. 3: Impaired skull bone suture originates different craniosynostosis syndromes.

A. Pathway and transcription factor alterations involved in different elements during skull precocious bone suture.

B. Clinical phenotypes in two different Craniosynostosis syndromes, Apert and Couzon, caused by constitutive activation of FGFRs.

WINGLESS-TYPE MMTV INTEGRATION SITE FAMILY MEMBER (WNT) SIGNALING.

WNT pathway components.

WNT family consists of structurally related genes, encoding for secreted signaling glycoproteins, which are extremely conserved in evolution. They are implicated in several developmental processes, including regulation of cell fate, early axis specification, organ development and patterning during embryogenesis (Hartmann and Tabin, 2001). Hence, aberrations in Wnt signalling lead to complex developmental diseases. WNT family members are defined by sequence homology to the Drosophila wingless (wg) and the murine int-1 proto-oncogene, hence the family name. The first Wnt gene was cloned 30 years ago (Nusse and Varmus, 1992); to date, other 18 Wnt genes have been identified in the mouse and human genomes (Milat and Ng, 2009) and see website http://www.stanford.edu/rnusse/wntwindow.htlm. Wnt proteins are traditionally classified into two classes, canonical and non-canonical, based on their distinct molecular signal transduction mechanism.

Canonical Wnts, such as Wnt1, Wnt3A, Wnt8 and Wnt10b, activate a cascade that results in the translocation of β-catenin to the nucleus, where it associates to the lymphoid-enhancer binding factor/T-cell specific transcription factors (TCF/LEF) that finally induces the expression of target genes (Logan and Nusse, 2004; Milat and Ng, 2009). The canonical pathway initiate when a canonical Wnt-ligand binds to one of the 10 known Frizzled (Fz) Receptors in mammals, which are G protein-coupled transmembrane receptors (Wodarz and Nusse, 1998). Although a comprehensive and conclusive view has not been achieved so far (Fuerer et al., 2008), various members of the density lipoprotein receptor (LRP) family, like LRP5 and LRP6, are essential co-receptors for Wnt signalling (Tamai et al., 2000; Wehrli et al., 2000). LRP phosphorylation by activated Wnt is critical and requires the cooperative roles of FZD, the cytoplasmic scaffolding proteins dishevelled (Dsh/Dvl) and axin (Bilic et al., 2007; Davidson et al., 2005). This event inactivates the glycogen synthase kinase 3 (GSK3) and therefore prevents phosphorylation and consecutive proteosomal-degradation of β-catenin (Behrens et al., 1996; Westendorf et al., 2004).

Non-canonical Wnts, including Wnt4, Wnt5a and Wnt11, activate transcription through β-Catenin-independent pathways, involving alternative intracellular second messengers. At least three alternative non-canonical Wnt pathways could be described. One is based on the intracellular release of Ca2+ that activates calcium-sensitive enzymes, which on their turn

activate specific transcription factors (Chen et al., 2005). This Wnt-cGMP/Ca2+-protein kinase C dependent pathway regulates cell migration during dorso-ventral patterning of the embryo, heart development and tumor suppression (Piters et al., 2008). The planar cell polarity (PCP) signaling represent another non-canonical Wnt pathway, required for embryonic morphogenesis; here Fz receptors binding by ligands results in the coactivation of Rho and Rac, two small GTPases that can regulate cytoskeletal architecture (Piters et al., 2008). Finally, the Wnt-protein kinase A (PKA) pathway is based on the increase cAMP levels, PKA activation and regulation of transcription factor CREB, implicated in mouse myogenesis (Kuhl et al., 2000; Semenov et al., 2007).

A large number of Wnt target genes have been identified to date, in over twenty studies based on genome-wide approaches in different cell lines and tissues. Distinct *in vitro* studies provided the evidence of "feedback targets" of Wnt: they are pathway components whose expression can be regulated by the signaling itself, indicating that feedback control is a key feature of Wnt signaling regulation. In particular, over 150 genes have been identified as direct transcriptional targets of the Wnt canonical pathways, as they contain Tcf/Lef binding sites, including basic regulators of cell proliferation in tumorigenesis or other contexts (c-myc, cyclin D, c-jun, etc.), growth factors (FGF9, FGF20, VEGF, BMP4), transcription factors (Runx2), and a wide number of genes implicated in cell adhesion and differentiation.

Other ligands can bind the Fzd–LRP5/6 receptor complex, thus antagonizing Wnt signal transduction pathway. These Wnt inhibitors are Dickkopfs (Dkk) proteins, which compete for the LRP5/6 receptor and prevent canonical signaling in epithelial-mesenchymal transition during mesodermal tissue development (Pinzone et al., 2009). In humans, an increase of DKK1 expression in leukocytes was associated to the presence of bone lesions in myeloma patients. Other Wnt inhibitors are the secreted frizzled related protein-1 (sFRP1) and the Wnt inhibitory factor 1 (WIF-1) in animal and cellular models (Milat and Ng, 2009). It is worth to mention the close relationships between the BMP- and the WNT-signaling at this level: as an example, Dkk1 and Nog cooperate in mammalian head induction; the expression of DKK1 is regulated by BMP-4 in limb development (Grotewold and Ruther, 2002). Furthermore, the multifunctional antagonist called Cerberus, a potent inducer of head formation during vertebrate development, has distinct binding sites for Wnt proteins and BMPs (Piccolo et al., 1999). Finally, USAG-1 might also play as a molecular link between Wnt and BMP signaling pathway (Yanagita et al., 2004).

WNT signaling in bone tissue.

Wnt receptor mutations lead to severe impairment of bone mass suggesting a critical role in skeletal tissue (Westendorf et al., 2004). Interference of this pathway at different stages leads to many clinical manifestations. Wnt or β-catenin deletion results in decreased osteoblastogenesis and increased osteoclastogenesis, whereas their overexpression leads to enhanced osteogenesis and impaired bone resorption (Glass et al., 2005; Holmen et al., 2005), suggesting the importance of Wnt in normal bone growth. β-catenin mutations appear to affect bone resorption by regulating, in differentiated osteoblasts, the expression of osteoprotegerin (OPG), which controls osteoclast differentiation (Glass et al., 2005). Also, conditional deletion of β-catenin in mouse embryo limb and head mesenchyme resulted in blockage of osteoblastic differentiation of mesenchymal precursors (Day et al., 2005). β-catenin is indeed crucial in determining the correct osteoblastic fate of mesenchymal progenitors in the developing embryo (Hill et al., 2005).

For bone development and homeostasis there is *in vitro* evidence that different Wnt proteins are produced by calvaria, primary osteoblast cell lines. Wnt7b gene is up-regulated during osteogenic differentiation of bone marrow stromal cells (Gregory et al., 2005; Kato et al., 2002). Wnt10b, Wnt1,Wnt2 and Wnt3a have an effect on bone physiology, by regulating bone marker gene expression osteocalcin, Runx2, Osterix, ALP. Stimulated osteoblastogenesis has been proofed in mice lacking either Wnt10b or Wnt5a, with impaired bone structure organization and reduced bone mass due to hypoplasia (Bennett et al., 2005). Wnt5a seems to act through CaMKII rather than TCF/LEF, thus confirming that both the canonical and non-canonical Wnt pathways play a role in osteoblastogenesis (Milat and Ng, 2009). Mice lacking Lrp5 display a low bone mass secondary to reduced osteoblast proliferation (Kato et al., 2002). A gain of function mutation in Lrp5 expressed in mature osteoblasts is associated to increased bone mass, due to inhibition of Wnt signaling (Yadav et al., 2008).

Moreover, LRP5 inhibits tryptophan hydroxylase (Tph1) expression, a rate-limiting enzyme in the gut-derived serotonin biosynthetic pathway, impairing serotonin synthesis; serotonin on its turn regulates the bone mass (Warden et al., 2005). Indeed, gut-specific deletion of LRP5 results in low bone mass, similarly to the phenotype observed in LRP5- null mice (Yadav et al., 2008). Osteoblast-specific deletions of LRP5 do not cause evident osteoblast defects. LRP5 may be involved in post-natal regulation of osteoblast differentiation and it is possible that LRP6, rather than LRP5, is the critical co-receptor for Wnt signalling in bone. The LRP6−/− genotype is lethal in mice, while heterozygous mice display reduced bone mass (Pinson et al., 2000).

Wnt signaling is also involved in the transcriptional modulation of the molecular events driving cartilage differentiation. Ectopic canonical Wnt signaling leads to enhanced ossification and suppression of chondrocyte formation during skeletogenesis (Day et al., 2005). On the other hand, during both intramembranous and endochondral ossification, β-catenin inactivation induces ectopic chondrocyte formation in place of osteoblast differentiation. Moreover, Wnt signaling is essential for skeletal lineage differentiation, preventing trans-differentiation of osteoblasts into chondrocytes, and control stem cell self renewal, proliferation and fate lineage specification, by regulating the balance between FGF and BMP signaling.
Dkk1 binds to LRP6 with high affinity in osteocytes and osteosarcoma cells, inhibiting β-catenin activation. When overexpressed, DKK1 induce osteopenia, while Dkk1 loss induces increased bone formation in mice. Other Wnt inhibitor molecules at the level of ligand- receptor bingiding are sFRP1 and WIF-1 also have important roles in bone homeostasis (Milat and Ng, 2009).

Over 70 mendelian syndromes presenting with skeletal abnormalities in humans are associated to mutations in Wnt signaling-related genes. This large group include complex developmental disorders with multi-system implication due to severe imbalance of body patterning in embryo. Besides incompletely characterized rare syndromes, some of the most significant WNT-associated skeletal phenotypes are characterized alternatively by limb malformations and defective/eccessive ossification. For example, loss-of-function mutations in LRP5 occurs in the osteoporosis-pseudoglioma syndrome (OPPG), an autosomal recessive syndrome characterized by low bone mass, ocular defects, and predisposition to fractures. LRP6 loss of function bone phenotype is much more severe than that associated to LRP5 loss (Pinson et al., 2000). In humans, a missense mutation in LRP6, with consequent impairment of Wnt signalling, has been associated to an autosomal dominant early coronary artery disease, to metabolic risk factors and to osteoporosis (Mani et al., 2007).

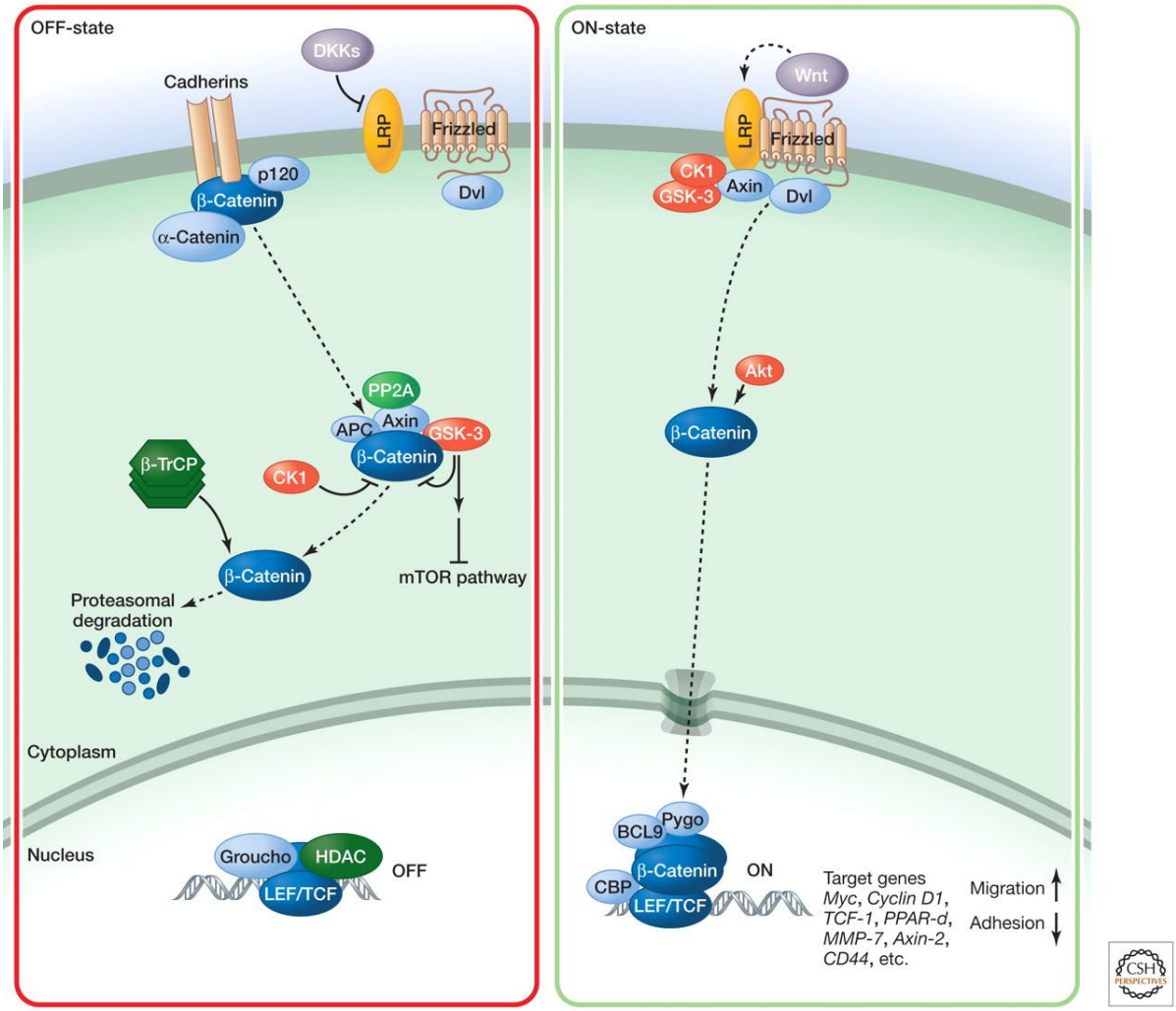


Fig. 4: Inactive and Active WNT signaling pathway.

Red circled panel is Off-state of Wnt pathway: a intracellular protein complex sequester in cytosol β-catenin and promotes its proteasomal degradation, thus inhibiting any Wnt target gene transcription. Green circled panel shows Wnt signaling "on –state": ligand binding activates signaling downstream events that dampen β-catenin degradation complex formation and allows nuclear translocation of β-catenin to finally transcribe Wnt target genes.

BONE MORPHOGENETIC PROTEIN (BMP) SIGNALING.

BMP pathway components.

The Transforming Growth Factor- β (TGF-β) superfamily includes over 30 multifunctional growth factors implicated in the regulation of a wide variety of biological processes, such as proliferation, differentiation, migration, and apoptosis. The role of TGF-β ligands is context-dependent, being affected by diverse environmental features, including tissue and cell type, cell differentiation stage and level of expression of interacting genes.

TGF-β superfamily members are grouped into 3 families on the basis of sequence homology and functional activities exploited through the activation of a specific signaling pathway: the TGF-β/activin/nodal family; the bone morphogenetic protein (BMP) family; the growth differentiation factor (GDF)/Muellerian inhibiting substance (MIS) family. As a rule, all molecules acting in the TGF-β signaling pathway are extremely conserved across species during evolution (Miyazono, 2000).

BMPs were originally identified and named after their ability to induce ectopic bone formation (Urist, 1965). The BMP family comprises over 20 distinct highly conserved secreted proteins, further categorized into multiple subgroups, according to functional and structural features (Miyazono et al., 2005; Wu et al., 2007). BMP actions are mediated through the interaction with different sets of transmembrane serine/threonine kinase receptor complexes, grouped in two types, BMPRI and BMPRII (Massague, 1998; Zwijsen et al., 2003). Upon BMP2 binding, the type-II receptor transphosphorylates the type-I receptor. On its turn, the activated type I receptor phosphorylates of selected members of the Sma and Mad related (SMAD) family of signal transduction proteins, namely Smad1, 5, and 8 (Kawabata et al., 1998; Nohe et al., 2002). The consequent activation of the Smad signaling cascade implies the formation of heterodimeric complexes with the common partner Smad Co-Smad or Smad4, which translocates to the nucleus and recruits distinct transcription factors to regulate target gene transcription (Wan and Cao, 2005; Wu et al., 2007); see schematic representation in Fig. 5.

As an alternative mechanism BMP2 can also bind to preformed heteromeric receptor complexes and activate a Smad-independent transduction cascade, which results in the induction of ALP activity via p38-MAPK (Guicheux et al., 2003; Kozawa et al., 2002; Nohe et al., 2002).

BMP functions cover several aspects of the cell differentiation program. In particular, mice deficient in BMP2 are not viable because of amnio-chorial defects and severe impairment of

cardiac development. The BMP4-null mutation is lethal between 6.5 and 9.5 days of embryonic development because of the lack of mesodermal differentiation and patterning defects (Wu et al., 2007).

BMP signaling is modulated at different levels: extracellular BMP antagonists compete for receptor binding on the cell surface; inhibitory SMADs Smad6 and Smad7 and other interacting molecules can interfere with the correct function of the intracellular Smad cascade; alternative signals from cell transduction pathways converge on the same downstream targets to cooperate or antagonize BMP functions. As an example, noggin acts early in gastrulation, where it antagonizes BMP-2, -4 and -7 and generates a dorsal-ventral BMP gradient which is crucial for the germ layer formation. Moreover, noggin has pleiotropic effects mainly in the formation of ectoderm and mesoderm derivatives (Krause et al., 2011).

Chordin is supposed to play a role during the very early embryo patterning, as it is expressed in the anterior primitive streak, in the node and subsequent axial meso-endoderm. Similarly to Noggin, Chordin deficiency is early lethal in mice and is associated to a ventralized gastrulation phenotype. Stillborn animals have normal early development and neural induction but display later defects in inner and outer ear development and abnormalities in pharyngeal and cardiovascular organization (Bachiller et al., 2000).

BMP family members display distinct spatio-temporal expression patterns, with consequent diversified roles in the morphogenesis of different structures during embryo development (Chen et al., 2004; Jena et al., 1997; Luo et al., 1995; Solloway et al., 1998). However, as their name properly reveals, BMP proteins are primarily expressed in bone and other extra skeletal tissues (Daluiski et al., 2001). Of the many different forms, BMP2,-4 and -6 are the main molecules expressed in bone; they regulate osteoblast cell differentiation and function via SMAD or MAP kinase signaling pathways (Miyazono, 1999). BMPs stimulate the maturation and function of chondrocytes with an increase in expression of type II and type X collagens (Canalis et al., 2003). They induce the osteoblastic commitment of mesenchymal cells, inhibit their differentiation along the myoblastic and adipogenic lineage and increase osteoclastogenesis (Katagiri et al., 1994; Okamoto et al., 2006; Pham et al., 2011).

BMP signaling in bone tissue.

BMPs play a pivotal role in all processes associated with limb development, among which the skeletogenesis. Nonetheless, the biological activities of different BMPs are not identical. In fact, BMP2, BMP4 and BMP7 (also known as osteogenic protein-1, OP-1) are the most extensively studied osteogenic BMPs, being involved in basic skeletal body patterning mechanisms (Bahamonde and Lyons, 2001). *In vitro*, BMP-2, BMP-4, and BMP-7 can induce multipotent mesenchymal cell differentiation into both osteo-chondrogenic lineage cells and osteoblast precursor cells, suggesting their essential contribute to direct and indirect ossification mechanisms occurring in vertebrates (Balint et al., 2003; Canalis et al., 2003).

BMP3 (osteogenin) appears to antagonize the osteogenic effects of BMP2 in stromal cells, likely acting via an activin-mediated pathway (Bahamonde and Lyons, 2001).

Disruption of BMP7 leads to multiple skeletal defects, lack of eye and glomerular development and subsequent renal failure and neonatal death (Jena et al., 1997). Preclinical studies have been demonstrating that these molecules can induce ectopic bone formation upon intramuscular implantation and efficient bone healing/regeneration, when delivered in the appropriate amount and bone defect site (Boden, 2005; Lattanzi et al., 2005).

BMP2 is usually considered a paradigmatic model for studying bone formation mechanisms, since the original demonstration of its efficacy in inducing ectopic bone formation in muscles (Wang et al., 1990). *In vitro* studies demonstrated that BMP2 is able to induce the osteogenic differentiation of mesenchymal cells and transdifferentiation of myoblast into osteoblasts (Ryoo et al., 2006). The BMP2 signaling proceeds downstream of the Smad cascade with the recruitment of bone specific transcription factors. The best characterized as the master osteogenic transcription factor is the Runt-related transcription factor 2 (Runx2), because mice lacking Runx2 show complete arrest in osteoblast maturation and consequent absence of bone (Komori et al., 1997). Runx2 binds the osteoblast-specific cis-acting element-2 (OSE2) in the promoter region of osteoblast-specific genes (Ziros et al., 2002), such as ALP, osteocalcin, osteopontin, bone sialoprotein and bone-specific collagens, thus inducing their expression. Runx2 acts during the initial steps of osteogenic differentiation, while the zinc finger-containing transcription factor Osterix (Osx) represents BMP2 responsive transcription factor in osteoblast terminal differentiation. As for Runx2, Osx-deficient mice display total absence of bone (Nakashima et al., 2002). Recent studies indicate that Runx2 and Osx mRNAs are not directly up-regulated by BMP2, suggesting the existence of crucial intermediators, possibly represented by selected members of the Distal-less homeobox family (Dlx). Dlx5 is a homeodomain-

containing transcription factor that is expressed in later stages during osteoblast differentiation and induces the expression of osteocalcin and the formation of a mineralized matrix (Ryoo et al., 2006). Dlx5-deficient mice display severe craniofacial abnormalities and delayed cranial ossification (Acampora et al., 1999).

Several BMP antagonists have recently been identified that inhibit BMP mediated signaling pathways by binding BMPs (Gazzerro and Minetti, 2007). There are three classified subfamilies based on the size of a cysteine-rich domain, known as cysteine-knot, that characterizes many TGFβ superfamily members: the Differential screening-selected gene aberrative in neuroblastoma (DAN) family; the twisted gastrulation; and chordin and noggin. The DAN family is further subdivided into subgroups, based on a conserved arrangement of additional cysteine residues outside of the cystine-knots: group 1 includes Protein Related to Dan and Cerberus (PRDC) and gremlin; group 2 are homologue of Xenopus Cerberus, Coco and Cer1; finally, group 3 counts of sclerostin and USAG-1. All these molecules are involved in embryo development at various levels, representing also crucial intersections between Wnt and BMP signaling (Yanagita, 2005).

With regard to bone formation, noggin and chordin are structurally and fuctionally related in BMP availability control in the extracellular compartment (Rosen, 2006). Noggin is a glycosylated chemokine protein, which is able to form a neutralizing complex that prevents BMPs from binding to BMPRs (Krause et al., 2011). In fact, noggin expression is essential for proper skeletal development, as over-stimulated BMP activity in noggin-null mice results in excess cartilage and failure to initiate joint formation, along with additional premature embryo lethality (Brunet et al., 1998; McMahon et al., 1998; Tylzanowski et al., 2006). Conversely, ectopic expression of noggin in developing embryos results in suppression of lateral somite differentiation and complete inhibition of chondrogenesis in limbs (Capdevila and Johnson, 1998). Transgenic mice overexpressing noggin in mature osteoblasts show a dramatic decrease in bone mineral density and osteoblast recruitment and function (Wu et al., 2003). Noggin expression is regulated through a feedback system: diverse BMPs induce the inhibitor expression in osteoblasts (Krause et al., 2011), while it is down-regulated by FGF-2 and FGF-9 in the mesenchyme during cranial suture fusion in mice. So a hypothesis for abnormal skeletal phenotypes with cranial and limb malformations, due to gain-of-function mutations of the FGF receptors, can be formulated partially on FGF dependent inappropriate inhibition of noggin expression (Warren et al., 2003).

In a similar way, chordin (CHRD) binds BMPs and sequester them in latent nonfunctional complexes. The formation of the Chrd-BMP complex completely prevents association of BMP2 with both BMPR1A and BMPR2 receptors, leading to early vertebrate embryo dorsal axis formation. Chrd binds predominantly to BMP2 and BMP4, although it has been demonstrated that BMP1 over-expression counteract the dorsalizing effects of chordin, suggesting that also BMP1 should be among the major chordin antagonists in early mammalian embryogenesis and in pre- and postnatal skeletogenesis.

Sost is expressed exclusively by osteocytes and inhibits the differentiation and mineralization of murine preosteoblastic cells. Transgenic mice overexpressing Sost exhibited low bone mass and decreased bone strength due to reduced osteoblast activity and bone formation (Winkler et al., 2003). The mechanism of action of Sost should be based on the inhibiton of BMP-induced SMAD phosphorylation and ALP activity (Yanagita, 2005). It also acts as an inhibitor of the Wnt signaling pathway, by binding and blocking the Wnt receptor LRP-5.

USAG-1, another cysteine-knot secretory protein, shares 38% identity to SOST amino acid sequence. Recombinant USAG-1 protein binds with high affinity and inhibits BMP-2, -4, -6, and -7, reducing ALP activity in mesenchymal cells and pre-osteoblasts (Murashima-Suginami et al., 2008).

Among the BMP-interacting molecules, it is worth to mention the recently discovered Lim mineralization protein (LMP), an intracellular LIM-domain protein acting as a potent positive regulator of osteoblast differentiation (Bernardini et al., 2010). In humans, three different splicing variants are transcribed from the LMP-coding gene (PDZ and LIM doamin-7, PDLIM7). Despite the truncation of nearly two third of the full-length isoform, LMP3 retains efficient osteogenic properties, demonstrated *in vitro* and in different animal models (Parrilla et al., 2010; Pola et al., 2004). *In vitro*, both LMP1 and LMP3 induce osteogenic differentiation of mesenchymal progenitors, fibroblasts and pre-osteoblasts, through the transcriptional activation of BMP2, BMP4 and BMP7 and TGFβ1 proteins (Bernardini et al., 2010). LMP1 osteogenic properties are also based on interactions with the Smad ubiquitin regulatory factor 1 (Smurf1), thus preventing Smads ubiquitination and potentiating BMP signaling (Sangadala et al., 2006). So far, the expression of human LMPs has been detected in the iliac crest bone, in teeth and in calvarial tissues and cells (Fang et al., 2010a).

Skeletal pathological phenotypes associated to BMP signaling.

Molecules directly involved in the BMP-TGFβ pathway are mutated in human syndromes, comprising skeletal malformations in their phenotypes, due to the actions carried out during mesoderm induction, tooth development, limb formation, bone induction and fracture repair. While heterozygous mutations in the BMP4 gene are associated to minor facial malformations, different point mutations in the human homolog of the murine Noggin can cause five different phenotypes, invariably carachterized by limb malformations and other skeletal anomalies due to impaired endochondral ossification (Rudnik-Schoneborn et al., 2010).

SOST was originally identified as thegene responsible for sclerosteosis, an autosomal recessive progressive sclerosing bone dysplasia (Brunkow et al., 2001). In scleosteosis, loss of SOST prolongs the active bone-forming phase of osteoblasts, resulting in increased bone mass.

Both in Europe and the United States the use of recombinant human BMP2 and BMP7 has been approved for selected clinical applications, as an alternative to autogenous bone grafts in the axial and appendicular skeleton. However, despite significant evidence of their potential benefit to bone repair there are not convincing clinical trials outcomes yet (Gautschi et al., 2007). The main limitation for inducing therapeutic bone formation consists of the need for delivery systems that provide a sustained, biologically appropriate concentration of the recombinant protein at the site of the defect (Parrilla et al., 2010).

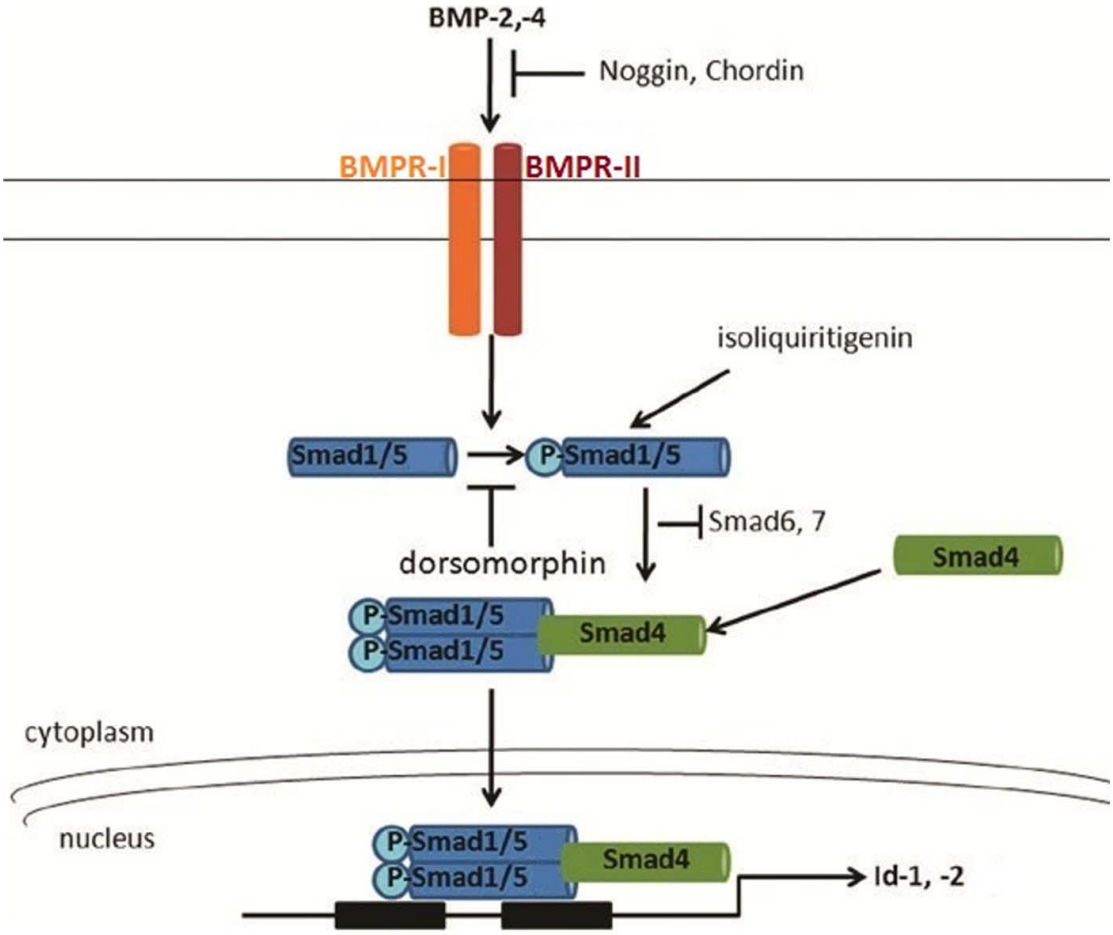


FIG. 5: BMP Pathway.

Schematic illustration of BMP pathway components function, from ligand binding to proper receptors, that dimerize and trigger receptorial Smad1 Smad5 or Smad 8 (not shown in figure 5) phosphorylation on specific Serines. till BMP target gene transcription. Activated Smads, heterodimerize with co-Smad (Smad4), enter the nucleus and bind to specific promoter regions fro BMP responsive gene transcription, such as Id1 and Id2. The figure shows also BMP agonist isoliqiritigenin, that bypasses receptor mediated activation of Smad1/5/8; BMP ligand antagonists chrodin and noggin; and BMP antagonist dorsomorphin, that blocks receptorial Smad phosphorylation.

HIPPO INTRACELLULAR SIGNALING.

Hippo pathway components.

In the past decade advances in understanding the mechanisms underlying pre-defined size in multicellular organism development, cell polarity and adhesion come from Hippo pathway characterization. Most of Hippo signaling components are conserved among species and nomenclature is still based on both Drosophila and mammal organism model studies (Pan, 2010; Sudol, 1994). The mammalian core key components of Hippo signaling cascade are Mst1/2, STE20 family kinases, homologous to Hippo (Hpo) in Drosophila; and Lats1/2 proteins, nuclear Dbf2 related (NDR) family kinases, homologous to drosophila Warts (Wts; Justice et al., 1995); Salvador (Sav in Drosophila and WW45 or Sav1 in mammals) and Mps one binder kinase activator- like 1A and 1B (mammals MOBKL1A and MOBKL1B respectively, while in Drosophila are known as Mats, mob as tumor suppressor) proteins associate to Mst1/2 and Lats1/2 and modulate their activity.

Upstream proteins have yet unclear mechanisms of function, but they generally link different external stimuli and cell architectural perturbations to the core components and in the end to proper transcriptional response in a diversity of contexts (Zeng and Hong, 2008; Zhao et al., 2011; Zhao et al., 2007). Among upstream controllers there are FERM domain containing proteins Expanded (Ex, or FRMD1-6 in mammals) and Merlin (Mer, also named Nf2, neurofibromastosis type 2 gene, in mammals), responsible for cell membrane and actin cytoskeleton association of Hippo core proteins (Hamaratoglu et al., 2006). Kibra interacts with both Mer and Ex in mammal and drosophila models, promoting their productive binding to Hpo and Sav (Genevet et al., 2010; Yu et al., 2010).

Hippo effectors downstream to core components are Yorkie (Yki) in Drosophila and the couple of PSD95/Sap90- Discs large- ZO1 (PDZ) domain containing YAP/ TAZ paralogs in Mammals. Lats1/2 and Mats1/2 phosphorylate Yap and Taz and trigger inhibition of their transcriptional co-activator role (Huang et al., 2005). The major transcription factor targets of Yap and Taz are four the TEA domain containing (TEAD1-4) proteins, correspondant to single Scalloped homolog in Drosophila (Goulev et al., 2008). They act on anti-proliferative and pro-apoptotic genes, involved in cell polarity, adhesion and organ size control; moreover, during self renewal of mouse ES cells maintenance, BMP induced Smad1 activation increases pSmad1 binding affinity

for TEAD factors, with common gene regulation by both mentioned pathways (Alarcon et al., 2009).

As a summary, when Yap and Taz are phosphorylated by active upstream Hippo pathway components, they are sequestered in the cytoplasm, with consequent inhibition of transcriptional activity; inactivation of Hippo signaling results in increased Yap and Taz nuclear translocation and TEAD- mediated transcriptional activation of cell context dependent target genes (Fig. 6).

Hippo signaling in bone tissue.

There are some experimental evidences that link Hippo pathway to bone biology in literature. The Hippo pathway effectors Taz and Yap negatively modulate WNT signaling in different cell models. In cell cultures from mouse kidneys Taz binds to Dvl in cytoplasm, preventing its phosphorylation and activation of Wnt/β-catenin signaling; in contrast, Taz level abrogation enhances Wnt3a stimulataion effect on the WNT pathway, increasing β-catenin nuclear translocation and expression of target genes (Varelas et al., 2010). An apparent discordant proof of Taz- Wnt signaling interplay is the Hippo effector transcriptional activation by the same Wnt/β-catenin pathway (Byun et al., 2014); but this could simply represent a feedback loop of Hippo regulation on Wnt pathway.

In human colorectal carcinoma cell lines cytosolic phosphorylated Yap binds β-catenin and prevent its nuclear translocation and consequent Wnt target gene expression (Imajo et al., 2012). Moreover, Yap can recruit Runx2, an essential transcription factor for bone formation and homeostasis, to sub-nuclear sites; the interaction results in Runx2 activity repression (Zaidi et al., 2004).

Even other modulators of Hippo pathway contribute to its possible role in osteoblast biology. An example is Ras association domain family protein 2 (Rassf2), whose knoct-out in mice impairs haematopoietic and bone remodeling processes (Song et al., 2012).

In general the hypothesis of Hippo pathway role in bone biology is sustained by the original discoveries of functions in limiting organ size through proliferation inhibition and apoptosis stimulation. From our lab research further evidences strengthen the perspective and involve the main actor of this Thesis, transcription factor Sox2 in direct regulation of YAP1 (Seo et al., 2013) and even Merlin/ Nf2 (Upal Basu Roy, personal communication).

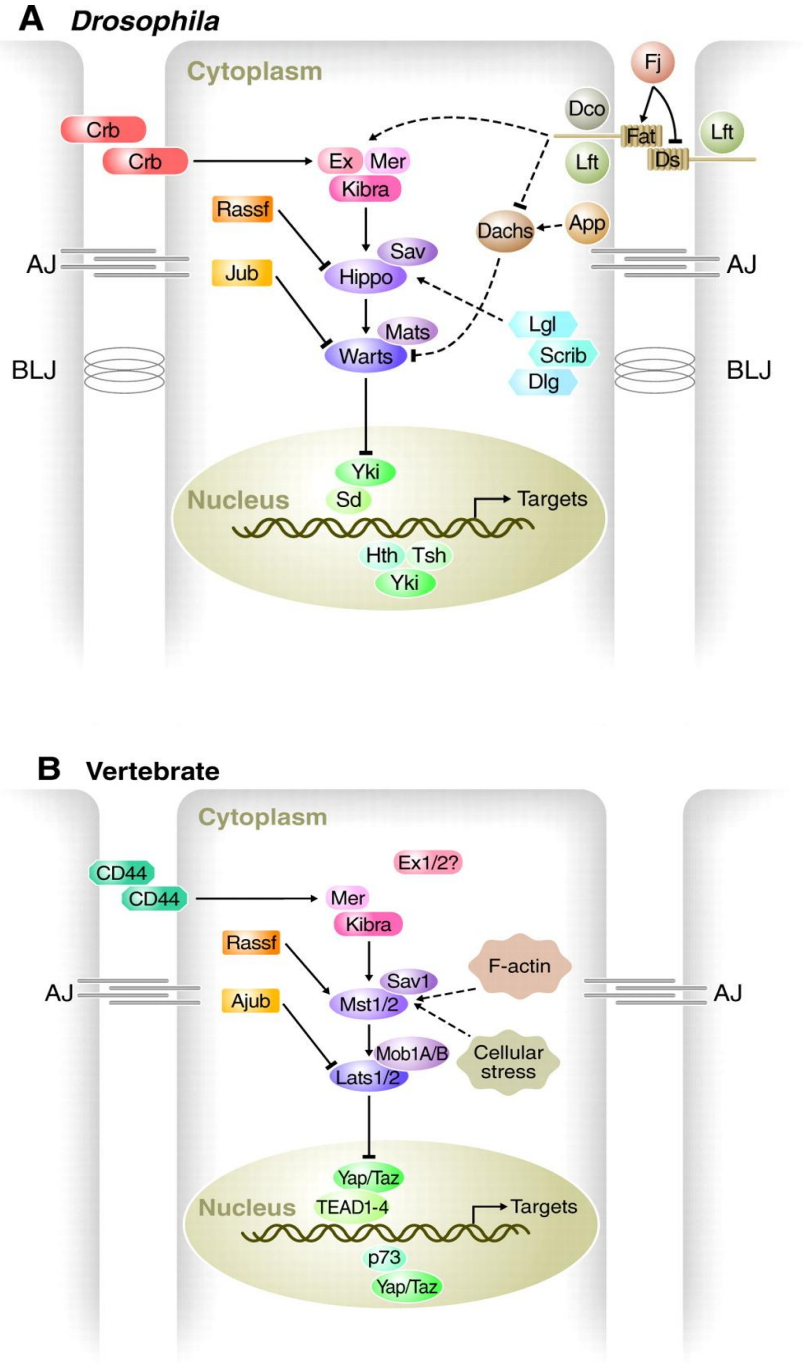


Fig. 6: Hippo Pathway in Drosophila and in Vertebrates.

Hippo signaling pathway components in Drosophila and correspondant elements in Mammals (see text above for details). To be noticed that when Hippo pathway is active, Hippo target genes are not expressed.

TRANSCRIPTION FACTOR CONTROL OF OSTEOGENESIS.

Very important contribution in the process of bone formation comes from different transcription factors, aside from hormones and growth factors. Several transcription factors have been identified that tightly regulate skeletal development (Karsenty, 2008; Karsenty et al., 2009; Komori et al., 1997), thanks to recent advances in mouse and human genetics. Based on overlapping between chondrocyte and osteoblast in the early stages of development, these transcription factors primarily function either in osteoblastic or in chondrocytic lineages. Besides transcription factors that regulate osteoblastogenesis after chondrogenesis initial stages, there are other regulators more specific for intramembraneous direct skeletal development. All the above mentioned events, starting from recruitment of mesenchymal cells to osteoblastic lineage, differentiation into pre-osteoblasts and then to osteoblasts and maturation of functional osteoblasts that secrete bony matrix events, are governed by different transcription factors. Some of the most important transcription factors, that regulate osteoblast proliferation, differentiation and apoptosis, are Runt-related transcription factor 2 (Runx2), Osterix (Osx), Activating transcription factor 4 (ATF4), Activator protein1 (AP1), a number of members of Homeobox and Helix-loop-helix proteins.

Runx2 belongs to the Runt family of transcription factors and it is considered as the master gene of bone formation (Karsenty, 2008). Common structural character among all members of the family is the highly conserved domain called runt domain, responsible for Runx2 interaction with nuclear proteins. Runx2 is the earliest and most important determinant of osteoblast differentiation (Komori et al., 1997) because it is involved in the recruitment of mesenchymal cells into osteoblastic lineage and later in differentiation of osteoprogenitor cells to mature osteoblasts. The expression of Runx2 occurs at very early stages of mesenchymal cell lineage and is observed in the developing embryo by E9.5 prior to bone formation (Lengner et al., 2002; Romero-Prado et al., 2006). Exogenous over-expression of Runx2 causes mesenchymal cells of other lineages to express osteoblast specific genes, that are directly regulated by Runx2 (Ducy et al., 1997). Moreover, in vivo studies in mice lacking Runx2 resulted in complete deprivation of osteoblasts in skeleton (Komori et al., 1997). Other research Laboratories have shown that an optimal expression of Runx2 is favorable to bone growth. Indeed Runx2 could negatively control osteoblast terminal differentiation: transgenic mice with constitutively high Runx2 expression in osteoblasts developed osteopenia with multiple fractures and their bones showed impaired mineralization and low number of mature osteoblasts, compared to wild-type mice.

During osteoblastogenesis Runx2 exert tight regulation of its activity and expression by many other transcription factors or protein-protein interactions or protein-DNA interactions (Karsenty, 2008).

One of Runx2 downstream acting transcription factors is Osx, a zinc finger-containing transcription factor, not expressed in Runx2 deficient mice, whereas Runx2 is expressed in Osx deficient mice (Nakashima et al., 2002). Moreover, Osx transcription is positively regulated by Runx2 and it directs the switch of pre-osteoblasts into immature osteoblasts (Celil and Campbell, 2005). The first evidence of Osx role in osteoblasts comes from studies in deficient mice, with perinatal lethality and impaired matrix mineralization (Nakashima et al., 2002). The outcome is observed only in bones derived from intramembraneous ossification, while in endochondral ossified bone there is still some matrix mineralization left; so Osx, unlike Runx2, seems not to be required for chondrocyte hypertrophy.

ATF4 presence in osteoblasts means that cAMP reponse elements binding (CREB) family of proteins is important in ossification processes (Xiao et al., 2005). There is peculiar protein accumulation in osteoblast cells, although the gene that encodes for ATF4 is expressed in many cell types. ATF4 transcription factor role is mainly limited to late osteoblast differentiation, when it takes palce its interaction with Runx2 to regulate the transcriptional activity of osteocalcin (OCN) gene, a late osteoblast differentiation marker. ATF4 also controls amino acid import into the osteoblasts, regulating the secretive function of these cells. ATF4 has a role also in osteoclasts, favoring differentiation and bone resorption (Elefteriou et al., 2005).

AP1 transcription factor is comprised predominantly in the group of proteins directly regulated by the Fos and Jun families. The generation of mouse models with loss- or gain-of function demonstrates the importance of AP1 proteins as regulators of bone formation (Wagner, 2002). c-Fos was the first transcription factor identified to control osteoblast proliferation and gene expression. Specifically, exogenous forced expression of c-Fos resulted in osteosarcoma and deletion of c-Fos led to osteopetrosis in mice, signifying the dramatic role of AP1 family of transcription factors (Grigoriadis et al., 1993). Similarly, other members of this family like Fra-1 and JunB also promote bone formation and overall bone mass, through increasing the number of osteoblasts (Kenner et al., 2004).

Homeobox proteins, such as Msx1, Msx2, distal-less homeobox (Dlx) 3, Dlx5 and Dlx6, play a role in osteoblast differentiation (Hassan et al., 2004; Shirakabe et al., 2001). These proteins act either as a transcriptional activator or repressor to regulate bone growth. Delayed skull ossification and reduced expression of Runx2 are the results of induced knock-out of Msx2 in mice (Shirakabe et al., 2001). Consistent with the in vivo findings, Msx2 regulates osteoblast proliferation and apoptosis in vitro (Lynch et al., 1998). Similarly, Dlx 3 and Dlx5 are always expressed during all stages of osteoblast differentiation, but the higher expression peak happens as osteoblasts mature. They also have indirect function by enhancing the expression of Runx2 and OCN (Holleville et al., 2007).

Another group of transcription factors are helix-loop-helix proteins, more specifically involved in osteoblast maturation. As an example, Twist-1 and Twist-2 are the major proteins that negatively regulate early osteoblast differentiation and proliferation: Twist-2 interacts with and reduces the expression of Runx2 in osteoblasts (Guenou et al., 2005).
In addition, recent advances in mouse and human genetic studies have led to the identification of novel transcription factors in osteoblasts (Govoni et al., 2009; Gutierrez et al., 2002). To date, the knowledge about the action of these factors on osteoblast function, including proliferation, differentiation and apoptosis, is still poor. A novel family of transcription factors involved in early limb development and osteoblast function is the Sry-like High mobility group (HMG)- box (SOX) family of transcription factors.

SOX FAMILY TRANSCRIPTION FACTORS.

SOX proteins.

The orginal member, that actually named to SOX protein family is Sry, sex determining factor, located on mammalian Y chromosome (Gubbay et al., 1990). Sry harbors a peculiar 80 amino acid long DNA binding domain, high mobility group (HMG) type, shared and evolutionary conserved among all Sox transcription factors. At the beginning the classification into Sox family required any protein to have at least 50% identity in primary sequence with HMG box of Sry; later a protein belongs to Sox family if it contains a HMG domain, in which there is the conserved RPMNAFMVM nucleotide string (Bowles et al., 2000).

SOX proteins are expressed only in animals, from nematodes, to insects, to all vertebrates (Soullier et al., 1999; Wegner, 1999) and they have been classified into 10 different groups, called from A to J, based on sequence homology (Fig. 7). SOX proteins belongs to a specific group because they share at least 90% of sequence identity in the HMG domain and a significative degree of homology outside the family identifying region. In mammals there are more than 20 SOX factors, while earlier in the evolution they decrease in number: one SOX factor belonging to each group in the other Vertebrates; 8 SOX proteins in Caenorhabditis elegans and only 5 in Drosophila melanogaster (Bowles et al., 2000; Cremazy et al., 2001).

Sox genes are interspersed throughout the mammalian genome, without showing genic cluster organization. Memebers of the same Sox group show similar genomic structure: coding regions of Sox B and C genes don't have introns, while the other Sox group genes have a more complex intronic- exonic organization, with different intronic element patterns inside the HMG region during evolution (Wegner, 1999).

SOX factor roles are fundamental in specific developmental stages of cell fate determination. Among the experimental evidences so far, Sry expression is restrained to those cells in genital ridges that originates Sertoli cells, while later it contributes to correct differentiation of male gonads. Another Sox factor involved in the whole differentiation process of Sertoli cells is Sox9; moreover Sox9 is important for cartilage tissue and glia cell development. Therefore it is not surprising that mutations in SOX genes usually result into human pathologies. Actually the knowledge is quite established for role of Sry in sex reversion, for Sox9 in sex reversion- related campomelyc dysplasia syndrome, for Sox10 in Waardenburg-Hrshsprung syndrome.

High Mobility Group (HMG) Box: a DNA binding domain conserved by evolution among the SOX transcription factor family members.

HMG box mainly let SOX proteins to bind DNA, without recognizing other DNA conformational structures, but specifically on a degenerated consensus sequence 7 nucleotide long: *5'-(A/T)(A/T)CAA(A/T)G-3'* (Wegner, 1999) on the minor groove of DNA double helix. Structural studies by cristallography helped in determining both HMG domain structure alone and bound to DNA (van Houte et al., 1995; Werner et al., 1995). As HMG domains in other proteins, Sox HMG region consists of 3 α-helices organized in a L- shaped tri-dimensional structure: antiparallel helix I and II build long arm of L-structure, the third helix the short arm. A highly evolutionary conserved hydrophobic core maintains HMG domain structure, even when bound to DNA. The prototypic Sry- DNA complex reveals how strong the conformational change in DNA is after Sry binding (Fig. 7): the nucleotide double helix is folded 70°- 85°, so it perfectly adapts its minor groove to the concave surface of HMG domain. Sox proteins, together with TATA binding protein (TBP) of transcriptional basal complex and some topoisomerases, are exceptions for way of binding DNA, since most of known transcription factors bind the major groove of genetic double helix. The peculiar properties allow Sox transcription factors to be considered architectural proteins: they modifiy the local chromatin structure, assembling transcriptional active multi-protein complexes (Wegner, 1999).
Moreover, Sox factors use their HMG box for the interaction with other partner proteins and for shuttling into and outside the nucleus, thanks to two different nuclear localization signals (Wilson and Koopman, 2002).

Molecular mechanism of action of SOX family transcription factors.

All Sox transcription factors recognize and bind in vitro the same consensus nucleotide motif, but each Sox protein selectively regulates the expression of a limited target gene set. Moreover, the same Sox factors can modulate different genes, based on the amount expressed in a specific cell type at a certain developmental stage. Finally, the same cell type can express in the same period of lifespan more than one Sox protein, each regulating a specific set of genes. Therefore the Sox specificity of action in different cell types at their differentiation step has to be strictly determined. One general mechanism of action, proposed after collection of many experimental

evidences about Sox protein partners and target genes, relies on at least two functional domains in most of Sox factors: a C-terminal transactivation or repressive transcriptional domain, that functions independently of the cell context, and a HMG domain optimal for binding DNA at low affinity. In vivo DNA binding of a SOX protein is not stable enough to activate or repress transcription of target genes alone. Only a partner factor, located or recruited onto a DNA site next to the one occupied by Sox factor, lead to the formation of a stable functional complex among DNA, Sox factor and partner protein (Kamachi et al., 2000). The ternary complex drive Sox to act as transcriptional activator or repressor, thanks to the partners available in the cell type, with proper localization, orientation and accessibility for their sites on DNA. To be noticed that, because of differences in the HMG domain, the Sox factors' binding ability with a specific partner also depends on protein- protein interactions outside the HMG-box (Wilson and Koopman, 2002).

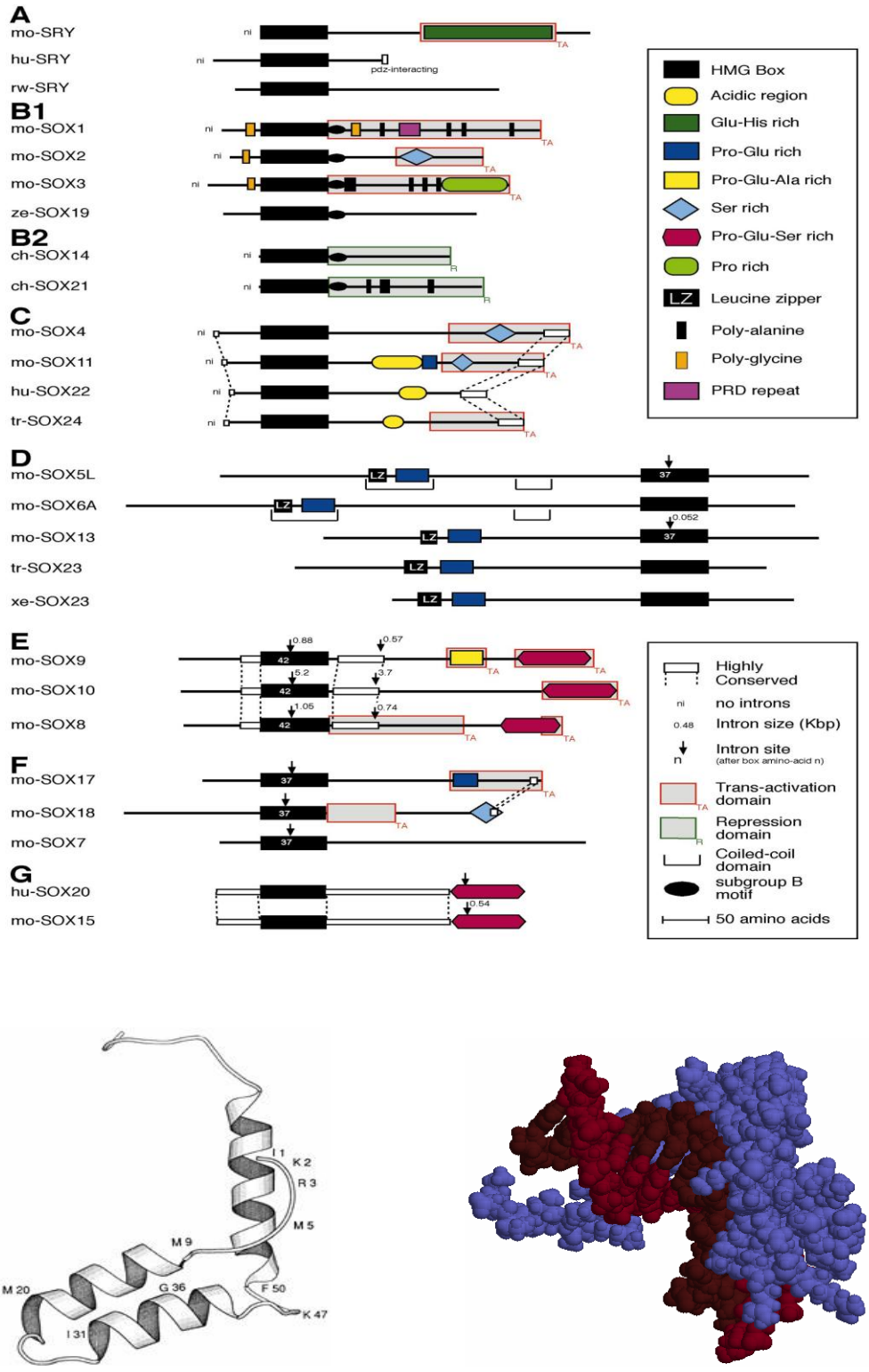


Fig. 7: Sox Family Transcription factor classification and structural details.

Upper panel shows Sox subfamily members with their structural and functional domains (Bowles et al., 2000).

Below on the left, Sry protein HMG-box schematic structure, with residues involved in DNA binding uncovered by site directed mutagenesis. On the right, tertiary structure of Sry HMG domain (red color) bound to minor groove of DNA double helix.

SOX protein interactors and target genes.

A number of studies in the past years indicated different cofactors binding Sox proteins, starting from cooperative partners in Sox factors transcriptional activity on their target genes, to adaptor and modulating nuclear transport proteins.

The mammalian key determinant for sex specification Sry can directrly interact with androgen receptor (AR), through its HMG domain (Yuan et al., 2001), and with WT1, another important transcriptional regulator of gonad development; functional interactions are also proofed with steroidogenic factor 1 (SF1) and Dax1 (Swain et al., 1998).

Sox9 regulates the expression of anti-mullerian hormone (AMH) gene in Sertoli cells during male sexual development by cooperation with SF1: Sox9 and SF1 bind on adiacent site on AMH gene promoter and they interact through the HMG box of Sox9 and C-terminal domain of SF1.

In chondrocytes Sox9 regulates the gene Col2a1, codifying for collagene type II. Sox9 binding sequence is located in the enhancer element COL2C2 and the transcriptional activity depends on cofactors: Sox9 interacts with histone acetyltransferase CBP/p300, whose induction of chromatin hyper-acetylation stimulates Sox9 transcriptional activity (Tsuda et al., 2003); Smad3 can even improve the functional interaction between Sox9 and CBP/p300 on Col2a1 promoter (Furumatsu et al., 2005); the transcription of Col2a1 gene is driven also by cooperation with Peroxisome proliferator-activated receptor coactivator 1 (PGC1), thanks to interaction on promoter site.

There are papers illustrating the dymerization of SOX E transcription factor subfamily members, following cooperative binding on adiacent DNA sites (Bridgewater et al., 2003); the dymerization relies on a short sequence immediately before the HMG domain, evolutionarily conserved in Sox E factors (Peirano and Wegner, 2000; Schlierf et al., 2002).

SoxD factors Sox5 and Sox6 contributes in chondorgenesis by homo- and hetero-dymerization through a coiled-coil domain mediated interaction while bound on promoters of target genes. A documented example comes again from study on Col2a1 promoter, where Sox5 or Sox6 can also synergistically activate transcription together with Sox9 (Lefebvre et al., 1998).

Sox6 cooperation could lead to transcriptional repression of target genes: it is the case of Sox6, just out of HMG box, region interaction with co-repressor CtBP and negative effect on FGF3 gene expression (Murakami et al., 2001).

In endothelial cells HMG-box and C-terminal region of Sox18 can interact with MCM1, Agamous, Deficiens, Serum response factor (MADS) domain of transcription factor Myocyte Enhancer Factor-2 C (MEF2C); the cooperation potentiates Sox18 transcriptional activity on endothelial target genes (Hosking et al., 2001).

B2 group of Sox factors (Sox14 and Sox21) share high homology in HMG domain and in the just following C-terminal region, coexpression in a lot of tissues and functional redundancy with B1 Sox proteins (Sox1, Sox2, Sox3 and Sox19). There is also more than one evidence for competition of Sox B1 and B2 factors on transcriptional regulation of common target genes, as activators and repressors respectively, cause of different sequence and consequent opposite functions in C-terminal domains. An example comes from regulation on δ-Crystallin (DC) gene (Uchikawa et al., 1999): Sox2 binds cooperatively the DC5 enhancer element with Pax6 and activate DC gene transcription; the competition of Sox14 or Sox21 for the same cooperative site with Pax6 results in DC gene transcriptional inhibition.

A couple of papers demonstrates interaction of some Sox factors with members of a large family of multifunctional scaffold/ adaptor proteins containing the 90 amino acid long PDZ domains. SMAD interacting protein 1/ Na+/H+ exchanger 3 regulatory factor 2 (SIP-1/NHERF2) the C-terminal end of Sry, but no other partners of the complex have been identified so far (Poulat et al., 1997); however, Sry and SIP-1/NHERF2 are co-expressed and act as transcriptional regulator and co-regulator in the nucleus of pre-Sertoli cells during testis determination in both human and mouse models (Thevenet et al., 2005). Another PDZ domain containing protein, Sintenin, can interact both with transcription factor Sox4 and the α subunit of interleukin 5 receptor (Geijsen et al., 2001). Il5 stimulates Sox4 driven transcription during B linphocytes differentiation.
It is not known yet if the interaction with PDZ proteins is a general characteristic of all Sox family members, but for sure different Sox proteins have been shown to bind the armadillo repeats in β-Catenin, thus crosstalking and resulting in opposite effects on Wnt pathway in a number of developmental contexts. Sox6 leucine zipper and glutamine rich region contacts β-catenin armadillo repeats 1-4 and antagonizes Wnt signaling in pancreatic β cells by repressor recruitment on cyclinD1 promoter activated by the β-Catenin/TCF complex (Iguchi et al., 2007). Sox9 in chondrocytes exerts negative role on Wnt dependent transcription with a third mechanism: direct interaction on armadillo repeats 4-10 of β-catenin competes for productive binding on TCF/LEF complex and promotes degradation (Akiyama et al., 2004). Even in gut epithelium Sox17 binds armadillo repeats 3-6 of β-Catenin, while complexed with TCF/LEF factors, thus competing with Wnt target gene activation by driving the ternary complex to increased proteasomal degradation (Sinner et al., 2007). In contrast with the negative role on Wnt pathway, during endoderm development in Xenopus Sox17 and β-catenin interact for cooperative activity of Sox17 target genes, including Hnf1β, Endodermin, Foxa1 and Foxa2 (Sinner et al., 2004). Increased Wnt signaling activation is also triggered by Sox4, known to

interact with and stabilize the β-Catenin/ TCF-LEF complex bound on Wnt target genes in gut epithelium and colon carcinoma cells (Sinner et al., 2007).

SOX protein activity regulation through cell localization and post- translational modifications.

Since Sox proteins have important developmental roles and complex regulation of expression patterns in different tissues, their modulation depends not only by main cell signaling pathways, such as BMP, FGF, WNT, but also post-translational modifications (PTMs), through sub-cellular localization and covalent molecule binding.

Sox factor HMG-box harbors two distinct nuclear localization signals (NLS), regulating protein localization and, as a consequence, the transcriptional activity.
Transportation into nucleus starts testicular differentiation in mammalian embryogenesis, when Sry C-terminal NLS in HMG domain binds importin-β1 (Forwood et al., 2001); mutations in that sequence impairs Sry nuclear transport, but not its DNA binding ability and still sex reversion is possible (Li et al., 2001). The same importin-β1 can bind Sox9 HMG box NLS on C-terminal side (Preiss et al., 2001): the sequence conservation in all Sox family subgroups could easily mean that importin-β1 mediated nuclear translocation is common mechanism.
Besides NLS, SoxE members contain also a nuclear export signal (NES) in their HMG domain. Proteins with both NLS and NES undergo to nucleo-cytoplasmic shuttling and the final localization relies on the balance of mechanisms pushing nuclear entry or exit. As an example, Sox9 is expressed in cytosol of both male and female gonad cells; when testicular development initiates, Sox9 moves to the nucleus to drive specific target genes transcription and its expression is maintained at high level, while in female cells the cytoplasmatic localization is soon lost because of decreased expression (Gasca et al., 2002).

Small molecules like phosphoric and acetyl groups often target Sox factors as PTMs, further extending complexity of their action effects in specific cell contexts, even interplaying one another. It is the case of Sox9 phosphorylation- dependent nuclear translocation: prostaglandin D2 (PGD2) phosphorylates Sox9 and increases the transcription factor nuclear localization through better interaction with importin-β1 (Malki et al., 2005) during mammalian gonadal development. Moreover, Sox9 interacts with catalyitic protein kinase A (PKA) subunit, that in

turn phosphorylates two Serine residues in Sox9 and triggers nuclear localization maintenance, higher DNA binding affinity and its transcriptional activity during chondrocytes development (Huang et al., 2000).

Even sumoylation is a PTM often associated with modulation of transcription factor activity, so in next section we focus on better understanding the molecular mechanisms and the effects of covalent binding of Small Ubiquitin-related Modifier (SUMO) on Sox family members.

POST- TRANSLATIONAL MODIFICATION (PTM) BY SUMOYLATION.

SUMO Proteins.

Sumoylation is very important for the normal function of all Eukaryotic cells and it consists of reversible covalent binding of a SUMO molecule to a specific substrate. SUMOs are about 11 kDa small proteins, with a globular β-GRASP folding, given by 4 β-sheets grabbing the only α-helix secondary structural element. Just one SUMO protein has been found in Saccharomyces Cerevisiae and in Schyzosaccharomyces pombe, Smt3 and Pmt3 respectevely; deletion of Smt3 gene drastically decreases cell viability, while deletion of Pmt3 gene results in cell growth impairment. In mammals there are four different SUMO molecules: SUMO-1 (also known as UBL1, Sentrin, PIC1, GMP1 or Smt3c), almost simultaneously isolated as a binding partner of the RAD51/52 nucleoprotein filament proteins mediating DNA strand exchange (Shen et al., 1996) and as component of promyelocytic leukaemia (PML) multiprotein nuclear complexes (Boddy et al., 1996); SUMO-2 (also known as sentrin 2, Smt3b, or GMP-related protein) and SUMO-3 (also named Smt3a or sentrin 3) have been demonstrated later to conjugate substrate proteins (Kamitani et al., 1998; Lapenta et al., 1997) and they share approximately 50% sequence identity with SUMO-1, while differing each other only for the last three N-terminal amino acids. SUMO-4 isoform is suspected to be a pseudogene, since only exogenously expressed mature form, obtained from informatic analysis as 95 aminoacid coding protein with 87% sequence similarity with SUMO-2, is conjugated to substrates in high stressed cell conditions (Bohren et al., 2004; Wei et al., 2008). SUMO-1 usually binds as a monomer to substrate and SUMO-2/3 can also build chains through internal lysine residues (Tatham et al., 2001).

SUMO polypeptides belong to Ubiquitin-like (Ubl) proteins: they have a different superficial charge distribution, but they share about 20% of homology with the 76 amino acid Ubiquitin, the same β-GRASP structure and in a similar manner they covalently bind to a Lysine residue on the substrate. This basic amino acid is usually inside a canonical consensus Sumoylation site ψKXE/D, where ψ corresponds to an aliphatic branched amino acid and X to any amino acid (Johnson, 2004; Xu et al., 2008). This consensus sequence has been extended to flanking regions, defining the phoshporylation dependent Sumoylation motif (PDSM) ψKxExxSP (Hietakangas et al., 2006) and the negatively charged amino acid- dependent Sumoylation motif (NSDM), with a string of acidic residues downstream the canonical consensus site (Yang et al.,

2006). Both the site variants expand a negatively charged region useful in facilitating the interaction of enzymes involved in SUMO covalent binding to substrate protein.

Sumoylation molecular mechanism.

Sumoylation results in the formation of an isopeptidic bond between a C-terminally exposed Glycine residue in SUMO and the ε-amino group of a Lysine in the acceptor substrate (Johnson, 2004; Wilkinson and Henley, 2010).

As illustrated in Fig. 8, first step of the enzymatic cascade consists of SUMO precursor activation through proteolytic cleavage of C-terminal Glycine protective region in SUMO by the heterodimer Sumo activating enzymes 1-2 (SAE1 or Aos1/ SAE2 or Uba2), belonging to the class of sentrin/SUMO specific protease (SENP) proteins. Then activated SUMO is moved from E1 to E2 conjugative enzyme Ubc9. Thiohester bond between carboxyl group of SUMO and the Cysteine in Ubc9 catalytic site is the preliminary step for SUMO to be transferred to the final substrate. In contrast with the similar enzymatic cascade for protein ubiquitination, Ubc9 is unique as mammalian E2 enzyme and can even complete isopeptidic SUMO binding to acceptor substrate, with no requirement of E3 ligases. Ubc9 can discriminate acceptor Lysine inside core consensus site rather than in a NSDM region: acetylated E2 enzyme catalyze SUMO binding on substrate minimal core consensus motif; deacetylation upon hypoxic stimulus drives Ubc9 activity to a preferred NDSM on target proteins (Hsieh et al., 2013).

However, a number of in vivo acting Sumoylation specific E3 ligases have been identified. The mammalian homologues of yeast Siz proteins are the protein inhibitor of activated STAT (PIAS) family of proteins, containing a Siz/PIAS (SP)-RING catalytic domain, similar to the HECT functional domain of E3 Ubiquitin ligases. PIAS proteins binds at the same time Ubc9 and the SUMO target protein, as a sort of enzymatic scaffold to enhance productive spatial mutual orientation of all the actors involved in this PTM. There are four human genes coding for five splice variants of PIAS proteins ubiquitously expressed: PIAS1, PIASx α and β (or PIAS2), PIAS3 and PIASy (or PIAS4). PIAS1 mediates Sumoylation of p73, thus confined in the nuclear matrix with consequent transcriptional repression (Minty et al., 2000); the same negative effect on transcription of respective target genes happens when PIAS1 Sumoylates androgen, estrogen, glucocoricoid and mineralcorticoid receptors (Tan et al., 2002). PIAS3 is specific for interaction with and Sumoylation of Nr2e3, transcription factor related to cone fotoreceptors and inhibited by PTM. Another Sumoylation promoted PIAS4 related negative effect is on p53, whose PTM

drives cell senescence processes (Bischof et al., 2006); PIAS4 also Sumoylates LEF1 and localize it in the PML subnuclear bodies (Sachdev et al., 2001).
In vertebrates other SUMO E3 ligases do nott have SP-RING functional motif, but can promote the SUMO attachment on proper substrates: it is the case of nuclear pore associated protein RanBP2, involved in histone deacetylasis 4 (HDAC4), sp100 and PML Sumoylation (Saitoh et al., 2006).
A third class of SUMO E3 ligases counts for Polycomb (Pc) protein group and for HDAC family members, like HDAC4 (Garcia-Dominguez and Reyes, 2009).
Another peculiar characteristic of Sumoylation is reversibility, obtained through enzymes belonging to the same group of activating SENPs (Mukhopadhyay and Dasso, 2007; Yeh, 2009). Human SUMO proteases are classified into 3 subgroups: SENP1 and SENP2 act both on SUMO1 and SUMO2/3 initial processing and deconjugation; SENP3 and SENP5 activity is more specific for SUMO-2/3 over SUMO-1; SENP6 and SENP7 prefer SUMO-2/3 poly chains only for deconjugation, not in activation phase.

SUMO doesn't always require covalent binding to its substrate. There is increasing evidence for non covalent Sumoylation importance (Kerscher, 2007): SUMO monomers or polymers can interact with a SUMO interacting motif (SIM), even known as SUMO binding motif (SBM) in the substrate, alter inter- and intramolecular structures to allow target protein to expose new sites for binding of other partners. The core amino acid sequence for SUMO non covalent binding is V/I-X-V/I-V/I, flanked by an acidic string of residues; this region can easily build hydrophobic interactions in parallel/antiparallel orientation between α-helix and β-sheet in SUMO (Hecker et al., 2006). As an example, c-Myb, a transcriptional regulator of proliferation and differentiation in progenitors during blood cell development, switches into oncogenic activity when its SIM in the trans-activation domain is mutated, thus not binding SUMO-2/3 anymore and loosing the physiological break to transcriptional activation (Saether et al., 2011).

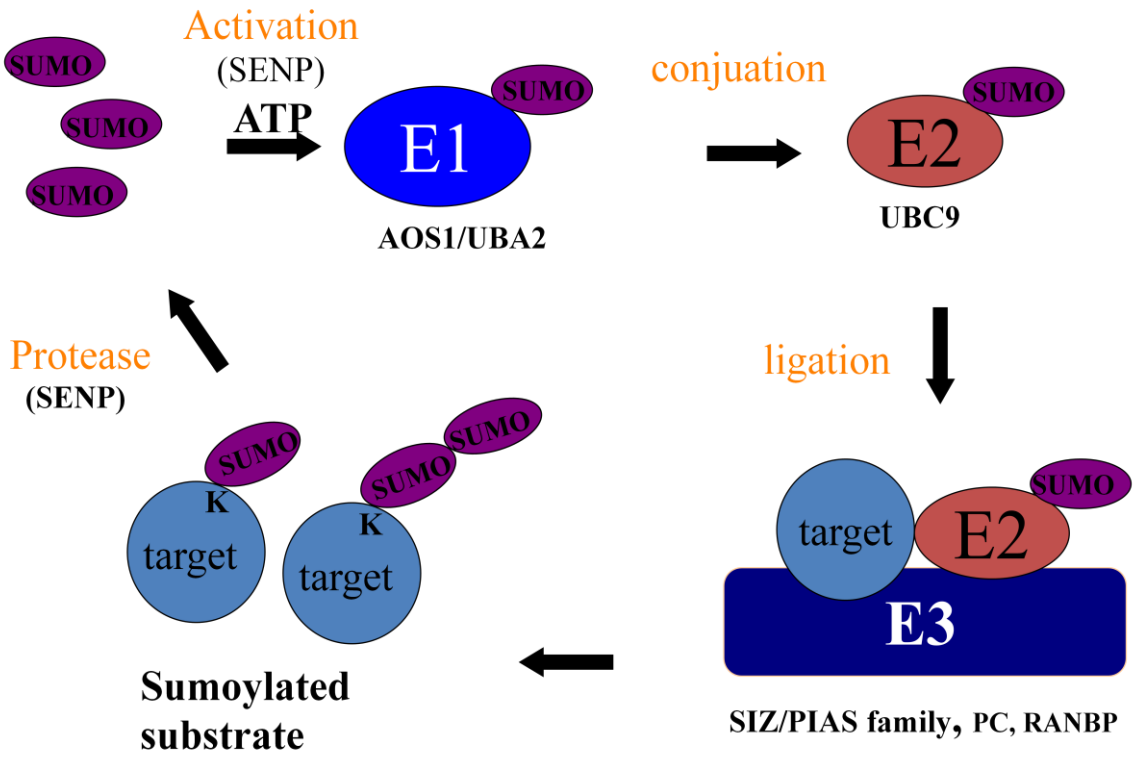


Fig. 8: Post-translational modification by Sumoylation.

Schematic representation of enzymatic steps from SUMO peptide activation, conjugation and ligation to a specific substrate, in a dynamic process that also involves removal of SUMO molecules from targeted proteins. See text for details.

Roles of Sumoylation.

20 years of research after Sumoylation discover have identified hundreds of protein targeted by this PTM (Wohlschlegel et al., 2006), with the most part localized in the nucleus. Despite the transient or reversible kinetic of reaction, the specific cell context, the very low fraction of each Sumoylated target protein compared to its total cell amount, SUMO binding plays a widespread set of effects. The molecular reasons consist of target specific roles of Sumoylation in localization, activity, stability and modulation of structural pattern for new possible interactions on modified substrates.

In mammals a lot of Sumoylated proteins are known transcriptional activators, repressors, co-activators or co-repressors, fine modulated in their normal role. Most cases show a negative effect of SUMO binding on transcriptional activity promoted by the targeted protein. The main mechanisms involved refer to Sumoylation- mediated recruitment of other repressive factors able to local chromatin remodeling; to formation of repression complex for non-covalent interactions due to SIMs and scaffold role of SUMO moiety; to spatial segregation of targeted proteins in not active cell compartments. Although the very low amount of modified substrate, Sumoylation only initiates the genic silencing, the following modifications have permanent effect. Quite well characterized factors whose transcriptional activation is impaired by SUMO conjugation are androgen receptor (Poukka et al., 2000), Lef1 (Sachdev et al., 2001), Elk1 (Yang et al., 2003), Sp3 (Ross et al., 2002) and Smad4 (Long et al., 2004). The above mentioned molecular hypotheses may occur at the same time for regulation of the same post-translational modified protein.

Anyway, p300 and Elk1, when Sumoylated, can recruit on gene target promoters HDAC6 and HDAC2 respectively; deacetylation of both histones and other co-bound factors leads to transcriptional repression (Girdwood et al., 2003; Yang and Sharrocks, 2004).

With a similar mechanism SUMO covalent binding on histone H4 negatively drives transcription of controlled genes through interaction with HDAC1 and HP1γ (Shiio and Eisenman, 2003).

Sumoylation allows localization of different targeted proteins, such as histone deacetylases HP1 and CtBP (Kagey et al., 2003), Lef-1 and Sp3 (Ross et al., 2002; Sachdev et al., 2001), into PML body and Polycomb body nuclear regions.

Nevertheless, in some cell models and contexts Sumoylation has been shown to activate transcription. Modified heat shock responsive proteins HSF1 and HSF2 (Goodson et al., 2001; Hong et al., 2001) and of Tcf4 through PIASy enzymatic action (Yamamoto et al., 2003) has

been demonstrated to better stabilize their respective target gene promoter binding with consequent activation of transcription. Moreover, p53 Sumoylation competes with Ubiquitination by Mdm2 and proteasomal degradation to drive reporter gene expression in vitro (Gostissa et al., 1999; Rodriguez et al., 1999).

In addition to transcriptional regulation, SUMO conjugation has a role in DNA integrity maintenance. There are two system, modulated by this PTM.
Thymidine DNA glicosylase (TDG) excide thymidine or uracyle when it happens a mismatch T-G or U-G in double strand DNA. In vitro experiment reveals that TDG excides the wrong nucleotide and strongly bind the DNA one-nucleotide missing site; SUMO conjugation is required for conformational change and decreased affinity to obtain enzyme release, while quick following de-Sumoylation facilitates TDG binding to another mismatch (Takahashi et al., 2005).
In S. cerevisiae SUMO is even involved in post-replication DNA ripair system, that acts on DNA during G2 cell cycle phase. Higher level of Sumoylation is detected on Lysine 164 of Proliferation cell nuclear antigen (PCNA) complex during S phase, in competition with the Ubiquitination on the same residue, required for proper recruitment of DNA repair enzymes. So SUMO prevents Ubiquitin role in post-replication DNA repair during cell cycle phases other than G2 (Frampton et al., 2006).

Chromatin structure and correct chromosomal segregation are dependent on proper Sumoylation apparatus function; however, molecular basis has not been well detailed so far.
S. pombe cells deprived of the endogenous Sumoylation system result in evident phenotypic alterations: limited cell growth, abnormal exposition to DNA damage agents, highly frequent loss of whole chromosomes, aberrant mitosis, and longer thelomers than wild-type fungi cells (Zhao and Blobel, 2005).
Some experimental evidences suggest SUMO importance in kynetocore function. Indeed, SUMO was firstly identified as a suppressor of a mutation occurring on MIF2 gene, in Vertebrates responsible for coding CENP-C centromeric associated protein: exogenous expression of SUMO rescues the wild-type phenotype of CENP-C null mutants (Chung et al., 2004).

Based on above illustrated roles, Sumoylation seems to be a nuclear localized PTM. Indeed, the first studies reported SUMO mediation of nuclear transport of targeted proteins because of isolation of Sumoylated RanGAP1, the higher modified substrate in vertebrate cells, necessarily

binding the nuclear pore to guarantee nuclear trafficking (Mahajan et al., 1997). However, recent findings also localize SUMO ligases and proteases in cytoplasmatic organelles and increase the number of cytosolic, plasma membrane, mitochondrial and endoplasmic reticulum proteins targeted by SUMO, with not nuclear altered functions. Even the same RanGAP1 Sumoylation occurs in cyotsol, then addresses nuclear translocation. At the level of plasma membrane ion channels K2P1 and Kv1.5 are less active upon in situ Sumoylation (Benson et al., 2007; Rajan et al., 2005), while glutamate receptor GluR6 internalization is enchanced by Sumoylation (Martin et al., 2007). At present Sumoylation is known also in mitochondria biology, where Sumoylated forms of DRP1, GTPase acting in mitochondrion fission, affects whole cell death regulation in response to ischaemia (Anderson and Blackstone, 2013).

Sumoylation system play roles also in correct mammal development.
SUMO1 Knock-out studies in mouse embryos state ubiquitin-like peptide have redundant functions and are dispensable for proper development (Zhang et al., 2008). Specifically SUMO1 effects are not lethal, but affect RanGAP localization and PML nuclear bodies formation; SUMO2/3 can compensate for SUMO1 loss (Evdokimov et al., 2008).
Mouse embryonic death, as enhanced apoptosis in blastocyst inner cell mass, impaired chromosomal segregation and nuclear integrity loss, is a consequence of E2 enzyme Ubc9 depletion (Nacerddine et al., 2005).
E3 ligase PIASy is dispensable for correct embryionic development (Wong et al., 2004), despite some light disruption of WNT and IFN intracellular pathways (Roth et al., 2004). PIASx Knock-out is not linked to embryo survival, but leads to reduced dendritic claw differentiation by MEF2A transcriptional repression (Shalizi et al., 2007) and mild spermatogenetic disturbs (Santti et al., 2005) in adult mice. Focusing on bone tissue cell biology, Ubc9 Knock-Down impairs BMP-induced osteoblastic differentiation in C2C12 mouse myoblast cells through Smad4 Sumoylation, since Smad4 prevented from Sumoylation shows increased transcriptional activity of a BMP responsive luciferase reporter plasmid. Moreover, Ubc9-mediated Sumoylation enhances BMP signaling in Saos-2 cells during osteoblastic differentiation through stabilization of Smad4 and increased nuclear accumulation of Smad1 (Shimada et al., 2008). Our research Lab and other groups demonstrated the role of Sumoylation as an important post translational modification to extend and fine tuning biological roles of Sox factor family members.

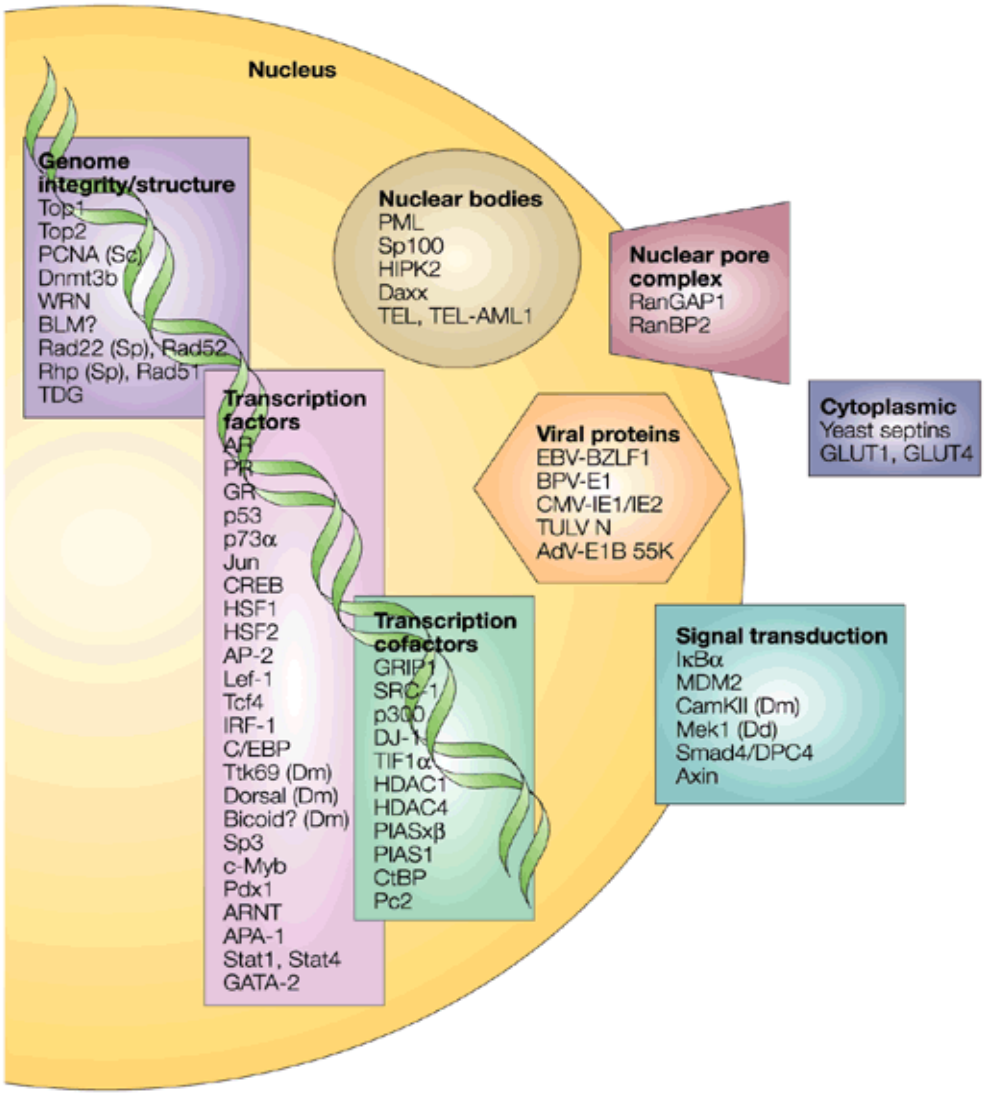


Fig. 9: Main Sumoylation target proteins.

Substrates of post- translational modification by Sumoylation grouped by cell localization or function.

SOX Factors and Sumoylation.

As previously discussed, one common molecular mechanism underlying the wide spectrum of biological effects of unmodified and Sumoylated target proteins is that Sumoylation inhibits transcriptional activity. Mutation of Sumo-acceptor lysines in Drosophlia melanogaster SoxN and human Sox3 dramatically increases their transcriptional potential on respective target genes in Central Nervous System (Savare et al., 2005).

Sox6 cooperates with Sox9 to activate the transcription of Col2a1 gene during chondrogenesis (Lefebvre et al., 1998). Sox6 is Sumoylated in vitro and in vivo by Ubc9 on two different Lysines and this post translational modification both represses transcription of Col2a1 gene on its own and impairs Sox6 binding to Sox9. Moreover, Sumoylation of Sox6 is not required for nuclear localization of the transcription factor, but it drives sub-nuclear re-localization of Sox6 into a more defined punctuate pattern overlapping with nuclear bodies stained by anti-PML antibodies.

Sumoylation has a remarkable impact on SoxE transcription factors and it helps explaining their functional diversity in evolutionary distant species, such as Xenopus and Mammals. In Xenopus embryonic development SoxE factors have equivalent and redundant functions and the Sox9 ortholog is Sumoylated on conserved Lysines 61 and 365. SoxE mutated on these key residues promote expression of markers of neural crest and its derivatives better than wild-type SoxE; moreover they inhibit the expression of typical genes of inner ear development (Taylor and Labonne, 2005). Opposite results are achieved using SoxE-Sumo1 fusion protein in Xenopus embryo development, since the construct mimics the constitutive post translational modification (Holmstrom et al., 2003; Long et al., 2004; Ross et al., 2002): inhibition of expression of neural crest markers and increased inner ear development and its characteristic marker expression. However neither non Sumoylable nor Sumo1 fusion mutants perfectly reproduce wild-type SoxE dependent phenotype; so the fraction of endogenous Sumoylated protein regulates and contributes to all the activities of wild-type prevalent forms (Taylor and Labonne, 2005).

Furthermore, Sumoylation has been shown to inhibit Sox9-dependent activation in synergy with partner transcription factors binding the same promoter region of target genes. This is the case of development and function of steroidoigenic tissues, where Mullerian Inhbiting Substance (Mis) gene transcription is decreased by Sumoylation of one or both orphan nuclear receptor Ad4 binding protein/ steroidoigenic factor 1 (AdBP4/SF-1) and the synergic partner Sox9; DNA binding activity and interaction between the partner transcription factors are not involved in the functional outcome (Komatsu et al., 2004).

SUMO E3 ligase PIAS1 (Protein Inhibitor of Activated Stat1) plays a role in Sox9 activity and stability regulation, but there are contrasting experimental evidences about. In COS-7 cells Sox9 dependent transcriptional activity of a Col2a1 promoter and enhancer reporter is increased by PIAS1 over-expression, along with Sox9 total amount (Hattori et al., 2006). Cotransfection with a SUMO1 expressing plasmid further enhances Sox9 transcriptional activation on the same target gene and makes sub-nuclear distribution of Sox9 more diffuse. On the other hand, interaction of Pias1 with murine Sox9 enhances Sumoylation of this transcription factor on Lysine 396. Sox9 Sumoylation inhibits the activity of both Vanin1 promoter and Col11a2 enhancer reporter plasmids in Cos-7 and 293T cells, while the opposite effect is obtained by Sox9 harboring a point mutation Lysine 396 into Arginine (Oh et al., 2007). Similar reporter assays are set up to evaluate the effects of Pias1 interaciton on both wild-type and mutated Sox9. Interestingly Pias1 can reduce transcriptional activity of both Sox9 and its Sumoylation deficient form, suggesting that the post translational modification is not strictly necessary for Pias1 repression of Sox9 transcriptional potential. However a SUMO-ligase deficient Pias1 mutant can't repress anymore Sox9 activity.

So, along with promoting Sumoylation of Sox9, Pias1 is required for Sumoylation of other unknown factors associated with Sox9 and / or Pias1 on Vanin1 promoter and Col11a2 enhancer in vivo.

This model has been recently proposed again (Lee et al., 2012) for Sox9 and the microphatalmia-associated transcription factor (Mitf) coregulation of activation of the dopachrome tautomerase (Dct) gene promoter during early vertebrate embryonic development, in the context of the switch regulation from neural crest inducing activity to inner ear formation (Lee et al., 2000; Ludwig et al., 2004). Sumoylation of both Mitf and Sox9 is required to modulate their synergistic activity on Dct promoter (Lee et al., 2012; Miller et al., 2005; Murakami and Arnheiter, 2005). In particular SUMO covalently bound to Sox9 provides a new structural Sumo interacting motif (SIM) (Hecker et al., 2006; Kerscher, 2007; Perry et al., 2008) to harbor the Groucho/TLE family Grg4 corepressor (Cinnamon and Paroush, 2008) on Dct promoter. This recruitment via multivalent interaction means displacing of CBP/P300 coactivator from Dct promoter and consequent inhibition of its transcriptional activity.

Another step in regulation of Sox9 activity consists of phosphorylation of Serine residues 64 and 181 in mice. Molecular consequences of this post-translational modification are the Sumoylation of Sox9 and its tighter cooperation with the zinc-finger type transcription factor Snail2. The biological effect is the induction of neural crest cell delamination (Liu et al., 2013).

Further evidence of SoxE factor Sumoylation comes from Sox10 transcription factor biology. The Sumoylation of human Sox10 transcription factor on Lysisnes 55, 246, 357, all conserved among different species, through its interaction with Ubc9 (Girard and Goossens, 2006). Functional consequences of Sox10 Sumoylation don't involve cellular localization, nuclear distribution, DNA binding properties of Sox10, but mostly its transactivation activity. Experiments in HeLa cells show increased transcriptional activity of promoters of two known Sox10 target genes, when Sox10 Sumoylation sites are mutated into Arginines: it's the case of both GJB1 gene, coding for the gap junction Connexin32 (Cx32) protein in Schwann cells of the peripheral nervous system, and MITF, during melanocytes development and differentiation. Moreover, absence of Sox10 Sumoylation supports the synergistic cooperation of cofactors Egr2 and Pax3 with Sox10 on Cx32 and Mitf promoters, respectively.

In our lab research Sox10 Sumoylation has been more detailed. Rat Sox10 interacts not only with Ubc9 and Sumo1, but with known E3 ligases Pias1, Piasxα, Piasxβ (Davide Ambrosetti, personal communication). The main Sumoylation sites result Lysines 55 and 357, but there are at least two other aminoacids that provide accessory sites for the same post-translational modification: Lysines 105 and 253. The enzymatic reaction is enhanced by cotransfection into 293T cells of SUMO1 and one of the three different E3 ligases mentioned above, together with Sox10 coding plasmid; the role of E2 enzyme Ubc9 seems to be limited to Sumoylation of the main Lysine residues or even to have a sort of inhibitory effect.

Sox10 Sumoylation outcome is transcriptional repression of activity of luciferase reporter plasmid carrying the -233/+150 nt promoter region of Connexin32, that harbors two Sox10 binding sites (Bondurand et al., 2001). Simultaneous transfection in 293T cells of WT Sox10 expression plasmid and reporter plasmid increases transcriptional activity of Connexin32 promoter region; cotransfection with only E3 ligase Pias1 doesn't affect transcriptional activity; while cotransfection with SUMO1 expression plasmid significantly decreases Sox10 driven transcription, even more inhibited by the presence of PIAS1, too. In the same luciferase assays conducted with K105R-K253R Sox10, the Connexin32 promoter region activity is not repressed, but almost two times higher than WT Sox10 induced activity. Furthermore Sumoylation doesn't perturb Sox10 diffuse nuclear localization (Davide Ambrosetti, personal communication).

THE TRANSCRIPTION FACTOR SOX2.

This Thesis aims to investigate the role in osteogenic lineage played by a specific SOX B1 subfamily member, Sox2. Sox2 is a fundamental transcription factor in stem cell biology maintenance, pre- and post-implantation development, cell reprogramming, tissue physiological or pathological homeostasis and regeneration. The master regulator Sox2 is a 319 amino acid long protein in mice and 317 in homo, with at least four different functional regions: HMG box is a key domain for both DNA binding and protein partner interaction; C-terminal transactivation domains cooperate with the transcriptional regulation activity; HMG box N-terminal region and the transcriptional activation N-terminal and C-terminal residues stabilize productive protein-protein interactions (Cox et al., 2010; Remenyi et al., 2003).

Self-renewal and differentiation governed by Sox2.

As other Sox factor family members, Sox2 is considered a “pioneer factor”: its contribution is necessary for both progenitor cell maintenance and differentiation regulation in the same embryonic derived lineage roll (Zaret and Carroll, 2011).
In mammalian pre-implantation development blastocyst stage has inner cell mass (ICM) and trophoectoderm (TE) elements. From pluripotent founders and a population of endodermal extraembryonic cells in ICM generate all embryonic lineages and cells for future yolk sac, respectively; TE multipotent stem cells give rise to extraembryonic ectoderm and later to placenta (Rossant and Tam, 2009). In the first lineage specification event Sox2 segregates into ICM, when initially present in both ICM and TE, but Sox2 deficient mouse embryos neither support embryonic stem (ES) cells from ICM nor trophoblast stem (TS) cells derivations (Avilion et al., 2003). Moreover, deletion of Sox2 in already established ES cells results in abnormal differentiation into trophoectoderm-like cells (Masui et al., 2007), but Sox2 over-expression perturbs equilibrium between self-renewal ability and differentiation power of ES cells (Kopp et al., 2008). The dosage dependent property implies a perfect balance of Sox2 with other factors regulating stem status maintenance in ES cells. The self renewal programs base on recruitment of coactivator p300 on proper target gene sets (Chen et al., 2008), while co-binding of Polycomb complex triggers repression of differentiation genes (Lee et al., 2006). A common feature of promoter of both silenced developmental genes and self-renewal genes (Boyer et al., 2005; Kim et al., 2008; Orkin and Hochedlinger, 2011) is Sox2 collaboration with octamer 3-4

(Oct3/4) transcription factor. Oct3/4 belongs to Homeo-domain containing subfamily in POU proteins, like Oct1 and Oct6, other Sox2 partners in different developmental models: the evidence suggests a widespread SOX-POU mechanism for regulation of tissue specific differentiation (Dailey and Basilico, 2001). Sox2 and Oct3/4 directly interact through their respective DNA binding domains, stabilized by interactions with the double nucleotide strands, where they recognize specific sites at the proper distance for cooperation (Ambrosetti et al., 1997; Ambrosetti et al., 2000). An auto-regulatory loop is activated by transcription of the same Sox2 and Pouf5f (Oct3/4) genes (Chew et al., 2005), together with other known genes for keeping ES cells undifferentiated: Fgf4, Utf1, Nanog, Fbx15 (Kuroda et al., 2005; Nishimoto et al., 1999; Rodda et al., 2005; Tokuzawa et al., 2003; Yuan et al., 1995).

After embryo gastrulation Sox2 expression is limited to the presumptive neuroectoderm, sensory placodes, brachial arches, gut endoderm and primordial stem cells (Avilion et al., 2003). The context dependent role of Sox2 is still strictly characterized by cooperation with specific lineage transcription factors and by antagonism with other positive or negative regulators to determine proper differentiation fate. In the absence of other stimulation, Sox2 drives neuronal commitment and subsequent discrimination into central nervous system (CNS) and peripheral nervous system (PNS), by controlling the proliferation and differentiation of fetal progenitor cells (Pevny and Nicolis, 2010). Oct3/4 itself becomes predominantly over Sox2 expression to activate transcription of genes characteristic of mesoendoderm precursor cells.

In the presence of the presomitic mesoderm development regulator T-box transcription factor 6 (Tbx6), Sox2 N1 enhancer, responsible for neuronal fate progression, is silenced and r transcription of paraxial specific genes can start (Takemoto et al., 2011).

Sox2 or paired box gene 6 (Pax6) prevalence drives eye precursor cells in optic cup towards neural retina or non-neurogenic retina development (Matsushima et al., 2011). Later in eye development, in future cristallin cells, Sox2 cooperates with Pax6 for the activation of enhancer DC5, controlling δ-crystallin gene expression. The two proteins alone cannot proficiently bind the enhancer, but only their interaction, stabilized by the DNA element, allows transactivation, with also L-Maf cofactor contribution (Kamachi et al., 2001; Shimada et al., 2003).

In addition to CNS precursor for eye and brain, Sox2 is also transiently expressed in in the Schwann cell lineage and blocks terminal differentiation of Schwann cell precursors, thus impairing axon myelination of PNS. In particular, Sox2 keeps precursors undifferentiated; cross-regulatory interaction between Sox2 and Mitf or Egr2 moves towards Schwann mature cells or melanocyte fate (Adameyko et al., 2012).

In the endoderm foregut development into organ specification Sox2 antagonizes Nkx2.1: anterior Sox2 expression results in future exophagus and stomach, while more ventral Nkx2.1 expression cover future trachea region (Que et al., 2009). Furthermore, the Sox2-Barx1-Srfp axis forms future stomach, while the Wnt-Cdx2 axis results in intestine formation; the two pathway interplay is responsible for correct establishing the boundary between glandular stomach and intestine in the organism (Zorn and Wells, 2009).

Although Sox2 has a negative effect in mesoderm lineage progression in vivo and during ES cell differentiation, specific Sox2 role in differentiation of mesoderm derivatives has been recently proposed for dermal papilla condensation in skin development and in osteogenic lineage.

Somatic tissue homeostasis, regeneration and disease.

Expanding evidences support the notion of importance of Sox2 in all adult tissues, at least in their stem and progenitor compartments, when Sox2 played a role in differentiating that tissue. A solid experimental proof comes from Sox2 expressing neuronal precursors, able to give rise to all kind of specialized cells in nervous system (Brazel et al., 2005). Moreover, immature Sox2 positive cells have been recently found in a number of other adult organs, like testes, forestomach and glandular stomach, trachea, exofagous, lens, anus, dental and cervix epithelia (Arnold et al., 2011).

Sox2 expressing adult stem cells also promote tissue repair processes, providing, for example, the undifferentiated cells necessary in peripheral nerve regeneration upon injury (Parrinello et al., 2010) and in trachea epithelium recovery upon chemical damage (Que et al., 2009).

Heterozygous mutation on Sox2 gene can result in anophtalmia-esophageal-genital (AEG) syndrome, characterized by ectodermal and endodermal tissue abnormalities, such as microphtalmia, hearing loss, brain deformations and trachea-esophageal fistula. Other Sox2 dose dependent pathologies affect pituitary and germ cell size and hormone production (Kelberman et al., 2006).

Sox2 acts as an oncogene in some epithelial cancers, due to locus amplification or gene over-expression. The molecular function can vary among proliferative trigger, cell survival maintenance or anti-differentiation (Rudin et al., 2012; Tompkins et al., 2011). Moreover, Sox2 expression contributes to metastatic ability of tumors of neural and neural crest origin.

Connections between Sox2 and TGFβ/ BMP pathway in cell reprogramming.

Ectopic heterologous expression of a transcription factor set, including Sox2, Oct3/4, Nanog, Klf4, drives reprogramming of several somatic cell types into induced pluripotent stem (IPS) cells, as pioneer studies in mouse and human fibroblasts demonstrated (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). In particular, Sox2 requirement is toward the end of reprogram processes, contributing to activation of itself transcription and of numerous other typical pluripotent associated target genes (Chen et al., 2011).

Alternatively, Sox2 induces different cell fates, based on specific cofactors and environmental stimuli: for example, Sox2 expression, alone or together with other neural transcription factors, can reprogram fibroblasts into neural stem cells (Han et al., 2012; Ring et al., 2012).

Nevertheless, the pluripotency induction is usually an inefficient or instable process, suggesting underlying regulation mechanisms. In this direction some studies identified additional transcription factors in IPS cell formation; other papers found small molecules interfering with cell signaling pathways and enhancing or substituting transcription factor contribution. Treatment with TGF-β receptor I kinase inhibitor results in blocked IPS cell formation from mouse embryonic fibroblasts. Therefore TGF-β signaling enables faster, more efficient IPS cell induction and the used inhibitor molecule replaces both Sox2 or c-Myc in cell reprogramming. In an independent similar experimental model, another TGF-β receptor I kinase inhibitor, called RepSox, enhances IPS cell formation by Sox2 and c-Myc replacement and concomitant up-regulation of Nanog, without affecting Sox2 expression (Ichida et al., 2009).

Coco, a secreted antagonist of TGF-β ligands, induces dormant metastasis-initiating cells from breast cancer to undergo reactivation in the lung. Coco exerts this function by blocking paracrine BMP signaling and thereby enhancing the self-renewal ability of metastasis-initiating cells, where Sox2 is expressed (Gao et al., 2012).

In colorectal cancer cells over-expressed Sox2 seems to regulate BMP signaling at different levels. Sox2 directly binds on promoters of a number of BMP pathway genes: ligands, receptors, co-receptors, receptor and inhibitory SMADs and other downstream mediators (Fang et al., 2010b). Although functional promoter characterization is still unclear, Sox2 inhibits BMP signaling in lung squamous cell carcinoma. The highly over-express lineage survival oncogene Sox2 directly targets BMP4 gene, maintained at low expression level. Reactivation of BMP4 halts cell proliferation and slows cancer growth (Fang et al., 2014).

During normal hair growth process Sox2 finely crosstalks with BMP signaling in dermal papilla niche. Sox2 expression controls migration speed of differentiating hair shaft progenitors by

balance of direct transcriptional control on Bmp6 ligand and Sostdc1 endogenous inhibitor promoters (Clavel et al., 2012).

Modulators of Sox2 acitvity: focus on SUMO post-translational modification.

TGF-β/ BMP pathway is among the extracellular regulators of Sox2 activity; while all above mentioned partner or cell specific transcription cofactors belong to the intracellular regulators. Post- translational modifications, such as Acetylation, Poly-ADP-ribosylation, phosphorylation and Sumoylation, are other important intracellular Sox2 regulators of both DNA binding dependent and independent activities. Despite demontrated functions of Sox2 in different in vitro and in vivo models of Vertebrate physiology and pathology, the relevance of post translational modifications of this transcription factor is still poorly investigated.

Regulation of Sox2 localization influences its activity. Sox2 can enter into nucleus of ES cells and neuronal progenitors by three overlapping mechanisms, each dependent on HMG-box sequence interaction with a specific importin: Imp9, heterodimeric Imp7/Impβ and Exp4 (Gontan et al., 2009). Nuclear export is obtained by in vivo P300/CBP mediated Acetylation on Lysine 75 in mouse Sox2, located in NES of HMG-box and evolutionary conserved in different species. Blocking the post-translational modification results in Sox2 nuclear retention and enhanced transcription of target genes. However, forced acetylated Sox2 level triggers ubiquitination and proteasomal degradation of the transcription factor, thus abrogating its transcriptional ability (Baltus et al., 2009).

PARP1, a DNA binding protein with NAD^+ dependent enzymatic activity, works as a cofactor in ES cells on Sox2-Oct3/4 enhancer of FGF4 gene. The enzyme directly binds to Sox2 a poly(ADP)ribosyl residue, thus displacing Sox2 from cooperation with Oct3/4 on FGF4 enhancer and blocking differentiation of ES cells (Gao et al., 2009).

On the other hand, a Sox2 post-translational modification maintaining ES cell self –renewal capacity is Threonine 118 phosphorylation by Akt1: both knock-out and over-expression experiments affecting Akt1 levels, show positive co-regulation of Sox2, together with its transcriptional cooperator Oct3/4 (Jeong et al., 2010).

As described for other Sox family transcription factors, Sox2 is also targeted by sumoylation. A fraction of total mouse Sox2 is sumoylated on Lysine 247, which harbors in a consensus sumoylation motif VKSE, conserved through different species, among which homo, mouse and

rat. Performing in vivo assays in HeLa cells the high molecular weight band at 55 kDa of sumoylated Sox2 is progressively enhanced by cotransfection of Sox2 expressing plasmid together with SUMO1 alone or SUMO1 and Ubc9 expressing plasmids. Site specific mutagenesis of Lysine 247 into Arginine or Alanine results in expression of Sox2 only with its unmodified form (Tsuruzoe et al., 2006). The same research group showed endogenous Sox2 sumoylation both in human gastric cancer cell line (AGS), where unmodified Sox2 and sumoylated Sox2 at 50 and 70 kDa bands are detecated, and mouse embryonic stem cells. sumoylation of Sox2 has a neagative role on transcription of Sox2 target genes. FGF4 enhancer transcriptional activity is impaired by sumoylated Sox2 cooperation with Oc3/4 cooperative partner than Sox2 not modified forms. The molecular explanation relies on sumoylated Sox2 altered formation of the Sox2-Oct3/4-target DNA ternary complex, as shown in electrophoresis shift mobility assay (EMSA) on a FGF4 enhancer oligonucleotide probe, rather than not sumoylated Sox2 forms and independently by Oct3/4 sumoylation.

There is a similar negative effect of sumoylation on transcription of Nanog, another cooperative Sox2-Oct3/4 terget gene; this is the result of two opposite mechanisms involving Oct3/4 and Sox2 independently (Wu et al., 2012). Nanog proximal promoter activation is increased by Oct3/4 Sumoylated form compared with K118R unmodified one in both NIH-3T3 and F9 EC cells, as shown also in other models (Wei et al., 2007; Zhang et al., 2007); while Sumoylated Sox2 decreases Nanog transcription in comparison with K247R mutant in the same cell lines. Sumoylation of Sox2 is enhanced by E3 ligase Pias2 in this cellular contest. Moreover, coimmunoprecipitation of Sox2 and Oct3/4 in NIH-3T3 cells suggests that Sumoylation of both transcription factors decreases their interaction, according to previous finding of interference in heterodimer formation on their DNA binding sites. No significative differences in subcellular localization nor nuclear patterns of modified and unmodified Sox2 and Oct3/4 proteins are detected in the experiments. This fine tuned regulation of Nanog expression should be important in highlighting mechanisms underlying induction of pluripotence from differentiated somatic cells. The post translational modification by Sumoylation affects the generation of induced Pluripotent Stem Cells (iPSCs). Klf4, Oct3/4 and Sox2, three of the most important transcription factors driving iPSCs formation, are all modified by SUMO covalent binding, resulting in inhibition of iPSCs induction (Tahmasebi et al., 2013). Klf4, Oct3/4 and Sox2, each harboring a mutation on the main Sumoylation site, all together synergize in increasing transcription of Nanog in mouse embryonic fibroblasts (MEFs), thus triggering their reprogramming into iPSCs. The same result is obtained by Sox2 mutated on the lysine that is the major sumoylation site,

although with less iPSC formation than coexpressing into MEFs Klf4, Sox2 and Oct3/4 with their main sumoylation sites mutated into arginines.

Despite previously reported Sox2 sumoylation enhanced by three phosphorylated serines, located three aminoacidic residues after the main SUMO modified lysine (Van Hoof et al., 2009), the Sox2 dependent iPSC formation is not affected when these serines are mutated into alanines or into aspartates (Tahmasebi et al., 2013) and the transcriptional potential is unchanged even after a slightly reduction of Sox2 sumoylated amount. Although the serine cluster is required for optimal sumoylation of Sox2, both mutations of these serines into alanines or into aspartates don't affect the number of colonies during iPSC formation.

OSTEOBLAST CLONAL VARIABILITY AND SOX2: A PRELIMINARY EXPERIMENT.

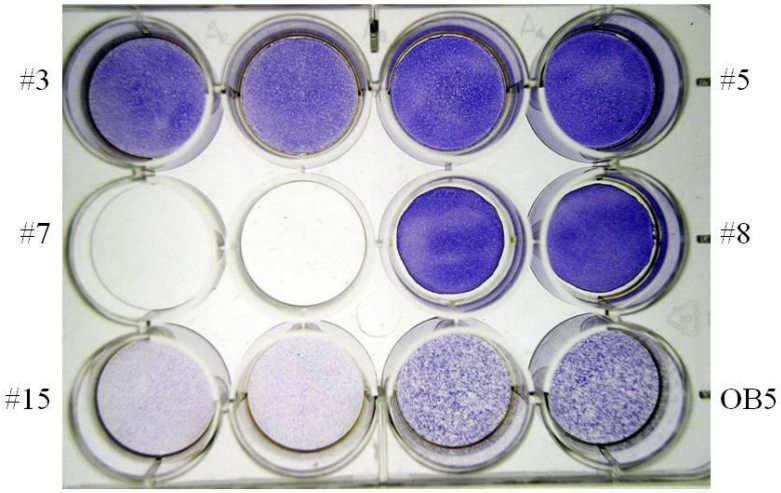
Sox2-Oct3/4 cooperation drives transcription of the Osteopontin gene (Botquin et al., 1998), a marker of osteoblast differentiation. This was the first experimental proof supporting a role for Sox2 in bone tissue. Mansukhani et al., (2000), studying the Craniosynostosis syndromes by microarray analysis, showed decreased differentiation capacity and a slight induction of apoptosis in osteoblast cells carrying mutations of the FGFR gene. These mutations result in the constitutive activation of the FGFR pathway in Craniosynostosis patients. Reproducing the pathological environment by FGF treatment of osteoblast cells, the block of differentiation was explained by down-regulation of the Wnt responsive genes and up-regulation of the Sox2 gene (Mansukhani et al., 2005). In addition, the authors selected, among the isolated calvarial primary osteoblasts (clones OB1-5), the clones constitutively expressing high level of Sox2. The microarray analysis of the expression pattern of these cells was in contrast with the previous observation and showed that the Wnt target genes were both up- and down-regulated. The different results may be due to conal variation.

Clonal variability is a problem in all cell cultures, as cells tend to modify the phenotype and homogeneity is obtained through continous clonal selection (Harris et al., 1987).

As we were interested in using a osteoblast primary line with a minimum of clonal variability, we examined the differentiation capacity of the OB5 clone and of the sublocones 3, 5, 7, 8, 15.

The results are shown in figure 11. Two assays were used to test cell differentiation: The ALP staining and the mineralization assay. Both assays show that each clone has a different staining intensity as compared to the original OB5 line, indicating a considerable clonal variability.

A.



B.

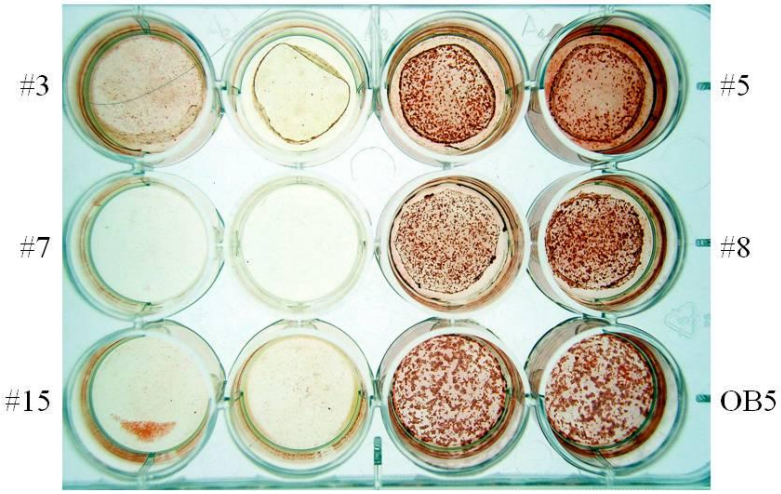


FIG. 11: Differentiation capacity of OB5 cells: comparison between OB5 clones

A. ALP staining to test the differentiation of the cells. Duplicate samples in the horizontal lane.

B. Mineralization test othe same OB5 clonesas in A . Duplicate samples in the horizontal lane.

RESULTS

SOX2 IN THE OSTEOGENIC LINEAGE.

Sox2 functions: Sumoylation modifies Sox2 structures.

The Sox2 protein is modified by sumoylation and this modification affects its activity by decreasing the binding of Sox2 to DNA. Mapping of the Sox2 sumoylation sites shows that only the lysine 247 is modified by SUMO1 (Tsuruzoe et al., 2006). Our preliminary results suggest that the PIAS protein enhances the Sumoylation of many SOX factors acting as a E3 SUMO ligase. Preliminary sumoylation assays conducted in the presence of PIAS1 confirmed that K247 is the main sumoylation site although the K247R Sox2 mutant can still be modified, suggesting the existence of at least one more site.

In order to search for new sumoylation sites in Sox2 we analyzed the Sox2 protein sequence (NP_035573.3) by using three different softwares i.e., SUMOplot (http://www.abgent.com/doc/Sumoplot/), SUMOsp 2.0 (http://Sumosp.biocuckoo.org; (Xue et al., 2006)), SUMOfinder (http://cbg.garvan.unsw.edu.au/Sumofi).

The results of the analysis are summarized in Table 1. Regardless of the program used for the analysis of the Sox2 sequence, the results show that at least three lysines can be covalently bound to SUMO1. Although only K247 is enclosed in the canonical consensus **ψKXE** motif, the K44, K89, and K123 sites obtained a very high score, thus indicating putative sumoylation sites.

Position	Sequence	Score SUMOplot	Score SUMOsp	Sumoylation site Type
K247	**V<u>K</u>SE**	**0,93**	**3,379**	**Type I: consensus ψKXE**
K44	**V<u>K</u>RP**	**0,82**	**2,309**	**Type II: non consensus**
K123	**M<u>K</u>KD**	**0,80**	**3,471**	**Type II: non consensus**
K89	**E<u>K</u>RP**	**0,39**	**1,559**	**Type II: non consensus**

TAB. 1: The 4 putative Sox2 sumoylation sites. From left to right the table lists putative sumoylable Lysines by position in mouse Sox2; their surrounding amino acids with the respective score in SUMOplot and SUMOsp softwares; and the type of consensus or not consensus sumoylation site.

In order to verify the role of these residues in the Sumoylation of Sox2 we generated Sox2 mutants by using the PCR technique. Lysine 44, 89, 123 or 247 were separately substituted by arginine. Each sequence was inserted in the pRK7-HA mammalian expression vector and assayed in a *in vivo* Sumoylation assay in 293T cells. In the assay, the expression plasmid of WT Sox2 and of each mutant was transfected alone or cotransfected with the vectors for the expression of SUMO1 and PIAS1. The protein extracts from transfected cells were analyzed by western blot using anti-HA mAbs.

The western blot of Fig. 12 shows that the cotransfection of the Sox2 and SUMO1 expression vectors resulted in a strong band migrating at a position corresponding to mono-Sumoylated Sox2 (lane 3). The addition of Pias1 induced a weaker second band migrating at a position corresponding to Sox2 modified in two residues (lane 7). Under all the experimental conditions used, the K247R mutant showed the presence of a unique weak band of Sumoylation suggesting that one residue, different from lysine 247, was modified (lanes 4, 6, 8).

The presence of a second Sumoylation site following the addition of Pias1 suggests that Pias1 promotes the Sumoylation of Sox2 by targeting additional residues and represents a novel observation. Thus, the analysis of Sox2 Sumoylation is complete only if conducted in the presence Pias1 in addition to Sumo1.

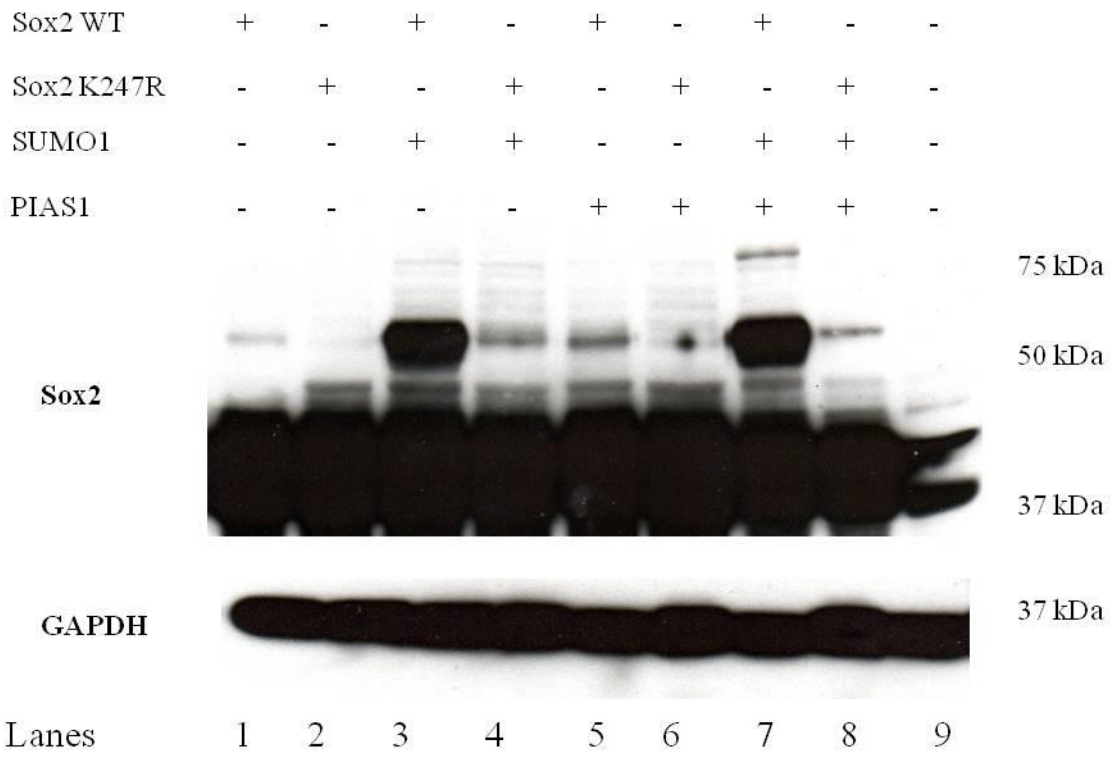


FIG. 12: *In vivo* Sox2 Sumoylation assay 293T cells.

The long exposure of the Western Blot allows to visualize the weaker bands. Lane 9 shows cells transfected with the empty plasmid only. The GAPDH protein represents the loading control.

For the identification of the other Sox2 Sumoylation site, we generated double mutants modified in K247 and in a additional putative Sumoylation site (K44R/K247R, K89R/K247R and K123R/K247R) (Figure 13A). We also constructed a mutant in which all 4 lysines were substituted, called Sox2K0. The mutants were cotransfected with SUMO1 and PIAS1 into 293T cells and assayed by western blot. Figure 13B shows that Sumoylation occourred in the K44R/K247R and in the K89R/K247R double mutants and was not detectable in the K123R/K247R mutant. Thus, the lysine 123 of Sox2 is the second residues modified by Sumoylation.

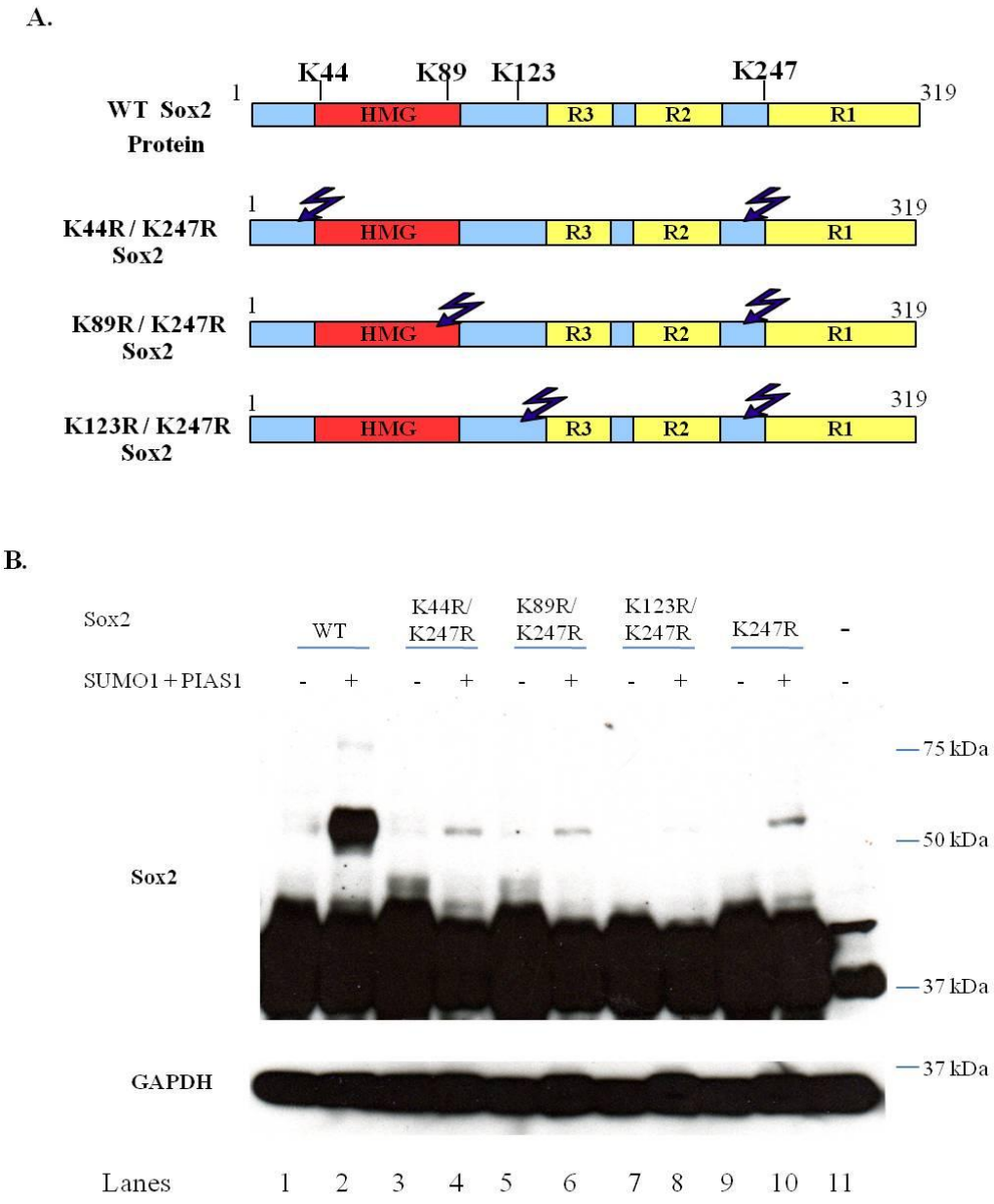


FIG. 13: Sumoylation assay of wild type and mutant Sox2 proteins..

A. Schematic representation of the wild type and mutant genes. The arrows indicate the lysine/arginine substitutions.

B. Sumoylation assay of the wild type and single/double lysine mutant Sox2 constructs as described in figure 13.

Role of sumoylation on the biological activity of Sox2.

In order to study the role of sumoylation on Sox2 biological function we compared the activity of WT and Sumoylation mutants of Sox2 on the transcription of the FGF4 gene and on the downregulation of the Wnt pathway.

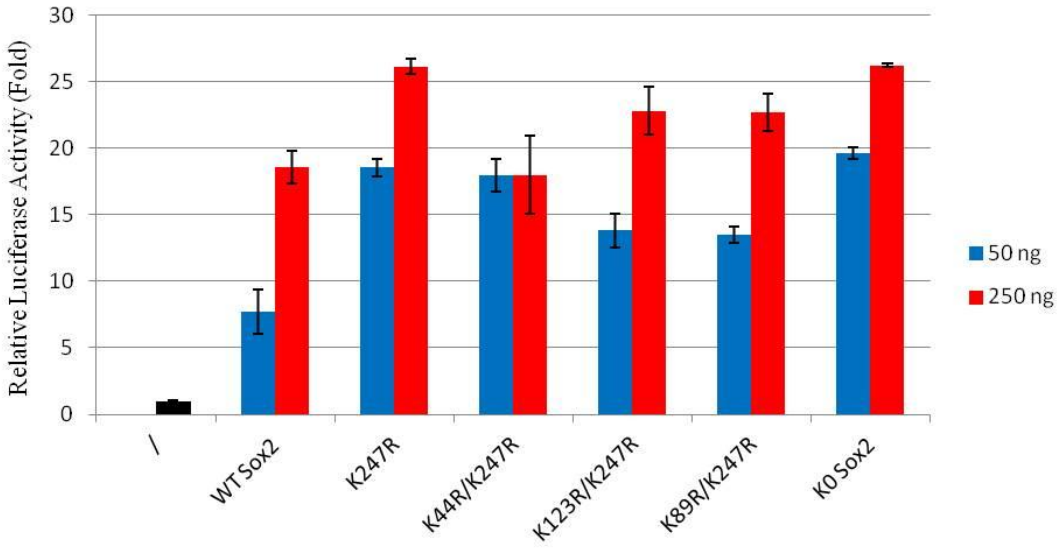
We used a reporter plasmid in which luciferase expression was driven by the minimal FGF-4 gene Promoter and 6 copies of the Sox consensus sequence (6x(O/S)-64-luc). Hela cells were transfected with the reporter plasmid alone or in combination with the vector for the expression of WTSox2 or for the expression of one of the Sox2 sumoylation mutants K247R, K44R/K247R, K89R/ K247R, K123R/ K247R and K0Sox2. The assay of Figure 14A shows that luciferase activity was induced when increasing amount of the WTSox2 expression plasmid was co-transfected with the reporter construct. A similar or slightly higher induction was observed for each of the sumoylation mutants (Fig. 14A). A possible explanation of these results is that the low level of Sox2 sumoylation observed in Figure 12, lane 1 is sufficient to inhibit, at least in part, the transcriptional activity. The slighthly higher transcriptional activity observed with the Sox2 mutants would be consistent with this hypothesis.

On this basis, we compared the activity of WTSox2 with that of the K247R mutant assayed both in the presence and in the absence of Sumo1 and Pias1 (Fig. 14B). The luciferase assay confirmed the observation that, in the absence of Sumo1 and Pias1, the K247R mutant induced luciferase expression consistently better than the WTSox2.

Surprisingly, the transcriptional activity of both WTSox2 and K247R mutant was significantly decreased by the co-expression of Sumo1 and Pias1. This could be explained by the presence in the K274R mutant of the weakly sumoylated lysine that we have observed in the experiment of figure12, lane 8. In fact, the sumoylation of this residue may be increased by the activity of SUMO and Pias1.

We may conclude that SUMO1 and PIAS1 promote sumoylation of WTSox2 and under these conditions, the transcriptional activity of Sox2 is inhibited.

A.



B.

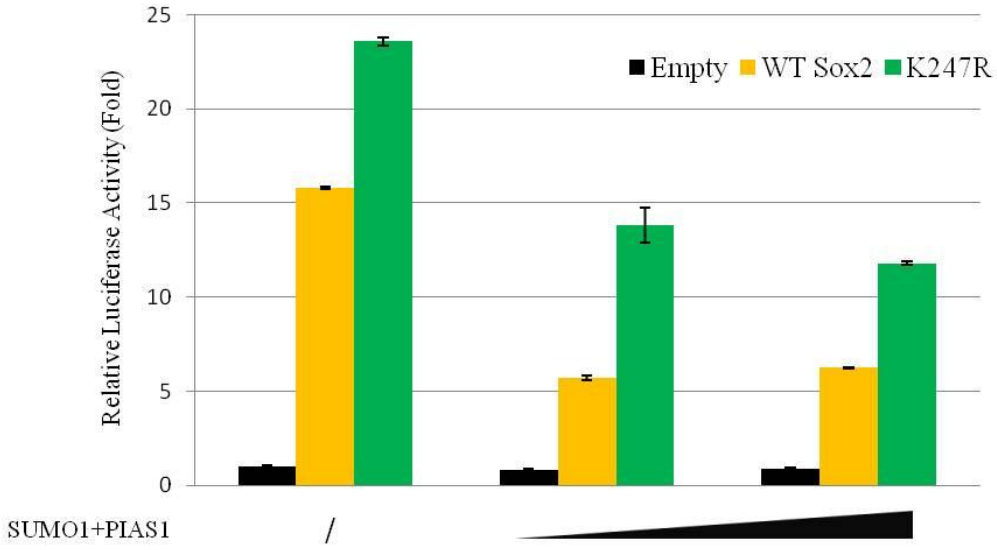


FIG. 14: Effect of Sumoylation on the transcriptional activity of wild type and mutant Sox2.

A. The transcriptional assay was carried out in HeLa cells.

B. 0, 20ng and 100 ng of each SUMO1 and Pias1 expression plasmids were transfected in HeLa cells together with the WT and K247R Sox2 mutant.

To analyze the effect of Sox2 on the Wnt pathway we carried out the following experiment. The binding of Sox2 to β-catenin inhibits its interaction with the LEF/TCF transcription factors, thus inhibiting the transcription of the Wnt target genes. We measured the binding of Sox2 to β-catenin by a luciferase assay carried out in 293T cells with a reporter plasmid containing the luciferase gene fused to multiple copies of the LEF/TCF binding site. To obtain the constitutive activation of the reporter gene we used a β-catenin deletion mutant that cannot be degraded by the proteasome.

Figure 15A shows that the expression of the β-catenin mutant efficiently triggers luciferase activity. As expected, co-transfection of β-catenin with an increasing amount of the WTSox2 expression vector inhibited luciferase gene expression. The presence of Sumo1 and Pias1 did not significantly modify the WTSox2 activity suggesting that Sumoylation does not affect this function. The histogram of figure 15B shows the effect of the expression of the WTSox2 as compared to the effect of the K247R Sox2 mutant. In agreement with the results of panel A Sumoylation did not affect the luciferase activity.

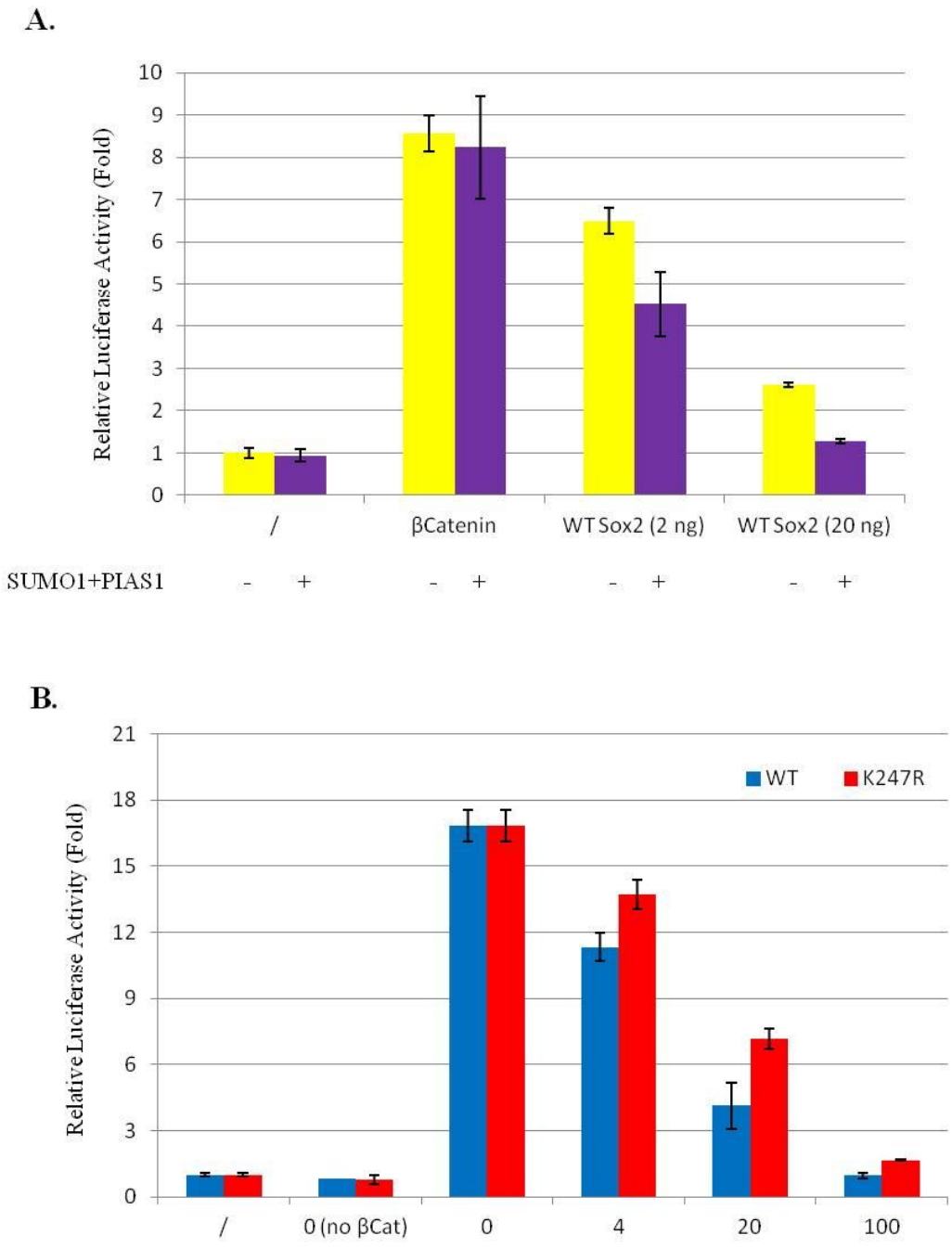


FIG. 15: Effect of Sox2 Sumoylation on WNT signaling on 293T cells.

A. Inhibition of β-catenin by WT Sox2 Sumoylation following 100 ng Sumo1 and 100 ng Pias1 addition.

B. Inhibition of β-catenin by WT and K247R Sox2.

SOX2 FUNCTION: IDENTIFICATION OF TARGET GENES IN THE OSTEOBLAST LINEAGE.

T-Rex Inducible System in OB1 cells.

The identification of target genes induced by the expression of a transcription factor can be difficult if the cell population used for the experiments is not homogeneous. This could be particularly true in osteoblasts, because of the high clonal variability among cell lines and even among subclones of a single cell line. Thus, we have set up a murine osteoblast cell line inducible for Sox2 expression. To this purpose we have chosen a T-Rex inducible cell line, in which Sox2 was constitutively repressed, in the absence, and induced, in the presence, of doxycycline (Dox) (Fig. 16).

OB1 cells were stably transfected with a vector expressing the Tet repressor gene (pcDNA6-TR). To test the activation of the system, each clone was transiently transfected with a luciferase reporter plasmid (pcDNA-TO-Luc) and treated for 24 h with doxycycline. We have selected clones #4, #6, #7, and #10 as those highly inducible and suitable for further experimentation.

To test the differentiation potential of the selected clones, we carried out Alkaline Phosphatase (ALP) staining of the cells. Clone # 6 showed the highest staining intensity with ALP (Fig. 17A). We also carried out a time course of induction for 24, 48 and 96 h. The luciferase expression of clone #4 was stable throughout the treatment, whereas clones #6, 7 and 10 showed a significant variability of the luciferase activity (Fig. 17B).

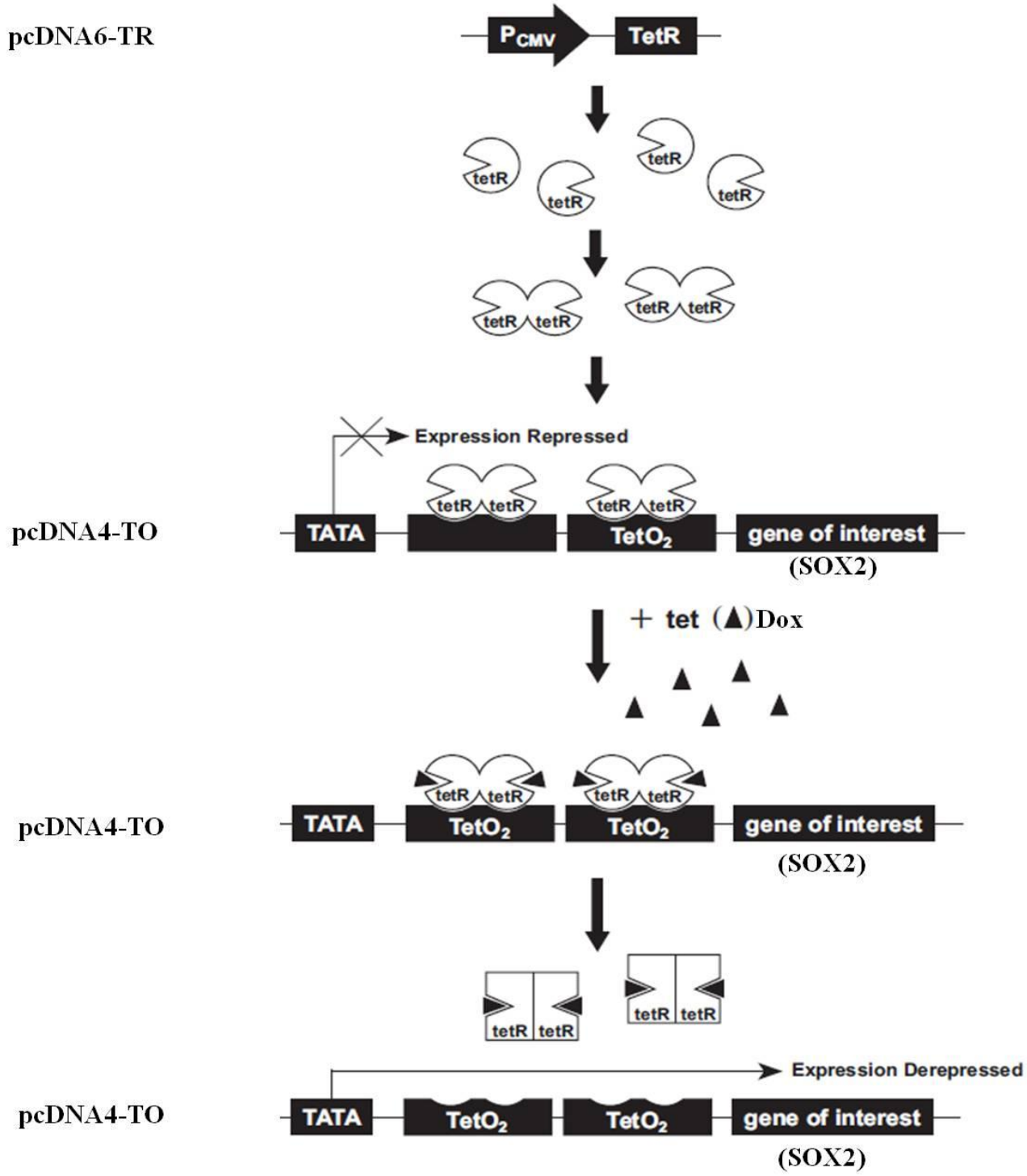
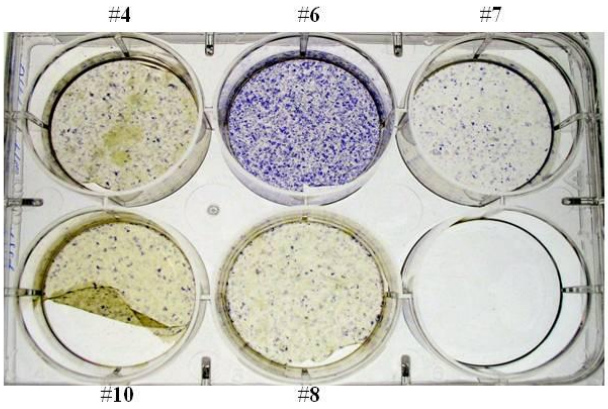


FIG. 16: Scheme of the T-Rex Inducible System to trigger Sox2 expression in the OB1 clone.

A.



B.

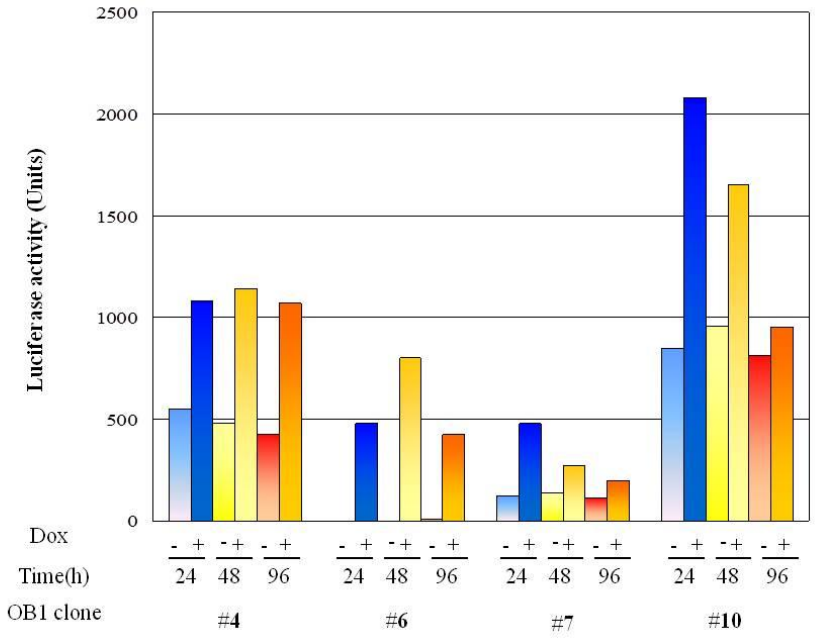


FIG. 17: Differentiation capacity and inducible transcriptional activity of the selected OB1 clones.

A. ALP assay of the selected clones.

B. Time course of doxycicline induction of luciferase activity for the selected clones.

As clone #4 (OB1#4) mantained throughout the time both the capacity to differentiate and to induce luciferase activity, we used it for further experimentation. OB1#4 cells were transfected with the CMV-TO-HA/Sox2 expression plasmid and a number of clones resistant to Zeocyne were selected. The cell extracts were analyzed by Western blot (Fig 18A). Only the protein extracts from the OB1#4#1 clone showed significant HA/Sox2 expression with anti-HA antibodies. The specificity of the signal was assayed using a protein extract from F9 cells which constitutively express Sox2. The anti-HA antibodies showed a band of the expected size only in the induced sample, the anti-Sox2 Abs showed bands in both induced and non-induced sample, with a stronger signal in the induced sample (Fig. 18B). The clone OB1#4#1 was used for further characterization in the following experiments. A doxycycline dose-response curve showed that 0.1 µg/ml of the drug was sufficient for full induction of HA/Sox2 in both 8 and 24 hour treatment (Fig. 18C). Sox2 expression was detectable from 6 (Fig. 18D) up to at least 96 hours (Fig 18E). Finally the removal of doxycycline after 24 hour treatment resulted in the disappearance of Sox2 within 6 h (Fig. 18F).

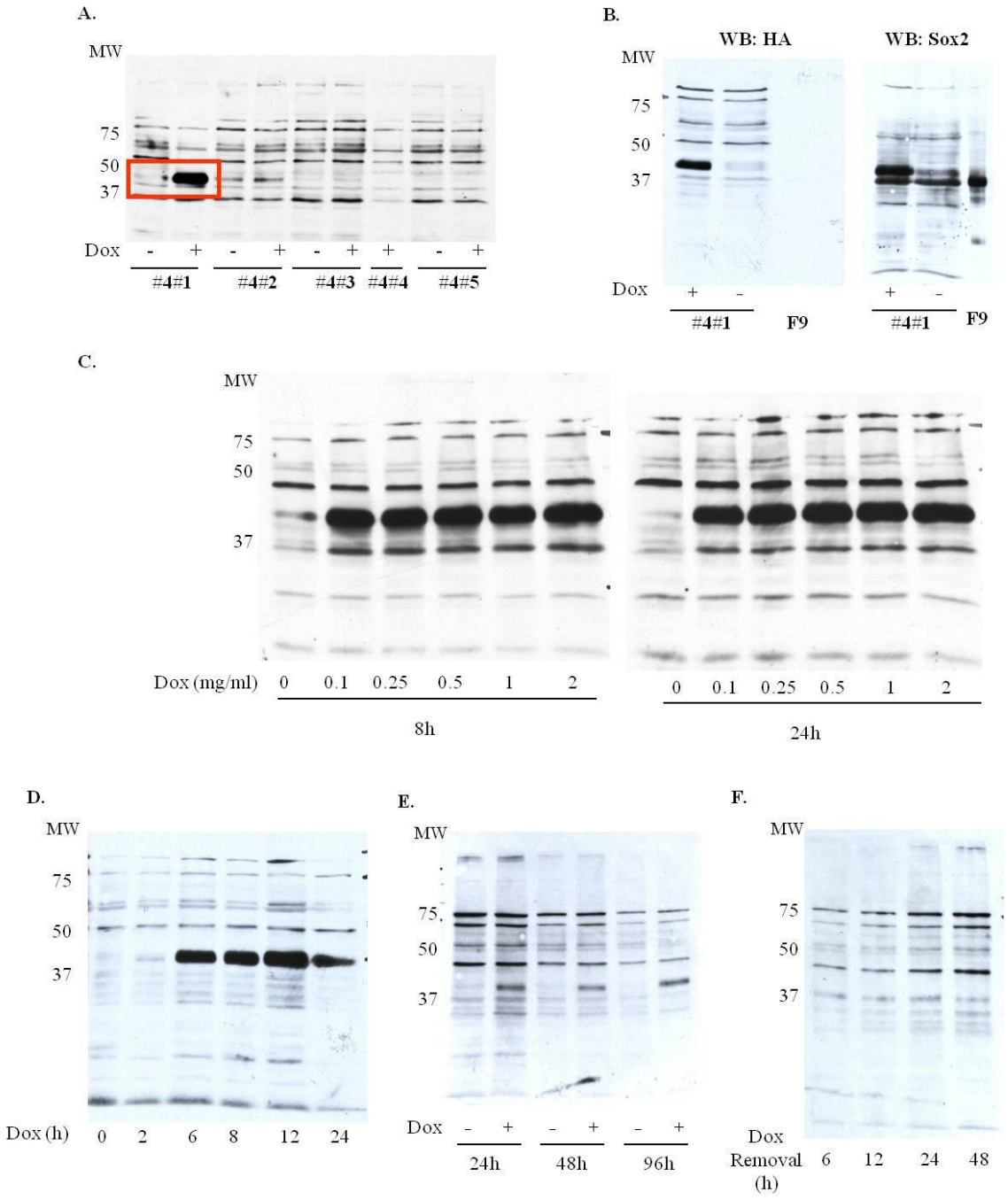


FIG. 18: Western blot analysis of the induction of HA-Sox2 in OB1#4 clones.

A. Selection of subclones inducible for SOX2. Clone #4#1 highlighted in red was used in the following experiments.

B. Western blot analysis of clone #4#1: staining with Anti-Ha and anti- Sox2 antibodies. A protein extract from the F9 cell line was used as control for the antibody specificity.

C. Western blot analysis of clone #4#1: doxycycline concentration curve for the induction of Sox2.

D. Western blot analysis of clone #4#1: doxycycline time course of induction of Sox2 from 2 to 24 h.

E. Western blot analysis of clone #4#1: doxycycline time course of induction of Sox2 from 24to 96 h.

F. Western blot analysis of clone #4#1: time course of decay of Sox2 induction following doxycycline removal, after 24h treatment.

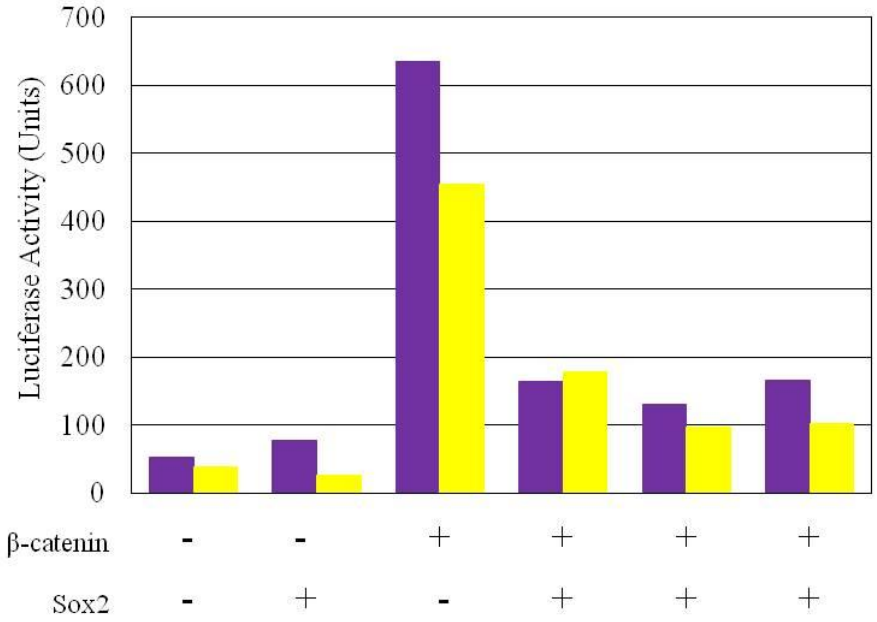
As we checked the biological activity of Sox2 using a HA-tagged protein, we decided to compare the effect of HA/Sox2 with that of Sox2 without tag in two different biological assays:

1) the inhibition of B-catenin activity by Sox2 in 293T cells;

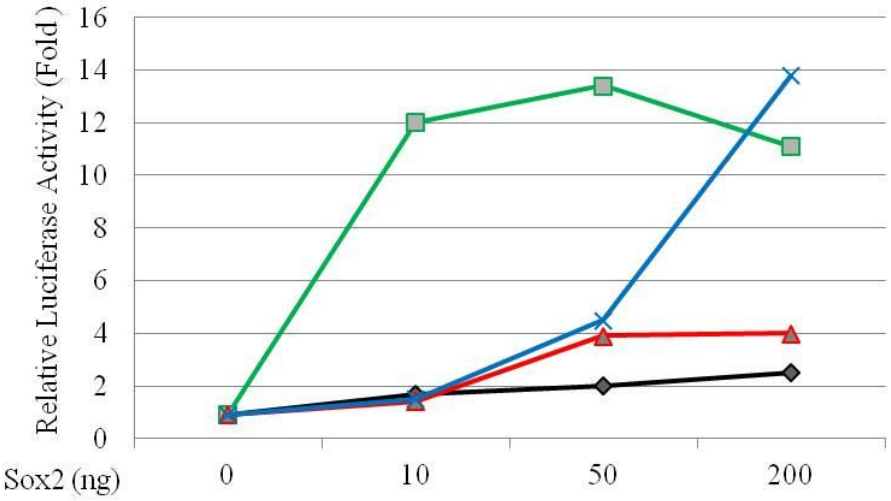
2) the activation of the FGF4 enhancer by Sox2 and Oct-3/4 in HeLa cells.

β-catenin activity was equally downregulated by both Sox2 and HA/Sox2 (Fig. 19A). In contrast, the FGF-4 enhancer was better activated by Sox2 as compared with HA/Sox2 (Fig. 19B). This difference is accounted for by the higher expression of Sox2 as compared with HA/Sox2 shown by the Western blot in Fig. 19C. We can conclude that the N-terminus HA tag did not grossly alter the transcriptional activity of Sox2.

A.


Luciferase Activity (Units)
700
600
500
400
300
200
100
0
β-catenin - - + + + +
Sox2 - + - + + +

B.


Relative Luciferase Activity (Fold)
16
14
12
10
8
6
4
2
0
Sox2 (ng)
0
10
50
200

C.

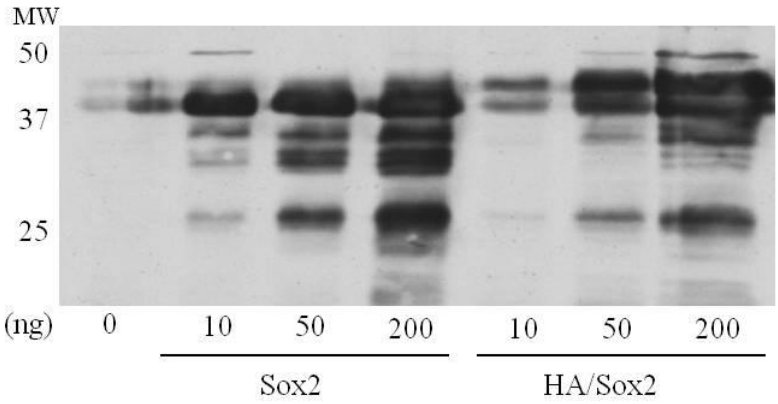

MW
50
37
25
(ng)
0
10
50
200
10
50
200
Sox2
HA/Sox2

FIG. 19: Comparison of the biological activity of HA/Sox2 with that of Sox2 without tag.

A. Inhibition of β-catenin activity by Sox2 in 293T cells. The yellow bars represent HA/SOX2 and the violet bars represent Sox2. Increasing amount (20- 100- 500 ng) of HA/Sox2 and Sox2 were added to the assay which contained a fixed amount (50 ng) of β-catenin.

B. Activation of the FGF4 enhancer by Sox2 and Oct-3/4 in HeLa cells. The red line refers to the addition of increasing amount of the Sox2 expression plasmid. The black line refers to the addition of increasing amount of the HA/Sox2 expression plasmid. The green line refers to the addition of increasing amount of the Sox2 expression plasmid and a fixed amount of Oct3/4. The blue line refers to the addition of increasing amount of the HA/Sox2 expression plasmid and a fixed amount of Oct3/4.

C. Western blot analysis of a protein extract from HeLa cells transfected with increasing amount of Sox2 and HA/Sox2, together with a fixed amount (10 ng) of Oct3/4.

Sox2 function: gene expression induced by Sox2 in osteoblasts.

To identify Sox2 target genes in osteoblasts we carried out a cDNA microarray experiment in OB1#4/#1 cells. Cells were treated with doxycycline for 0, 6, 12, 24 and 48 h and the mRNA expression pattern was analyzed by DNA microarray according to the Affymetrix protocol. Raw data were filtered to remove experimental points and, when necessary, whole chips affected by intrinsic high variability. The analysis resulted in a list of 158 genes differentially expressed following doxycycline induction of Sox2; 120 genes were up-regulated and 38 genes were down-regulated. As previously shown in other cell models, Sox2 is a transcriptional activator, rather than a repressor . Among the genes differentially expressed, it seemed interesting to separately classify the genes related to different functions using the bioinformatic softwares DAVID (Huang da et al., 2007) and GSEA (Subramanian et al., 2005). The results show that:

- approximately 47% of the classified genes are involved in bone biology (Table 2A);
- approximately 50% of the classified genes are involved in apoptosis (Table 2B);
- approximately 62% of the classified genes are involved in the regulation of differentiation (Table 2C);
- approximately 61% of the genes are involved in cell growth (Table 2D);
- approximately 31% of the genes are involved in cytoskeletal function (Table 2E);
- approximately 41% of the genes are involved in the Wnt, BMP or Hippo cell signaling pathway (Table 2F).

The percentages shown above clearly suggest that several genes have multiple and overlapping functions. The observation that Sox2 regulates directly many genes involved in osteogenesis strongly supports the idea of an important role in the differentiation of osteoblasts cells. Furthermore, the gene analysis suggests a more general and central role of the Sox2 transcription factor in a number of biological processes involved in cell differentiation. These data correlate with the described key functions of Sox2 in stem cells maintenance and with the described role on the Wnt pathway. The regulation by Sox2 of the genes involved in the BMP and Hippo pathways are investigated in the following experiments (Fig. 23-26).

Real-time RT-PCR was used to verify the regulation by Sox2 of a number of differentially and highly expressed genes i.e. Sox2, Gng2, Il-6, Fam110c, Cd200 and Mtm1 (Fig. 20). These genes were selected because they seem to have a role in bone tissue biology .

Table 2A. Genes involved in BONE BIOLOGY

GENE SYMBOL	GENE NAME	RNA (FOLD CHANGE)			
		Doxycycline (h)			
		6	12	24	48
Acvr2a	Activin receptor type 2A Precursor	1.05	0.96	1.01	**1.51**
Acvr2b	Activin receptor type 2B Precursor	**1.71**	**1.68**	1.15	1.13
Bmp2k	BMP 2 inducible protein kinase	1.06	1.34	**1.61**	1.06
Cd200	OX 2 membrane glycoprotein Precursor CD200 antigen	**1.94**	**1.96**	**2.09**	**1.81**
Cd74	MHC class II associated invariant chain CD74 antigen	0.96	1.28	**1.89**	**2.41**
Cyp26b1	cytochrome P450, family 26, subfamily b, polypeptide 1	1.43	**1.65**	**1.51**	1.08
Fetub	Fetuin B Precursor	1.21	**1.94**	**1.63**	1.24
Gfra4	GDNF family receptor alpha 4 Precursor	1.15	1.15	1.00	**1.64**
Ifi204	Interferon activable protein 204	1.12	1.27	**1.56**	1.09
Il6	Interleukin 6 Precursor	1.13	**1.83**	**2.18**	1.60
Inhba	Inhibin beta A chain Precursor	1.32	1.48	**1.63**	1.49
Jag1	Protein jagged 1 Precursor	**2.11**	**2.66**	**1.76**	**1.50**
Klra6	Killer cell lectin like receptor 6	**1.83**	1.40	0.98	0.69
Mmp16	Matrix metalloproteinase 16 Precursor MMP3	1.10	1.32	1.29	**1.59**
Mras	Ras related protein M Ras Precursor	**1.63**	1.03	1.43	**2.46**
Nt5e	5 nucleotidase Precursor CD73 antigen	0.79	0.99	1.03	**1.50**
Plekhg2	Pleckstrin homology domain containing family G member 2	1.16	0.91	1.14	**1.93**
Slc39a1	Solute carrier family 39 member 1	**2.31**	**3.23**	**1.72**	**2.96**
Smoc1	SPARC related modular calcium binding protein 1 Precursor	1.16	1.16	**1.72**	1.38
Stc2	Stanniocalcin 2 Precursor	0.96	1.15	**1.63**	1.45
Tspan7	Tetraspanin 7	1.23	1.41	**1.52**	1.32
Tspan13	Tetraspanin 13	1.33	**2.92**	**1.87**	**1.64**
Vcam1	Vascular cell adhesion protein 1 Precursor	**1.50**	1.39	1.17	1.15
Vcan	Versican core protein Precursor Large fibroblast proteoglycan	1.21	1.45	**1.69**	1.37
Cml1	Probable N acetyltransferase Camello like protein 1	0.84	0.90	**0.63**	0.69
Elk1	ETS domain containing protein Elk 1	**0.59**	0.70	0.82	1.29
Fastk	Fas activated serinethreonine kinase	1.18	1.36	**0.60**	1.07
Irs2	Insulin receptor substrate 2	0.82	1.02	1.07	**0.58**
Ngef	Ephexin 1 Neuronal guanine nucleotide exchange factor	0.96	**0.66**	**0.60**	0.91
Sdc4	Syndecan 4 Precursor	**0.63**	0.69	0.97	0.96
Srf	Serum response factor	**0.66**	0.72	1.00	1.05
Tnfrsf12a	Tumor necrosis factor receptor superfamily member 12A	**0.66**	**0.60**	0.81	0.96

Tabella 2B. Genes involved in Apoptosis.

GENE SYMBOL	GENE NAME	RNA (FOLD CHANGE)			
		Doxycycline (h)			
		6	12	24	48
Acvr2a	Activin receptor type 2A Precursor	1.05	0.96	1.01	**1.51**
Acta1	Actin, alpha skeletal muscle	0.83	0.80	**1.54**	1.20
Adap1	centaurin, alpha 1	**1.63**	1.44	0.90	0.97
Aqp8	Aquaporin 8	0.70	0.86	0.86	**1.73**
Cd1d1	T cell surface glycoprotein CD1d1 Precursor	1.14	1.32	**1.72**	**1.65**
Ela2	Leukocyte elastase Precursor Elastase 2	**1.56**	1.16	1.10	1.07
Epha7	Ephrin type A receptor 7 Precursor	**3.64**	**3.83**	**2.00**	1.46
Ifit1	Interferon induced protein with tetratricopeptide repeats 1	0.92	1.60	1.28	1.54
Ltf	Lactotransferrin Precursor	**1.71**	**1.52**	0.85	1.42
Mras	Ras related protein M Ras Precursor	**1.63**	1.03	1.43	**2.46**
Mtx1	Metaxin 1	1.07	0.95	0.91	**1.51**
Oasl2	54 kDa 2 5 oligoadenylate synthetase like protein 2	1.08	1.23	1.05	**1.56**
Slc39a1	Solute carrier family 39 member 1	**2.31**	**3.23**	**1.72**	**2.96**
Srpx	Sushi repeat containing protein SRPX Precursor	1.03	0.93	1.22	**1.52**
Zswim2	Zinc finger SWIM domain containing protein 2	1.08	1.09	0.91	**1.54**
Cd59a	CD59A glycoprotein Precursor	1.23	**2.04**	**1.76**	1.28
Col6a2	Collagen alpha 2[VI] chain Precursor	0.85	1.02	1.45	**1.65**
Cyp26b1	cytochrome P450, family 26, subfamily b, polypeptide 1	1.43	**1.65**	**1.51**	1.08
Dpysl3	Dihydropyrimidinase related protein 3	1.15	1.39	**1.54**	1.21
Dusp6	Dual specificity protein phosphatase 6 MKP 3	**1.53**	**1.57**	1.27	1.11
Nt5e	5 nucleotidase Precursor CD73 antigen	0.79	0.99	1.03	**1.50**
Slc18a2	VAT2 Solute carrier family 18 member 2	**1.58**	**1.54**	1.21	1.02
Smoc1	SPARC related modular calcium binding protein 1 Precursor	1.16	1.16	**1.72**	1.38
Stc2	Stanniocalcin 2 Precursor	0.96	1.15	**1.63**	1.45
Steap4	Six transmembrane epithelial antigen of prostate 4	1.10	**2.80**	**1.72**	1.16
Usp18	Ubl carboxyl terminal hydrolase 18	1.25	**1.53**	1.28	**1.97**
Vcam1	Vascular cell adhesion protein 1 Precursor	**1.50**	1.39	1.17	1.15
Anp32a	Acidic leucine rich nuclear phosphoprotein 32 family member A pp32	**0.61**	0.86	0.97	0.97
Fastk	Fas activated serinethreonine kinase	1.18	1.36	**0.60**	1.07
Tnfrsf12a	Tumor necrosis factor receptor superfamily member 12A	**0.66**	**0.60**	0.81	0.96
Elk1	ETS domain containing protein Elk 1	**0.59**	0.70	0.82	1.29
Irs2	Insulin receptor substrate 2	0.82	1.02	1.07	**0.58**
Msln	Mesothelin Precursor Pre pro megakaryocyte potentiating factor	0.88	0.95	0.68	**0.64**

Table 2C. Genes involved in Cell Differentiation.

GENE SYMBOL	GENE DESCRIPTION	RNA (FOLD CHANGE)			
		Doxycycline (h)			
		6	12	24	48
Acvr2a	Activin receptor type 2A Precursor	1.05	0.96	1.01	**1.51**
Acvr2b	Activin receptor type 2B Precursor	**1.71**	**1.68**	1.15	1.13
Arl4a	ADP ribosylation factor like protein 4A	1.02	1.41	**1.55**	1.19
Eda	Ectodysplasin A	1.03	1.42	**1.93**	1.40
Epha7	Ephrin type A receptor 7 Precursor	**3.64**	**3.83**	**2.00**	1.46
Evx2	Homeobox even skipped homolog protein 2 EVX 2	**1.51**	1.25	0.88	0.81
Fmnl1	Formin like protein 1	0.84	0.62	1.14	**1.78**
Gng2-ps1	Guanine Nucleotide Binding Protein Gamma 2 -pseudogene 1	**6.10**	**30.11**	**13.98**	**7.16**
Gspt2	Eukaryotic peptide chain release factor GTP binding subunit ERF3B	**1.59**	1.38	1.21	0.98
Insl6	Insulin like peptide INSL6 Precursor	1.48	**1.79**	**1.62**	**1.65**
Mkx	Homeobox protein Mohawk	**1.72**	**1.55**	1.17	1.06
Mras	Ras related protein M Ras Precursor	**1.63**	1.03	1.43	**2.46**
Mtm1	Myotubularin	**1.71**	**2.05**	1.39	1.01
Parm1	Prostatic androgen receptor mediator 1	**1.57**	**3.31**	**4.22**	1.41
Prima1	Proline rich membrane anchor 1 Precursor	0.91	0.82	1.00	**4.18**
Smoc1	SPARC related modular calcium binding protein 1 Precursor	1.16	1.16	**1.72**	1.38
Acta1	Actin, alpha skeletal muscle	0.83	0.80	**1.54**	1.20
Aldh1a7	cytosolic 1 Aldehyde dehydrogenase family 1 member A7	1.44	**1.56**	**2.33**	1.33
Cbfa2t3	Protein CBFA2T3	**1.84**	1.12	0.70	1.09
Cd200	OX 2 membrane glycoprotein Precursor CD200 antigen	**1.94**	**1.96**	**2.09**	**1.81**
Dusp6	Dual specificity protein phosphatase 6 MKP 3	**1.53**	**1.57**	1.27	1.11
Ela2	Leukocyte elastase Precursor Elastase 2	**1.56**	1.16	1.10	1.07
Ifi204	Interferon activable protein 204	1.12	1.27	**1.56**	1.09
Mbnl3	Muscleblind like protein 3	1.14	**1.59**	**2.18**	1.35
Rnf128	E3 ubiquitin protein ligase RNF128 Precursor	1.34	**1.98**	**2.13**	1.40
Slc18a2	VAT2 Solute carrier family 18 member 2	**1.58**	**1.54**	1.21	1.02
Slc39a1	Solute carrier family 39 member 1	**2.31**	**3.23**	**1.72**	**2.96**
Steap4	Six transmembrane epithelial antigen of prostate 4	1.10	**2.80**	**1.72**	1.16
Usp18	Ubl carboxyl terminal hydrolase 18	1.25	**1.53**	1.28	**1.97**
Vcam1	Vascular cell adhesion protein 1 Precursor	**1.50**	1.39	1.17	1.15
Aldh3a1	Dimeric NADP preferring Aldehyde dehydrogenase family 3 member A1	0.98	0.92	**0.61**	**0.64**
Anp32a	Acidic leucine rich nuclear phosphoprotein 32 family member A pp32	**0.61**	0.86	0.97	0.97
Atoh8	Protein atonal homolog 8	**0.58**	0.83	1.31	1.14
Gsta3	Glutathione S transferase A3	0.85	0.73	0.83	**0.56**
Klf1	Krueppel like factor 1	**0.53**	0.75	**0.67**	1.03
Slc7a5	Solute carrier family 7 member 5	**0.64**	0.71	0.69	1.32
Tslp	Thymic stromal lymphopoietin Precursor	**0.64**	0.70	0.92	1.01
Fastk	Syndecan 4 Precursor	**0.63**	0.69	0.97	0.96
Dhrs3	Retinal Short chain dehydrogenasereductase 3	1.21	1.09	**0.61**	0.83
Hbegf	Proheparin binding EGF like growth factor Precursor	**0.66**	**0.67**	0.74	0.99
Il1rl1	Interleukin 1 receptor like 1 Precursor	0.78	0.72	**0.65**	0.78

Table 2D. Genes involved in Cell Growth.

GENE SYMBOL	GENE NAME	RNA (FOLD CHANGE)			
		Doxycycline (h)			
		6	12	24	48
Acvr2b	Activin receptor type 2B Precursor	**1.71**	**1.68**	1.15	1.13
Cd200	OX 2 membrane glycoprotein Precursor CD200 antigen	**1.94**	**1.96**	**2.09**	**1.81**
Cd320	CD320 antigen Precursor Transcobalamin receptor	0.85	0.93	0.97	**1.58**
Cd59a	CD59A glycoprotein Precursor	1.23	**2.04**	**1.76**	1.28
Dusp6	Dual specificity protein phosphatase 6 MKP 3	**1.53**	**1.57**	1.27	1.11
Epha7	Ephrin type A receptor 7 Precursor	**3.64**	**3.83**	**2.00**	1.46
Fanci	Fanconi anemia group I protein homolog	1.01	1.00	**1.60**	0.81
Hltf	Helicase like transcription factor	1.40	**1.66**	**1.56**	1.13
Ifi204	Interferon activable protein 204	1.12	1.27	**1.56**	1.09
Ltf	Lactotransferrin Precursor	**1.71**	**1.52**	0.85	1.42
Mras	Ras related protein M Ras Precursor	**1.63**	1.03	1.43	**2.46**
Parm1	Prostatic androgen receptor mediator 1	**1.57**	**3.31**	**4.22**	1.41
Slc39a1	Solute carrier family 39 member 1	**2.31**	**3.23**	**1.72**	**2.96**
Smoc1	SPARC related modular calcium binding protein 1 Precursor	1.16	1.16	**1.72**	1.38
Vcam1	Vascular cell adhesion protein 1 Precursor	**1.50**	1.39	1.17	1.15
Vcan	Versican core protein Precursor Large fibroblast proteoglycan	1.21	1.45	**1.69**	1.37
Acvr2a	Activin receptor type 2A Precursor	1.05	0.96	1.01	**1.51**
Bmp2k	BMP 2 inducible protein kinase	1.06	1.34	**1.61**	1.06
Brwd3	Bromodomain and WD repeat containing protein 3	**1.56**	1.44	**1.70**	1.24
Cd74	MHC class II associated invariant chain CD74 antigen	0.96	1.28	**1.89**	**2.41**
Cdc14a	Dual specificity protein phosphatase CDC14A	**1.51**	1.25	1.26	0.91
Cyp26b1	cytochrome P450, family 26, subfamily b, polypeptide 1	1.43	**1.65**	**1.51**	1.08
Fetub	Fetuin B Precursor	1.21	**1.94**	**1.63**	1.24
Inhba	Inhibin beta A chain Precursor	1.32	1.48	**1.63**	1.49
Klra6	Killer cell lectin like receptor 6	**1.83**	1.40	0.98	0.69
Nt5e	5 nucleotidase Precursor CD73 antigen	0.79	0.99	1.03	**1.50**
Stc2	Stanniocalcin 2 Precursor	0.96	1.15	**1.63**	1.45
Steap4	Six transmembrane epithelial antigen of prostate 4	1.10	**2.80**	**1.72**	1.16
Elk1	ETS domain containing protein Elk 1	**0.59**	0.70	0.82	1.29
Hbegf	Proheparin binding EGF like growth factor Precursor	**0.66**	**0.67**	0.74	0.99
Il18rap	Interleukin 18 receptor accessory protein Precursor	**0.65**	0.79	**0.64**	1.09
Irs2	Insulin receptor substrate 2	0.82	1.02	1.07	**0.58**
Msln	Mesothelin Precursor Pre pro megakaryocyte potentiating factor	0.88	0.95	0.68	**0.64**
Sdc4	Syndecan 4 Precursor	**0.63**	0.69	0.97	0.96
Tnfrsf12a	Tumor necrosis factor receptor superfamily member 12A	**0.66**	**0.60**	0.81	0.96
Tslp	Thymic stromal lymphopoietin Precursor	**0.64**	0.70	0.92	1.01
Aldh3a1	Dimeric NADP preferring Aldehyde dehydrogenase family 3 member A1	0.98	0.92	**0.61**	**0.64**
Klf1	Krueppel like factor 1	**0.53**	0.75	**0.67**	1.03
Srf	Serum response factor	**0.66**	0.72	1.00	1.05

Table 2E. Genes involved in Cytoskeletal Functions.

GENE SYMBOL	GENE NAME	RNA (FOLD CHANGE)			
		Doxycycline (h)			
		6	12	24	48
Arl4a	ADP ribosylation factor like protein 4A	1.02	1.41	**1.55**	1.19
Dtna	Dystrobrevin alpha	1.30	1.34	**1.58**	1.28
Fam110c	Protein FAM110C	**1.76**	**1.87**	**2.37**	**1.77**
Fgd3	FYVE, RhoGEF and PH domain containing protein 3	0.79	0.75	0.90	**1.70**
Fmnl1	Formin like protein 1	0.84	0.62	1.14	**1.78**
Mtm1	Myotubularin	**1.71**	**2.05**	1.39	1.01
Myo7b	Myosin VIIb	**3.61**	0.71	**8.68**	0.95
Prima1	Proline rich membrane anchor 1 Precursor	0.91	0.82	1.00	**4.18**
Smoc1	SPARC related modular calcium binding protein 1 Precursor	1.16	1.16	**1.72**	1.38
Vcam1	Vascular cell adhesion protein 1 Precursor	**1.50**	1.39	1.17	1.15
Cml1	Probable N acetyltransferase Camello like protein 1	0.84	0.90	**0.63**	0.69
Fastk	Fas activated serinethreonine kinase	1.18	1.36	**0.60**	1.07
Ngef	Ephexin 1 Neuronal guanine nucleotide exchange factor	0.96	**0.66**	**0.60**	0.91
Sdc4	Syndecan 4 Precursor	**0.63**	0.69	0.97	0.96
Srf	Serum response factor	**0.66**	0.72	1.00	1.05
Cd74	MHC class II associated invariant chain CD74 antigen	0.96	1.28	**1.89**	**2.41**
Icam5	Intercellular adhesion molecule 5 Precursor	0.92	0.82	1.00	**1.70**
Mtx1	Metaxin 1	1.07	0.95	0.91	**1.51**
Srpx	Sushi repeat containing protein SRPX Precursor	1.03	0.93	1.22	**1.52**
St8sia4	CMP N acetylneuraminate poly alpha 2,8 sialyltransferase	**1.64**	1.49	1.32	1.10

Table 2F. Genes involved in the WNT or BMP or HIPPO Pathways.

GENE SYMBOL	GENE NAME	RNA (FOLD CHANGE)			
		Doxycycline (h)			
		6	12	24	48
WNT pathway					
Arl4a	ADP ribosylation factor like protein 4A	1.02	1.41	**1.55**	1.19
Armcx1	Armadillo repeat containing X linked protein 1	**1.52**	**1.84**	1.15	0.98
Eda	Ectodysplasin A	1.03	1.42	**1.93**	1.40
Ela2	Leukocyte elastase Precursor Elastase 2	**1.56**	1.16	1.10	1.07
Gng2-ps1	Guanine Nucleotide Binding Protein Gamma 2 -pseudogene 1	**6.10**	**30.11**	**13.98**	**7.16**
Mapk1ip1	MAPK interacting and spindle stabilizing protein 1	0.85	0.73	1.06	**1.58**
Anp32a	Acidic leucine rich nuclear phosphoprotein 32 family member A pp32	**0.61**	0.86	0.97	0.97
Hbegf	Proheparin binding EGF like growth factor Precursor	**0.66**	**0.67**	0.74	0.99
BMP pathway					
Acvr2a	Activin receptor type 2A Precursor	1.05	0.96	1.01	**1.51**
Acvr2b	Activin receptor type 2B Precursor	**1.71**	**1.68**	1.15	1.13
Bmp2k	BMP 2 inducible protein kinase	1.06	1.34	**1.61**	1.06
Icam5	Intercellular adhesion molecule 5 Precursor	0.92	0.82	1.00	**1.70**
Ifi204	Interferon activable protein 204	1.12	1.27	**1.56**	1.09
Inhba	Inhibin beta A chain Precursor	1.32	1.48	**1.63**	1.49
Mmp16	Matrix metalloproteinase 16 Precursor MMP3	1.10	1.32	1.29	**1.59**
Parm1	Protein PARM 1 Precursor	**1.57**	**3.31**	**4.22**	1.41
Smoc1	SPARC related modular calcium binding protein 1 Precursor	1.16	1.16	**1.72**	1.38
Vcan	Versican core protein Precursor Large fibroblast proteoglycan	1.21	1.45	**1.69**	1.37
Atoh8	Protein atonal homolog 8	**0.58**	0.83	1.31	1.14
Cml1	Probable N acetyltransferase Camello like protein 1	0.84	0.90	**0.63**	0.69
Elk1	ETS domain containing protein Elk 1	**0.59**	0.70	0.82	1.29
Sdc4	Syndecan 4 Precursor	**0.63**	0.69	0.97	0.96
Tnfrsf12a	Tumor necrosis factor receptor superfamily member 12A	**0.66**	**0.60**	0.81	0.96
Hippo pathway					
Cdc14a	Dual specificity protein phosphatase CDC14A	**1.51**	1.25	1.26	0.91
Inhba	Inhibin beta A chain Precursor	1.32	1.48	**1.63**	1.49
Vcan	Versican core protein Precursor Large fibroblast proteoglycan	1.21	1.45	**1.69**	1.37
Slc7a5	Solute carrier family 7 member 5	**0.64**	0.71	0.69	1.32

Tab. 2 A-F: Functional annotations of osteoblast genes up- and down-regulated by Sox2 induction in the OB1#4#1 clone.

The genes were classified according to the putative function. In black are the figure referring to RNAs whose concentration is unchanged. In red are the RNAs downregulated less than 0.67 fold. In green are the RNAS upregulated more than 1.5 fold.

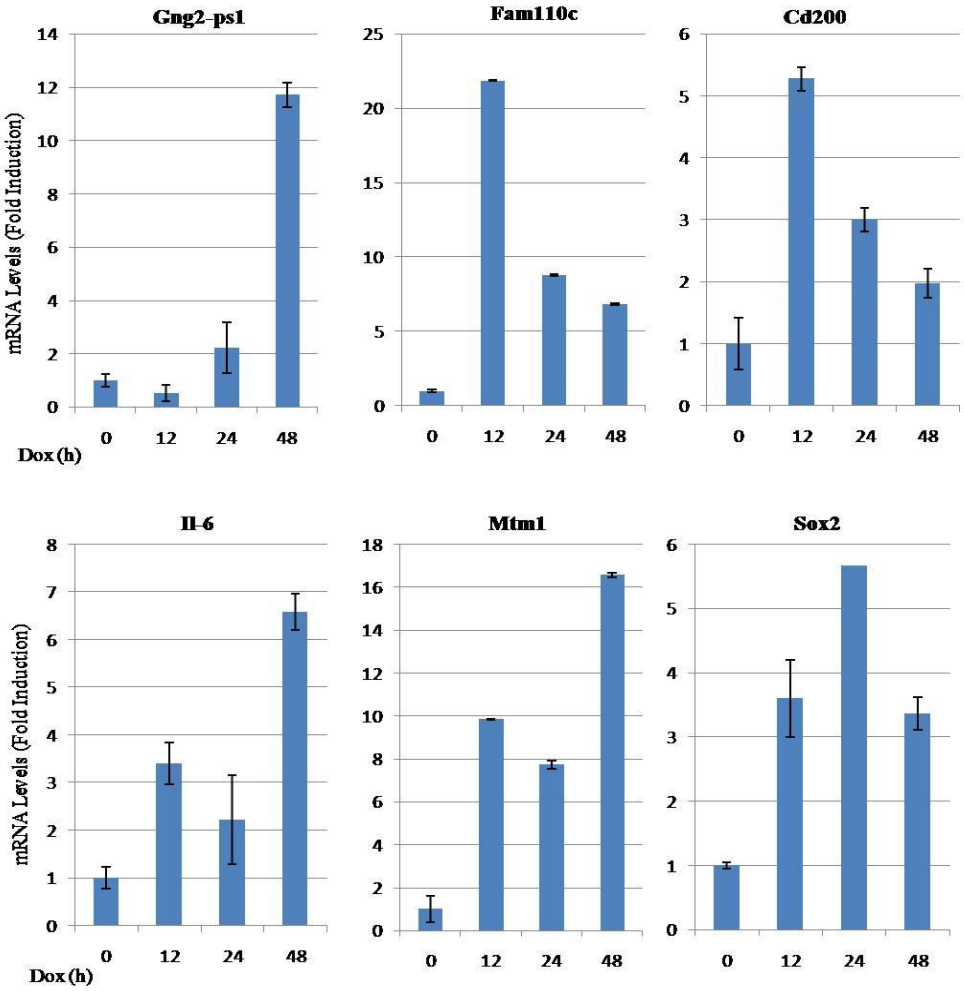


FIG. 20: Time course of expression of some of the genes upregulated in the Microarray experiment.

The mRNA fold induction is measured by Real-Time PCR and is related to the amount of β-catenin, which is a fixed parameter (Mansukhani et al., 2005), and to doxycycline treatment in OB1#4#1 cells.

Analysis of the differentially expressed genes.

The binding of Sox2 to the promoter of differentially expressed genes shows that Sox2 directly regulates their function(s). Thus, we have analyzed the promoter region of such genes searching for Sox2 binding sites through the PSCAN software at http://www.beaconlab.it/pscan (Zambelli et al., 2009). At least one Sox2 binding site was identified within the 1000 bp analyzed in 54% of the up-regulated and 42% of the down-regulated genes (data not shown). This observation strongly support the hypothesis that these genes are regulated by Sox2.

A role of Sox2 on the regulation of at least some of the genes derived from the microarray analysis was underlined by the comparison of the above results with those obtained by Basu-Roy and colleagues (personal communication) in OB1 cells infected with a lentiviral vector carrying the Sox2 gene. In the latter case the infected cells were analyzed using the ChIP-seq technique. Table 3 shows that approximately 10% of the genes differentially expressed in the microarray experiment contain Sox binding sites.

The Prostatic Androgen-Repressed Message 1 (Parm1), Tumor Necrosis Factor Receptor Superfamily Member 12a (Tnfrsf12a) and Vascular Cell Adhesion Molecule 1 (Vcam1) genes were previously described by Seo et al., 2011 as Sox2 differentially expressed genes in a microarray experiment carried out in primary calvaria- derived osteoblast cell line infected by Cre adenovirus. The Cre adenovirus expression allows the knock-out of the Sox2 gene. Under these conditions, Vcam1 and Parm1 were down-regulated, while Tnfrsf12a was up-regulated.

Sox2 targets	ChIP-seq enriched Sox2 peaks			mRNA levels (Fold Ind) Doxycycline (h)			
Gene symbol	Fold Enrichment	Chr	Sox2 binding position (Start-End nucleotides)	6	12	24	48
Il1rl1	**9.96**	1	40492608-40493204	0.78	0.72	**0.65**	0.78
2310043J07Rik	**9.88**	5	136527009-136527880	1.02	0.91	**0.65**	1.08
Cdc14a	**8.12**	3	116124984-116128513	**1.51**	1.25	1.26	0.91
Dusp6	**6.61**	10	98725335-98726347	**1.53**	**1.57**	1.27	1.11
	5.88	10	98730285-98731129				
	5.8	10	98727625-98729394				
Parm1	**5.76**	5	91951820-91952457	**1.57**	**3.31**	**4.22**	1.41
5730494M16Rik	**5.7**	18	25330380-25331157	**1.90**	**1.74**	1.22	1.10
Epha7	**5.6**	4	28739822-28741421	**3.64**	**3.83**	**2.00**	1.46
Zfp692	**5.58**	11	58115245-58116523	0.93	0.77	1.00	**1.55**
Col6a2	**5.53**	10	76085262-76086268	0.85	1.02	1.45	**1.65**
Vcam1	**5.32**	3	115825430-115826173	**1.50**	1.39	1.17	1.15
Slc7a5	**5**	8	124425357-124426536	**0.64**	0.71	0.69	1.32
Irs2	**4.56**	8	11008569-11009469	0.82	1.02	1.07	**0.58**
Arl4a	**4.51**	12	40763594-40764547	1.02	1.41	**1.55**	1.19
Tnfrsf12a	**3.94**	17	23813337-23814860	**0.66**	**0.60**	0.81	0.96
Mtx1	**3.5**	3	89017402-89019903	1.07	0.95	0.91	**1.51**
Npm3-ps1	**3.3**	6	85018336-85019873	**0.63**	0.80	1.08	0.85

Tab. 3: Comparison between the the Sox2 target genes of table 2 and the genes selected by ChIP analysis.

The colour scheme is the same as in Table 2. The blue strips indicate the genes selected for further experiments.

The interaction of Sox2 with the Parm1, Vcam1 and Tnfrsf12a promoters was confirmed by a ChIP-PCR experiment similar to that previously described (Seo et al., 2011; Seo et al., 2013). Figure 21A shows a PCR analysis of the DNA from cells infected with the lentivirus vector, carrying or not the Sox2 coding sequences, immunoprecipitated by Sox2 antibodies. The IgG immunoprecipitation experiment represents the negative control. The presence of fragments amplified by primers specific for the Parm1, Vcam1 and Tnfrsf12a genes demonstrates that the promoter of these genes interacts with Sox2.

If these genes are differentially expressed in Sox2 overexpressing cells they may be regulated in the opposite way in Sox2 Knock-out cells. Thus we analyzed the expression of the Parm1, Vcam1 and Tnfrsf12a genes in OB1 cells infected by GFP-Cre adenovirus as compared with OB1 cells infected by the GFP adenovirus only (Basu-Roy et al., 2010; Seo et al., 2011). Figure 21B shows the results of a Real-Time PCR experiment measuring the expression of the three genes. As expected, the Vcam1 and Parm1 genes were downregulated in Sox2 KO cells. Interestingly, the Tnfrsf12a gene was downregulated in Sox2 KO cells as well as in cell over-expressing Sox2. This suggests a complex regulation of this gene which does not depend exclusively on Sox2 expression.

The consistent results obtained with the different experimental approaches allow to conclude that in osteoblasts at least two genes, Parm1 and Vcam1, are regulated by the Sox2 transcription factor. The regulation of the Tnfrs12a gene by Sox2 may be mediated by a combination of factors.

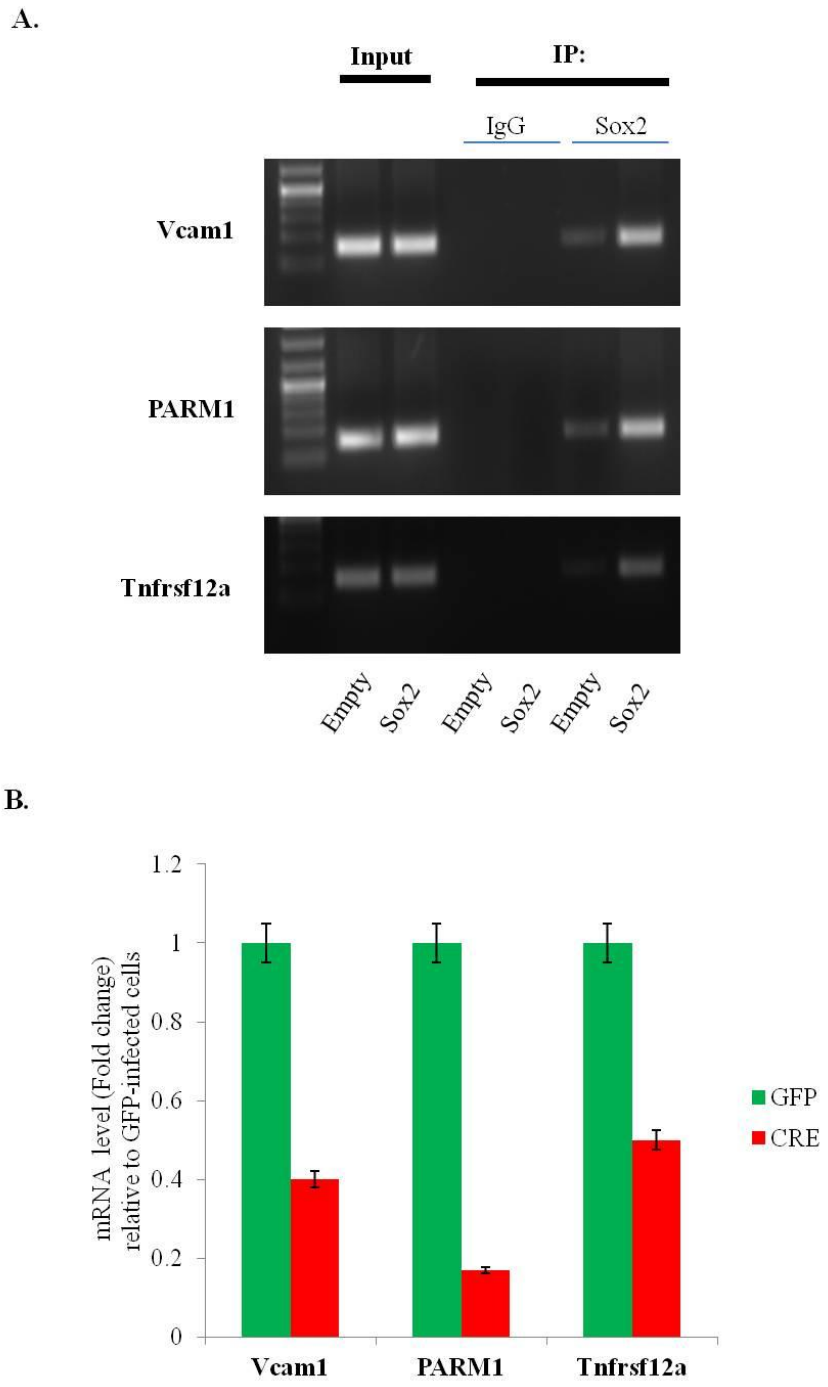


FIG. 21: Binding of Sox2 to the promoter of the Vcam1, Parm1 and Tnfrsf12a genes.

A. PCR analysis of the DNA fragments obtained through the ChIP technique of OB1 cells infected by the FUCRW lentivirus or the FUCRW-Sox2. The immunoprecipitation with IgG represents the negative control for the amplification.

B. Real-Time PCR of the Vcam1, Parm1 and Tnfrsf12a genes from OB1 Sox2 floxed cells (Basu-Roy et al., 2010; Seo et al., 2011) infected by the CRE lentivirus vector or by the GFP lentivirus vector.

SOX2 INHIBITS THE BMP PATHWAY IN THE OSTEOGENIC LINEAGE.

Sox2 is not sufficient to inhibit the osteoblast differentiation mediated by BMP.

It is now accepted that FGF inhibits osteoblast differentiation and that FGF triggers Sox2 expression. The finding that several Sox2 targets are genes involved in bone biology suggests that Sox2 itself is sufficient to block osteoblast differentiation. To test this hypothesis we induced osteoblast differentiation in OB1#4#1 cells and in two control cell lines, OB1 and OB5, by adding 100 ng/ml rhBMP2 to the osteogenic medium (Fig. 22). We measured differentiation using the ALP assay. The ALP enzymatic activity staining showed that, in the presence of BMP, OB1#4#1 cells have already started differentiating within 3 days and, within 7 days, differentiation increases (panel A-B) . As expected the positive control OB5 cells differentiate faster, whereas the OB1 cells differentiate slowly (panel A-B). Surprisingly, Sox2 induction did not inhibit differentiation in the OB1#4#1 cells.

These results show that in this experimental system Sox2 is not sufficient to block osteoblast differentiation triggered by BMP treatment. This is in contrast with the finding that the expression of several genes involved in the BMP pathway are modulated by Sox2 in both a positive and negative manner (Tab. 2F). Thus, we decided to investigate the possible effect of Sox2 on the BMP pathway.

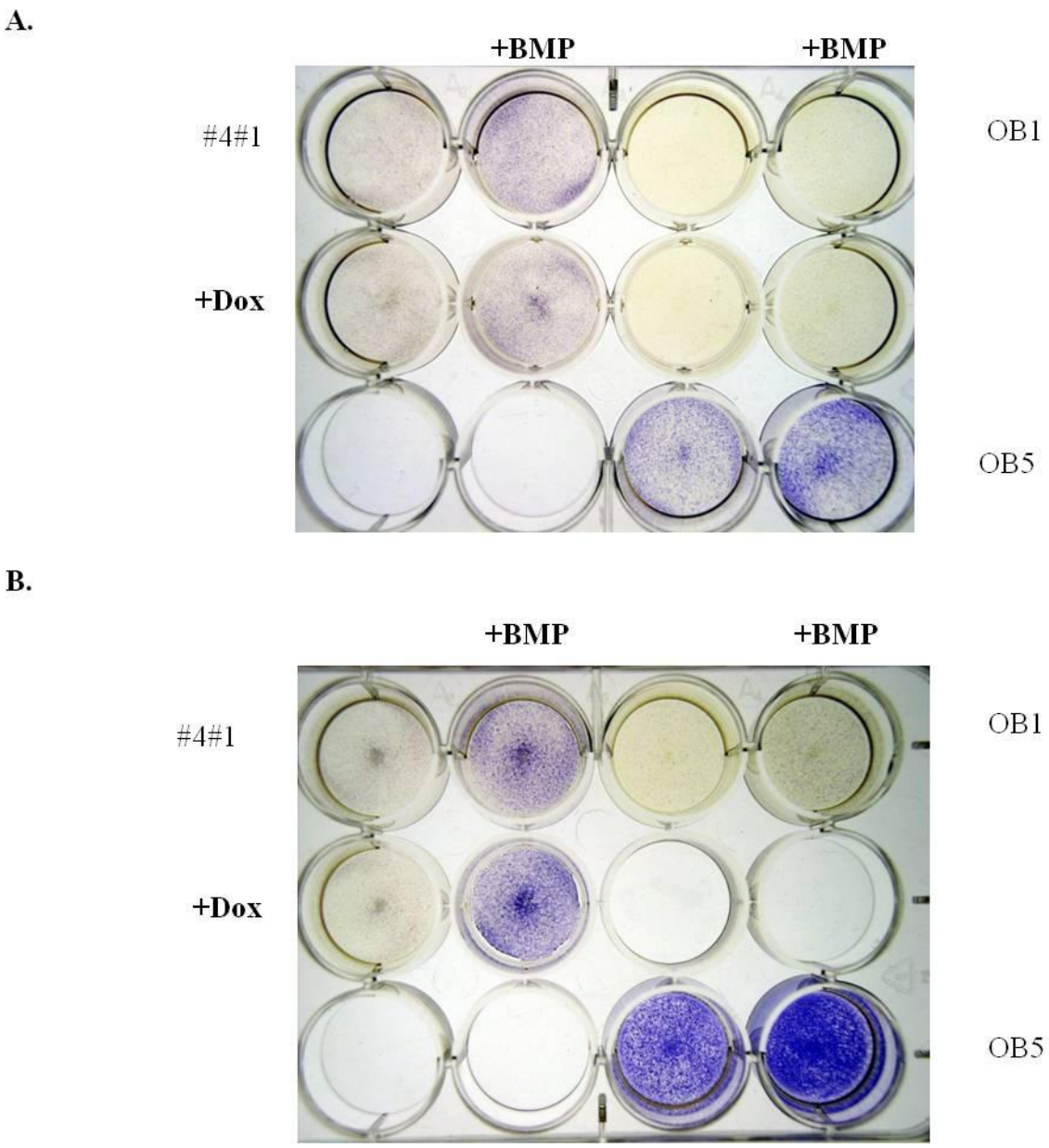


FIG. 22: Differentiation of OB1#4#1 cells by BMP addition with and without doxycicline treatment.

A. OB1#4#1 Cells differentiated for 3 days. The addition of BMP to OB1 and OB5 cells are control samples.

B. OB1#4#1 Cells differentiated for 7 days. The addition of BMP to OB1 and OB5 cells are control samples.

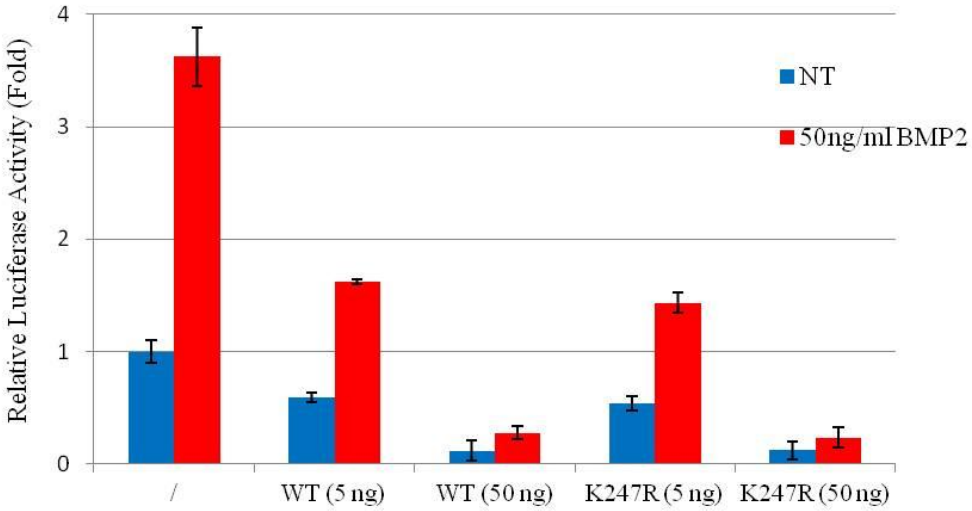
Sox2 inhibits BMP signaling in 293T cells.

In order to analyze the BMP function we used a plasmid, in which the expression of the luciferase reporter gene was driven by two copies of the BMP Responsive Element (BRE) (kind gift from Dr. Rifkin, NYULMC, New York, US). In this experiment we used the 293T cell line instead of osteoblast cells because 293T cells are efficiently transfected. Fig. 23A shows a luciferase assay carried out in 293T cells transfected with this plasmid and with (red bars) and without (blue bars) addition of 50 ng/ml of rhBMP2. Increasing amount of the Sox2 expression plasmid was cotransfected with the reporter gene.

In the absence of Sox2 the luciferase activity increased approximately 3.5 fold following BMP treatment. Sox2 inhibited the luciferase activity showing a negative effect of Sox2 on the BMP pathway. Using the K247R Sox2 sumoylation mutant instead of the wild-type protein did not change the negative effect on the luciferase activity.

The effect of Sox2 on the BMP reporter gene suggested an effect of Sox2 on the SMAD proteins which bind to the BMP responsive element. To this purpose, we analyze by western blot the protein extract of 293T cells transfected with the empty expression vector, the vector containing Sox2, the vector containing the Sox2 sumoylation mutant K247R or the sumoylation mutant K0 (Fig. 23B). The SMAD1 and SMAD5 proteins were detected by using mAbs specific for the phosphorylated serines. The phosphorylation of the SMAD1/5 (pSMAD1/5) proteins with or without BMP treatment is always the same and does not depend on the Sox2 construct used for the transfection. We conclude that, in 293T cells, Sox2 is a negative regulator of BMP target gene expression without affecting the initial step of SMAD activation. The absence of Sumoylation in Lysine 247 does not modify the inhibition of the BMP pathway mediated by Sox2.

A.



B.

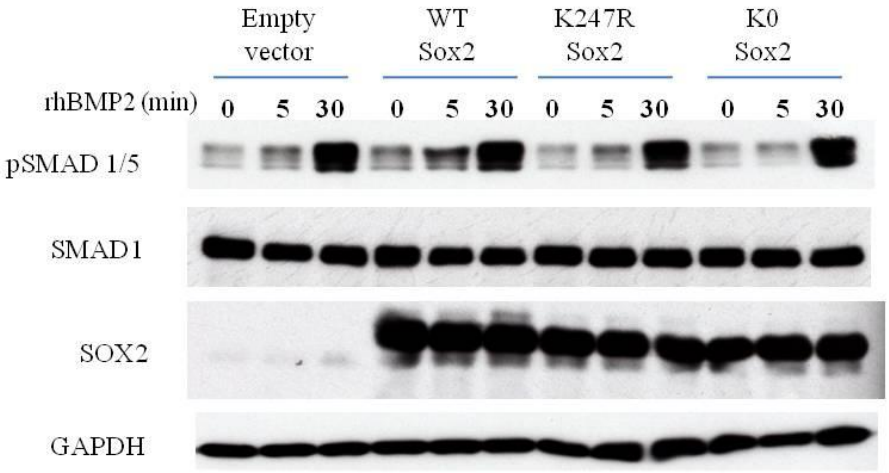


FIG. 23: Transcriptional role of WT and mutant Sox2 on the BMP target genes in 293T cells.

A. Samples treated (red bars) and not treated (blue bars) with 50 ng/ml rhBMP2.

B. Western Blot analysis of 293T cells transfected as indicated and starved overnight before addition of 50 ng/ml rhBMP2 for increasing time.

Sox2 inhibits BMP signaling in C3H10T1/2 cells.

The effect of Sox2 on BMP pathway was further analyzed using the C3H10T1/2 cell line. These mesenchymal cells can be differentiated in osteoblasts and seemed a more suitable system to study bone biology. To generate a stable cell line efficently responding to BMP, we transfected C3H10T1/2 cells with the BRE-luciferase construct and selected for 15 days stable G418 resistant clones. Two independent pools each containing 8 clones were isolated and tested for the BMP response.

The experiment in Fig. 24A shows that, following BMP treatment, pool 1 generate higher luciferase response then pool 2 .

The Western blot in Fig. 24B shows that, as expected, both pools responded to BMP treatment with the phosphorylation of the SMAD proteins whether in low or high serum concentration. Overnight starvation in 0.2% FBS containing medium, before BMP treatment, eliminated the basal SMAD activation.

Thus, we chose pool1 cells to test the effect of Sox2 on the BMP response.

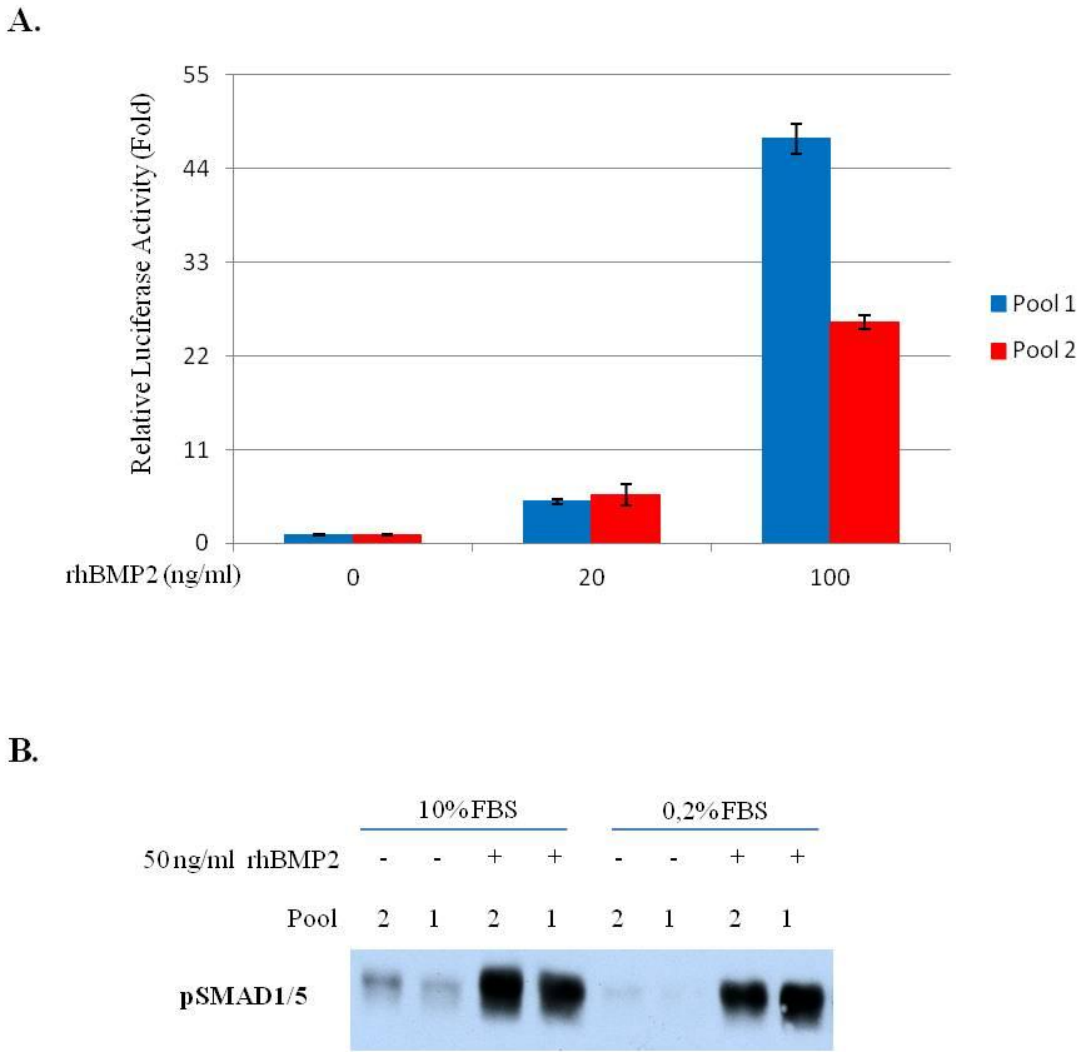


FIG. 24: Activation of the BMP pathway in selected pools of C3H10T1/2-BRE cells.

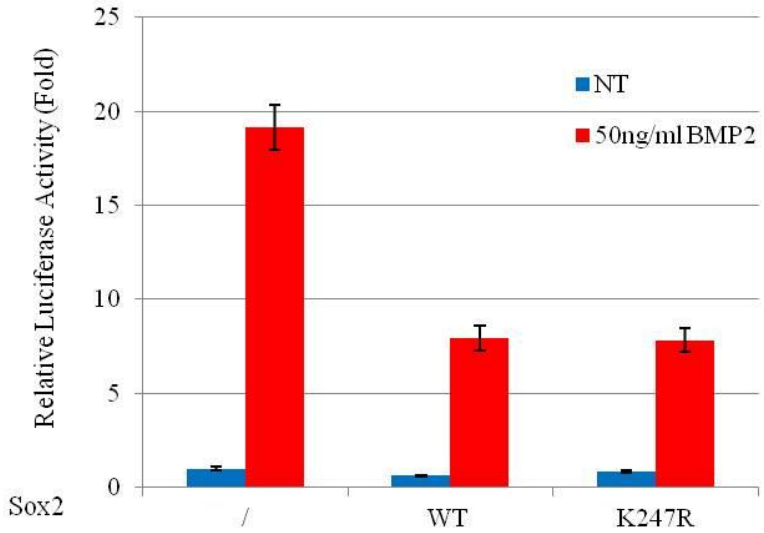
A. Transcriptional activation of the Luciferase reporte gene following addition of increasing amount of rhBMP2.

B. Western blot analysis of SMAD phosphorylation in samples treated and untreated with rhBMP2.

The Pool1 cells were infected with the FUCRW lentiviral expression vector alone, the FUCRW lentiviral vector containing WT Sox2 or K247R Sox2 coding sequences. In agreement with the results obtained in 293T cells, the activation by BMP of the luciferase reporter gene is inhibited by both Sox2 and the Sox2 sumoylation mutant (Fig. 25A).
Pool1 cells were treated with BMP and the protein extracts were analyzed by Western blot (Fig 25B). The results show that BMP triggered the phosphorylation of the SMAD proteins and that neither Sox2 nor the Sox2 sumoylation mutant modified the reaction.

We can conclude that, Sox2 acts as a negative regulator of the BMP pathway in osteoblast precursor as well as in 293T cells. It is interesting that this function is detectable in two different cell lines suggesting that is not a cell specific effect.

A.



B.

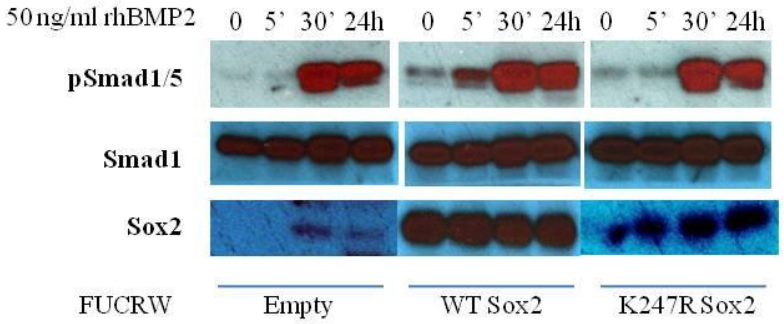


FIG. 25: Transcriptional role of WT and mutant Sox2 on the BMP target genes in C3H10T1/2-BRE cells.

A. Effect of WT and mutant Sox2 transcription factors on the BMP dependent transcription of the luciferase reporter gene in C3H10T1/2-BRE cells infected with the empty lentivirus vector and with wild type and mutant Sox2 lentivirus vectors.

B. Western blot analysis of a protein extract from C3H10T1/2-BRE cells infected with the empty lentivirus vector , and with wild type and mutant Sox2 lentivirus vectors.

SOX2 INHIBITS THE HIPPO PATHWAY.

Sox2 plays an additional role in bone tissue biology by regulating the expression of some of the components of the Hippo pathway, such as YAP1 (Seo et al., 2013). Furthermore, the results of Table 2F show that Sox2 regulates the expression of Hippo target genes and preliminary ChIP experiments suggested that Sox2 binds to the Promoter of the Hippo up-stream regulator Nf2 (Basu-Roy, personal communication). Thus, we decided to investigate the possible role of Sox2 on the expression of the Nf2 gene.

Primary calvarial mouse osteoblasts were infected with lentivirus carrying either the empty vector or the vector expressing the WT Sox2. The western blot analysis of the infected cells showed that Nf2 expression is inhibited when Sox2 is activated (Fig 26A). This result suggest that Sox2 downregulates the expression of the Nf2 gene. In order to confirm this observation we generated a luciferase reporter plasmid containing a copy of the Promoter of the Nf2 gene. As shown in Fig. 26B, a luciferase assay carried out in 293T cells showed a dose response activation of the Promoter (blue bars) as compared to the control reporter vector without the Promoter (red bars). The experiment of Figure 5C shows that the cotransfection of the reporter gene with increasing amount of the Sox2 expression vector resulted in the inhibition of the luciferase activity. The control samples show that the luciferase activity of the control reporter construct was not inhibited by the expression of Sox2 (Fig. 26C).

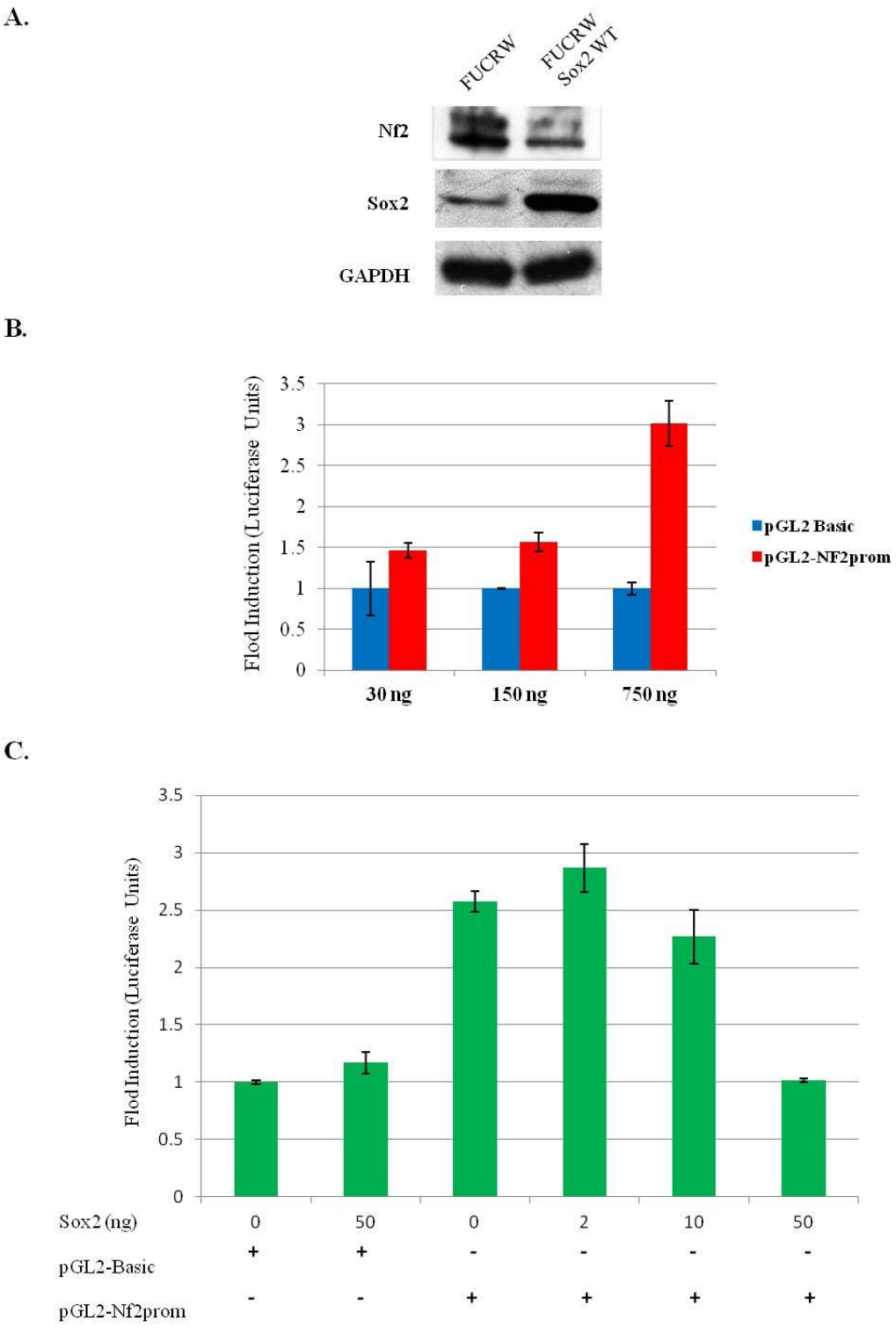


FIG. 26 A role of WT Sox2 on the Hippo Signaling pathway.

A. Western Blot analysis of a protein extract from primary osteoblast cells infected with Wt Sox2 lentivirus and with the empty vector. **B**. Transcriptional activation of increasing amount of the luciferase reporter plasmid containing the NF2 promoter in 293T cells. **C**. Transcriptional activation of of the luciferase reporter plasmid containing the NF2 promoter in 293T cells. The co-transfection with the WT Sox2 expression plasmid is indicated.

DISCUSSION

THE SUMOYLATION OF THE SOX2 TRANSCRIPTION FACTOR.

The transcription factor Sox2 in mouse is modified by Sumoylation on one lysine only, K247, corresponding to K245 in the human protein (Tahmasebi et al., 2013; Tsuruzoe et al., 2006; Van Hoof et al., 2009). However, the Sox2 sumoylation assay carryed out so far, did not include E3 ligases that may be necessary to obtain the maximum level of sumoylation. In fact, the Sox9 and Sox10 factors require the interaction with the PIAS1 E3 ligase to trigger a high level of sumoylation. (Hattori et al., 2006; Oh et al., 2007). Thus, we added Pias1 to the Sox2 sumoylation assay. Our results show clearly that Pias1 is essential to obtain full sumoylation of the Sox2 transcription factor. In fact, under these experimental conditions, the Sox2 mutant K247R is sumoylated on K123. In agreement with this observation, the addition of Pias1 inhibits the transcriptional activity of both the WTSox2 and the K247R mutant suggesting that the further modification of lysine 123 triggers the downregulation of transcription. The transcriptional activity of Sox2 in Hela cells may be influenced by the presence of Oct3/4 (Tai et al., 2005). However, the sumoylation of Oct3/4 has the opposite effect to that shown by Sox2: it enhances its transcriptional activity (Wei et al., 2007; Wu et al., 2012). Thus, we conclude that Oct3/4 can not explain the downregulation observed.

As the members of the Sox family of transcription factors share the HMG-box and this domain directly interacts with Pias1 (Davide Ambrosetti, personal communication), it is possible that Pias1 is necessary for the complete sumoylation of all family members.

SOX2 TARGET GENES IN OSTEOBLASTS.

The bone tissue is extremely plastic, as hundreds of genes control its homeostasis, interacting in a complex developmental network, along with post-translational and epigenetic mechanisms that can modify the genome performance. Hormones, cell signaling pathways and a number of transcription factors are the actors playing in the bone stage and the whole screenplay is still far from an

exhaustive overview. The physiological complexity results in several pathological implications affecting bone development, formation and integrity. Given the importance of transcription factors in the regulation of bone homeostasis, the interest of our laboratory for the FGF pathway dated from its involvement in craniosynostosis, the premature skull bone suture pathology. At the molecular level, some craniosynostosis syndromes are due to constitutive active FGF pathway. Mimicking pathological conditions in cultured calvarial osteoblasts through FGFR activating mutation or exogenous FGF treatment, we noticed increased apoptosis, a block cell differentiation by inhibition of Wnt target genes and abnormal up-regulation of the Sox2 transcription factor (Mansukhani et al., 2005; Mansukhani et al., 2000).

We have already shown in the preliminary experimental section that clonal variability may generate ambiguous results. To avoid the problem of heterogeneity we decided on two different strategies:
1) we set up an OB1 cell system in which Sox2 expression is under the control of an inducible promoter, a versatile system easy to manipulate;
2) we used lentiviruses carrying the Sox2 coding sequence to infect osteoblast cells. This system gives high efficiency of integration into the cell genome.
We identified new direct Sox2 targets in osteoblasts, combining the two experimental approaches: the identification of genes differentially expressed by Sox2 and of genes interacting with Sox2. The microarray analysis of the inducible osteoblast clone generated 158 genes whose expression changes in response to Sox2 induction; approximately 75% of the genes are up-regulated. By analogy with other cell models, Sox2 in osteoblast cells acts mainly as a transcriptional activator (Ambrosetti et al., 1997; Ambrosetti et al., 2000).
Most of the differentially expressed genes can be classified in functional categories related to bone biology (Tab 2 A-F).
It is interesting that the FGF ligand triggers Sox2 expression and that in the mouse and human models of craniosynostosis, the activated FGFR2 mutants induce the constitutive expression of Sox2 (Mansukhani et al., 2005). Thus, it is possible that some of the Sox2 target genes that we have selected are involved in the ethiopathology of the disease. Interestingly, the intrinsic effect of activating FGFR2 mutants in primary osteoblasts results in a increased capacity of cell differentiation and proliferation (Holmes et al., 2009). In fact, at least 10 of the Sox2 target genes that we have identified are involved in bone biology, cell differentiation and cell growth, i.e. Smoc1, Vcam1, Acvr2a, Fastk, Mras, Slc39a1, Tnfrsf12a, Ifi204, Elk1, Acvr2b. They may represent good candidates to play a role in osteoblast physiology and pathology.
The gene expression changes in the Sox2 inducible osteoblast cells could be direct or indirect consequence of Sox2 over-expression. To identify Sox2 direct target genes, we searched, by the

PSCAN bioinformatic tool, the 1000 bp region overlapping the transcriptional start site of each putative target and found that about half of the promoter regions harbor at least one putative Sox binding site. Thus, at least half of the differentially expressed genes should be genuine Sox2 targets. Interestingly, there is an overlap between the differentially expressed genes in osteoblasts with those, Sox2 binding, selected by the ChIP-seq experiment (Upal Basu-Roy, personal communication). About 10% of the genes whose expression is modified in the Sox2 inducible cells are also interacting with Sox2 on the chromatin (see Table 3). We conclude that these genes are direct Sox2 targets in osteoblasts.

Parm1, Vcam1, and Tnfrsf12a previously described as Sox2 targets were further analyzed for the following reasons. During cardiomyogenesis TGFβ induces Parm1 expression (Isodono et al., 2010) sustaining BMP signaling through an increase of BMP2 and BMP4 transcription and phosphorylation of SMAD1/5/8 (Nakanishi et al., 2012). Vcam1 has an osteoclastic function (Hopwood et al., 2007) that involves the WNT and BMP pathways. In addition, Vcam regulates the migration of mesenchymal cells which is inhibited by the knocked-down of Vcam1 (Nishihira et al., 2011). Tnfrsf12a blocks BMP2 induced osteogenic differentiation through the MAPK/Erk pathway and increases the expression of the RANKL receptor in pre-adipocyte mesenchymal cells (Ando et al., 2006). In rat cardiac fibroblasts Tnfrsf12a promotes cell proliferation and collagen synthesis (Chen et al., 2012).

The Chip-PCR experiment of Figure 21A is consistent with the binding of Sox2 to the promoter of the three genes. Consistently the knock-out of Sox2 by Cre-mediated excision results in the downregulation of the expression of Parm1 and Vcam1 (Fig. 21B). As already discussed in the results section, the regulation of the Tnfrsf12a gene seems to be complex and does not depend exclusively on Sox2.

The FGF signal is sufficient to inhibit osteoblast differentiation. In contrast, Sox2 expression, despite the numerous molecular effects on a number of cell systems, does not inhibit osteoblast differentiation, as assayed on the Fig. 20.

Thus, Sox2 may require the expression of coactivators to fullfil this complex function.

SOX2 INHIBITS THE BMP PATHWAY WITHOUT AFFECTING SMAD ACTIVATION.

The BMP pathway is central to the process of osteoblast differentiation. In our inducible system we have found both down-regulated genes (Atoh8, Cml1, Elk1, Sdc4, Tnfrsf12a) and up-regulated genes (Acvr2a, Acvr2b, Bmp2k, Icam5, Ifi204, Inhba, Mmp16, Smoc1, Vcan) involved in the BMP pathway or BMP target genes (Tab. 2F). In the complex BMP pathway regulation in osteoblasts Sox2 exerts both positive and negative roles.

There are only a few reports on the negative role of Sox2 in BMP signaling (Clavel et al., 2012). In this work we show that WT Sox2 inhibits the BMP signal and the same inhibitory effect occurs with the K247R Sox2 sumoylation mutant plasmid (Fig. 24A). It is interesting that the phosphorylation of the SMAD1/5/8 proteins is not affected by the expression of Sox2 or of the sumoylation mutants (Fig. 24B).

Inhibition of the BMP pathway by Sox2 may involve:

1) SMAD4 displacement or sequestration from its promoter binding;

2) activation or stabilization of the inhibitor complex SMAD6/7;

3) inhibition of the cytoplasmic-nuclear import-export of the SMAD proteins (Miyazono et al., 2005).

The finding that Sox2 inhibits the BMP pathway is an interesting observation with many possible implications. For example, inhibition of the BMP target genes by Sox2 may be an important mechanism in the reprogramming of somatic cells into induced pluripotent stem cells, since RepSox, a TGF-β receptor I kinase inhibitor molecule, is known to be involved in iPS cell formation (Ichida et al., 2009).

SOX2, HIPPO PATHWAY AND ADIPOGENESIS.

The Hippo pathway is modulated by Sox2 at the level of transcriptional coactivator YAP1 (Seo et al., 2013) and of upstream mediator Nf2 (Upal Basu Roy, personal communication). In this work we show that Sox2, in primary osteoblasts, downregulates the expression of Nf2, a negative regulator of the Hippo pathway (Fig. 27A). We also show that, in 293T cells, Sox2 is a transcriptional repressor of the endogenous Nf2 promoter activity (Fig. 27B).

This is a rare example of a mechanism in which Sox2, which is usually a transcriptional activator, functions as a repressor. It is possible that Sox2 recruits a transcriptional repressor factor on the Nf2 promoter.

It is noteworthy that the Sox2 targets of Table 2F include genes involved il the Hippo pathway. i.e., the Slc7a5 gene, which is downregulated, while the Cdc14a, Vcan, and Inhba genes are upregulated by Sox2 induction (Tab 2F). The involvement of Parm1 and Tnfrsf12a with fat tissue differentiation is also consistent with the role of the Hippo pathway in the differentiation of mesenchymal stem cells into adipocytes (Seo et al., 2013). In fact, Parm1 increases PPARγ2, leptin and adipogenin expression, inducing mouse adipocyte differentiation (Song et al., 2009); Tnfrsf12a inhibits murine and human adipocyte differentiation (Tiller et al., 2009).

In conclusion, Sox2 may regulate the Hippo pathway through both direct and indirect mechanisms. We may say that Sox2 induction affects only a relatively small number of target genes, but these genes are important in bone cell biology. Sox2 also controls and coordinates the complex interplay among different pathways that contribute in the determination of osteogenic and adipogenic fate.

In summary, the combined approach of an inducible cell system and viral infection was determinant to overcome the problem of clonal variability. We confirmed the preponderant role of Sox2 as a transcriptional activator, rather than a repressor, and we annotated the expression variations into clusters reflecting potential key functions in cell growth, differentiation, migration and adhesion control. Among the potentially very interesting genes in osteoblast biology, we demonstrated that Vcam1, Parm1 and Tnfrsf12a are new direct Sox2 targets.

MATERIAL AND METHODS

CELL LINES.

Characteristics and culture conditions.

In the experiments of the Thesis we used the following cell lines:

The 293T cell line, originally referred as 293tsA1609neo, is a highly transfectable derivative of human embryonic kidney (HEK)-293 cells, and contains the SV40 T-antigen. 293T cells are used for FGF4 and β-catenin transcriptional assays and for in vivo Sumoylation assays; for BMP and Nf2 transcriptional assays; as packaging cells to produce active lentiviruses carrying WTSox2 and K247R mutant.

HeLa is an immortalized human tumor cell line, isolated from cervix adenocarcinoma of a 31 year-old black woman, Henrietta Lacks, who named the cell line.

Primary osteoblasts are maintained in culture only for a limited number of passages. They originate from calvaria of newborn mice, dissected free of surrounding muscles and soft tissues and washed in PBS containing penicillin and streptomycin. Isolated calvaria were sequentially digested in aMEM (GIBCO BRL), containing 0.1% collagenase and 0.2% dispase at 37 °C. Digested fractions were collected every 10 min and fractions 2–5 were pooled. Cells were collected by centrifugation and resuspended in aMEM supplemented with 10% FCS (Mansukhani et al., 2000).

OB1 – OB5 are murine cell lines derived from primary calvarial osteoblast, infected with a pBabe-puro retrovirus expressing the Polyoma large T-Antigen (Su et al., 1999). The cells were infected for 1 h in the presence of 8 mg/ml of polybrene with a virus stock produced in 293 cells as described previously (Bellosta et al., 1997). Clones were selected using 4 mg/ml of puromycin for 2 weeks. Immortalized clones OB1-5 were characterized according to morphology, histochemical staining for alkaline phosphatase and for their ability to express osteocalcin upon differentiation. Expression of Polyoma large T-Ag was determined by immunofluorescence using an anti-Polyoma large T-Ag rat serum.

OB1TR#4HA/Sox2#1 cells are the inducible system for Sox2 expression in osteoblasts (see section below for more details). They are used for microarray experiment for checking expression profile changes upon Sox2 induction.

F9 are adherent, epithelial- like, mouse embryonic carcinoma cell line. We used them as a constitutive Sox2 high expression control.

C3H10T1/2 is a murine embryonic cell line, with a mesenchymal pluripotent status that allow to undergo to both osteogenic and adipogenic lineages when properly stimulated. Clone 8 was isolated by C. Reznikoff, D. Brankow and C. Heidelberger in 1972 from a line of C3H mouse embryo cells. It derives from sarcoma and has adherent epithelial morphology. They are the model for BMP pathway experiments.

C3H10T1/2 –BRE: stable cell line carrying the BMP responsive luciferase reporter plasmid; we performed BMP pathway experiments on this cell model (see more details in following sections).

All cell line are grown at 37°C in High Glucose Dulbecco's Modified Eagle's Medium, (DMEM, EuroClone), supplemented by 5% (for HeLa and 293T cells) or 10% (for all osteoblast and mesenchymal derived cell lines) of North American fetal bovine serum (FBS, EuroClone), 4.5 g/l L-Glutamine (Sigma-Aldrich) and 1X streptomycin/penicillin (Sigma-Aldrich); we call this as "complete growth medium" . Human cells cultures are maintained in humidified atmosphere with 5% CO_2, while murine cell lines better growth at 7% - 10% CO_2.
Starvation refers to cell growth in DMEM only added by 0.2% FBS overnight before BMP experiments in 293T cells and C3H10T1/2 cells.

Establishment of a stable C3H10T1/2 –BRE cell line.

C3H10T1/2 cells (400,000 cells per well) were transfected in six-well plates with 4 µg of pGL3-$(BRE)_2$-lucNEO with Lipofectamine 2000 reagent (Life Technologies; see other section for more protocol details). The day after transcfection we started selection by treatment of cell growth medium with 500 µg/ml neomycin (also called G418, Sigma-Aldrich) for 15 days, changing the

medium each 2-3 days. Cells not carrying plasmid dye and resistant clones start growing. We selected two different pools, each of 7-8 clones, and we called C3H10T1/2-BRE Pool 1 and Pool2. We tested BMP responsive reporter activation by different amount of rhBMP2 (R&D Systems) from 20 ng/ml to 100 ng/ml, upon starvation. Then we checked SMAD1/5/8 activation by Western Blot (see further protocol details).

OSTEOGENIC DIFFERENTIATION.

OB1TR#4HA/Sox2#1 cells are plated 30,000-50,000 in a 24-well plate in normal complete medium, so that within about 36h incubation they reach confluence. Now the complete medium switch to differentiation medium: complete medium supplemented by 10 mM β-Glycerol phosphate (Sigma Aldrich) and 100 µg/ml Ascorbic Acid (Sigma Aldrich). Change the cell medium every 2-3 days, without disturbing the differentiating cell layer.

rhBMP2 treatment is added to osteogenic medium only for OB1TR#4HA/Sox2#1 differentiation. After 2- 15 days cells could be stained for Alkaline Phosphatase (ALP) Assay to measure early osteobalst differentiation:

- Aspirate culture medium and wash cells with 1X PBS;
- Add 1 ml/well of Fixation Solution (20 ml ddH_2O, 30 ml Acetone, 400 µl of concentrate citrate solution (Sigma Adrich)) and incubate at room temperature (RT) for 1 min;
- Wash cell with 1X PBS
- Add 1 ml/ well of Staining Solution (48 ml ddH_2O and 2 ml of Naphthol AS-MX phoshpatase alkalin solution 0.25%
- Incubate the plate in the dark for 30 min + not over than 30 more minutes at RT: development of purple-violet stained areas indicate positive reaction;
- Stop the reaction by adding ddH_2O.

To check later differentiation status, incubation in osteogenic medium for 20 days can proceed to measure osteoblast mineralization by 75 µg/mlAlizarin Red S staining: overnight incubation with the reagent directly on cultured plates is enough to detect mineralized nodules.

MAMMALIAN EXPRESSION PLASMIDS AND LENTIVIRUSES.

We summarize all expression plasmid used in the experiments performed in this Thesis:

pRK7-HA-Sox2 (later named WTSox2 or Sox2): Sox2 murine coding sequence (cds) is amplified by BamSox2DIR and EcoSox2REV from pCEP4Sox2 template expression plasmid. After restriction enzyme digestion, Sox2 cds is cloned into pRK7-HA plasmid (Fig. 27), once opened on correspondent BamHI and EcoRI sites and properly dephosphorylated.

Point mutants of pRK7-HA-Sox2 (K44R; K89R; K123R; K247R): to find out additional Sumoylation site(s) in Sox2 other than K247, we based on bioinformatical analysis and performed site direct mutagenesis to obtain Sox2 K247R, K44R, K89R, K123R mutants (see next sections for more details).

Double mutants pRK7-HA-Sox2 (K44R/ K247R; K89R/ K247R; K123R/ K247R): double Sox2 mutants are obtained by enzymatic digestion and proper ligation of the fragments between PvuII-EcoRI in pRK7-HA-Sox2 K247R plasmid with each of the BamHI-PvuII parts in pRK7-HA-Sox2 K123R, pRK7-HA-Sox2 K89R or pRK7-HA-Sox2 K44R. In this way the last C-terminal 140 amino acids of Sox2, harboring Lysine 247 to Arginine mutation, combine with each of other N-terminal coding sequence portions, resulting in three Sox2 double mutants K44R/ K247R, K89R/ K247R and K123R/ K247R.

K0 (= all 4 putative K to R substitutions) Sox2: In the matter of Sox2 K0, all four putative Sumoylable Lysines changed into Arginines: a further K89R substitution has been obtained by site directed mutagenesis on K123R/ K247R Sox2; BssHII- EcoRI digested fragment from the triple mutant is ligated with the BamHI-BssHII region from K44R/ K247R Sox2 plasmid digestion, to achieve the four punctual mutations on Lysines of interest.

pRK7-SUMO1: in ou lab, Rattus norvegicus SUMO1 full cds is obtained by PCR with primers 5'-SUMO and SmaSUMO rev and cloned into BamHI-SmaI restriction sites in pRK7 vector.

pcDNA3.1-Pias1: another CMV promoter expression plasmid with the E3 ligase Pias1 cds in frame is a gift from Dr. Ciarrocchi.

pGL2-Basic: the Promega reporter vector whose luciferase activity is not under the control of any regulatory region, just to be used as background for transcriptional assays (Fig 27).

pGL2-64enhFGF4-6x(O/S): FGF4 expression in blastocyst inner cell mass and in embryonic carcinoma cells is driven by combinatorial activity of Sox2 and Oct3/4 transcription factors, binding to adiacent sites on FGF4 enhancer region, HMG-box and POU domain respectively (Ambrosetti et al., 1997; Ambrosetti et al., 2000). A reporter plasmid used to test this synergistic activity has the firefly luciferase gene under the control of six in tandem repeats of 35 bp long FGF4 enhancer, containing the Sox2 and Oct3/4 DNA binding sites, followed by FGF4 promoter region between -64 nt and +101 nt from TSS of the gene. Specifically we cloned the region 6x(O/S)-64enhFGF4 from pCAT3-6x(O/S)-64 into the SacI-BglII restriction sites in pGL2-Basic Multiple cloning site (MCS).

pGL2-Nf2prom: firefly luciferase reporter to measure the activation ability of almost 2400 bp of the promoter region of mouse gene Nf2, -2000 bp from TSS to +400 bp. The Nf2 regulatory element comes from amplification from genomic DNA template using high fidelity X-tra Taq (Genspin) and PfuI (Promega); the fragment has been cloned into NheI-BglII restriction sites in pGL2 backbone.

pGL3-$(BRE)_2$ / pGL3-$(BRE)_2$-lucNEO: the gene for resistance to Neomycin (Neo^R) or G418 has been cloned (or not) into SalI-BamHI restriction enzyme sites of pGL3-luciferase backbone vector, whose luciferase gene is under the control of double repeat of BRE from Id1 gene promoter, cloned in the NheI site in vector multiple cloning site.

pRL-TK: plasmid with a renilla luciferase under the control of a Timidine Kinase promoter, useful to normalize transient transfection efficency in transcriptional assays involving firefly luciferase reporter as pGL2 or pGL3 vectors.

TOPFLASH M50 (Korinek et al, 1997) and pCAN-ΔN89β-Catenin: the Wnt responsive reporter plasmid, whose luciferase activity is driven by 8 TCF/LEF binding sites, and the truncated active form without the first N-terminal 90 amino acids of β-catenin are a gift from Dr- L.Howe (Strang Cancer Prevention Center, New York NY).

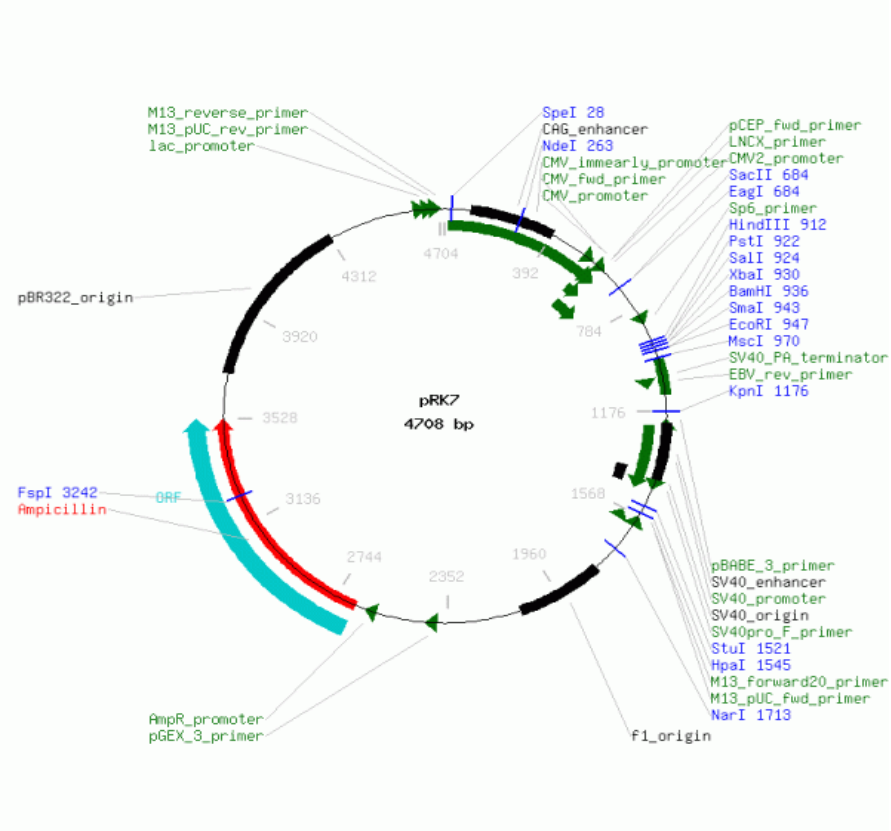
wtOct3 2: it is the conding plasmid for Oct3/4, the transcriptional cooperator with Sox2.

pcDNA 6-TR: it drives constitutive expression of TetR gene (Tetracycline Repressor gene) through the CMV strong promoter, to maintain "off" the expression of gene of interest (in our case Sox2) driven by the TO on the other plasmid of the inducible system (Fig. 29).

pcDNA 4-TO; pcDNA-TO-Luc; CMV-TO-HA/Sox2 (Fig. 28): it is the inducible expression plasmid of our system, whose TO keeps inactive expression of the Luc or HA/Sox2 cds till the repressor is encoded by pcDNA6/TR, till there is no doxycycline in cell growth medium.

pLP1 / pLP2 / pVSVG (all from Life Technologies, Fig. 29): they are the helper plasmids providing Gag/Pol, Rev and envelop proteins, respectively, to obtain vital lentiviruses, according to our protocol (see section below).

FUCRW-WTSox2 / K247R: they are the lentiviruses and carry a RFP cds to be easily localized in cells (as gross infection titre), together with the gene to be integrated in host cell genome and strongly expressed. Here Wt and K247R Sox2 cds come from previously described pRK7-HA constructs. PCR of Sox2 cds are obtained by primers XbaI Sox2cds DIR+REV or EcoRI Sox2cds DIR+REV and then cloned into FUCRW backbone (Fig 29). We used infection parameter M.O.I. around 10



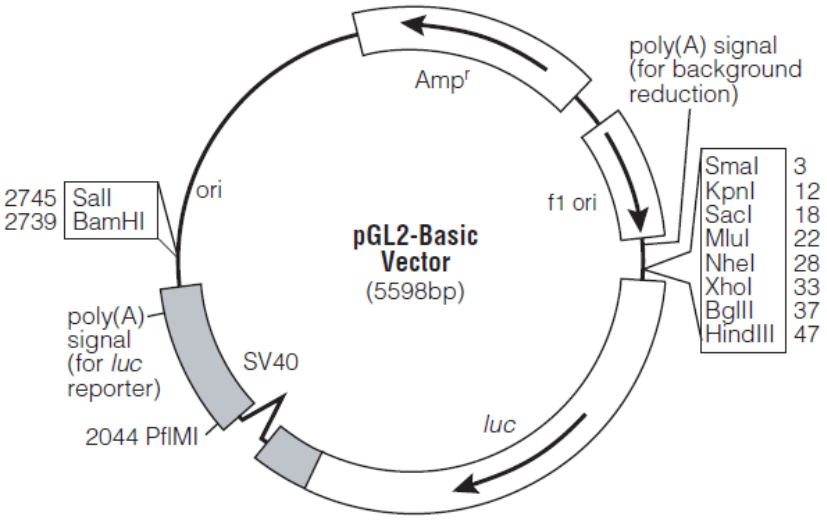


FIG. 27: pRK7 (Addgene) and pGL2-Basic vector map (Promega)

pRk7 is the mammalian expression vector in which we cloned Wt Sox2 and K to R substitution mutants; HA epitope has been inserted between HindIII and BamHI sites, then in frame Sox2 cds in BamHI-EcoRI site.

pGL2-Basic is the luciferase reporter backbone for assays in OB inducible system and on Hippo pathway in 293T cells.

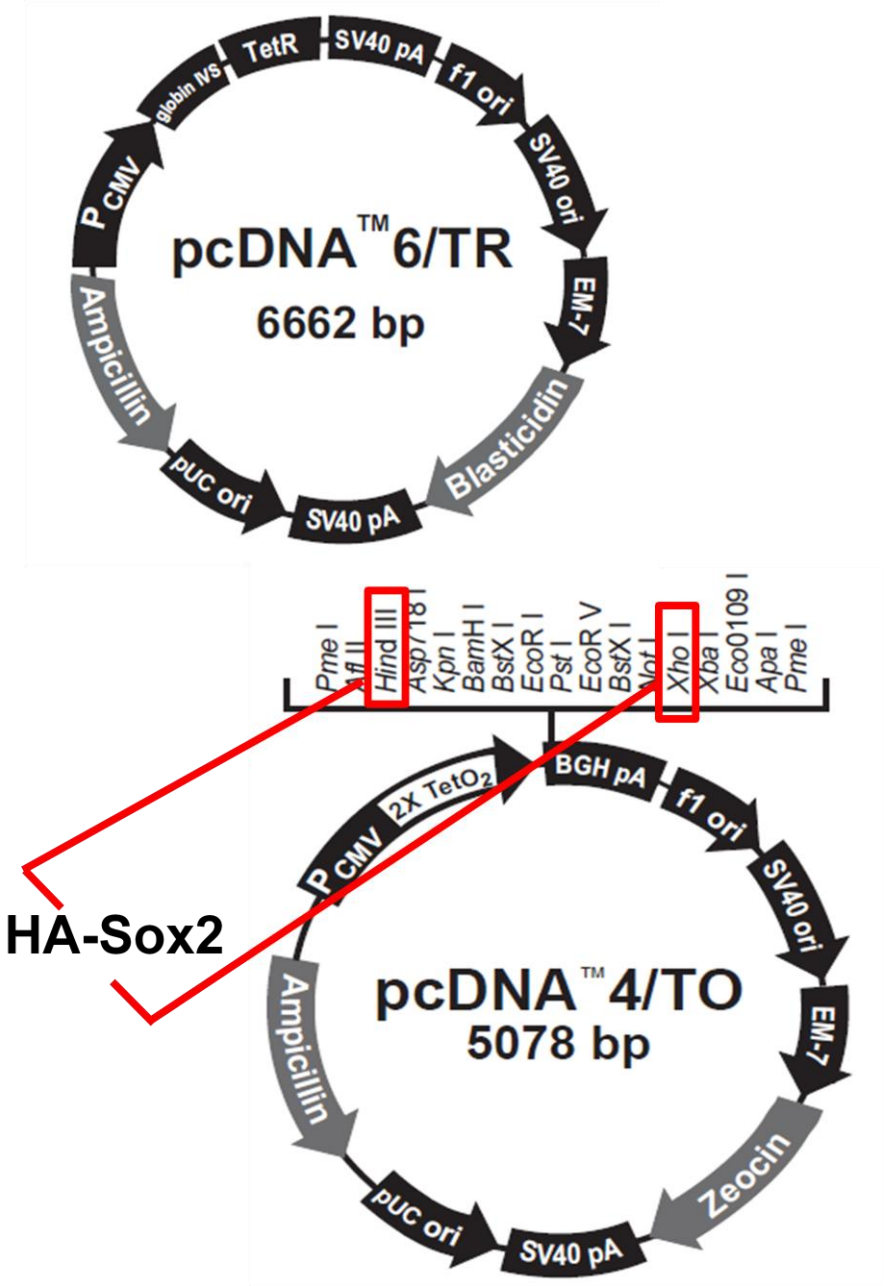


Fig. 28: Inducible system constitutive plasmids

pcDNA6/TR codes for Tet repressor, that binds the 2xTetO2 region downstream the CMV promoter in the inducible pcDNA4/TO plasmid. HA/Sox2 cds is inserted between HindIII and XhoI restriction enzyme sites (CMV-TO-HA/Sox2).

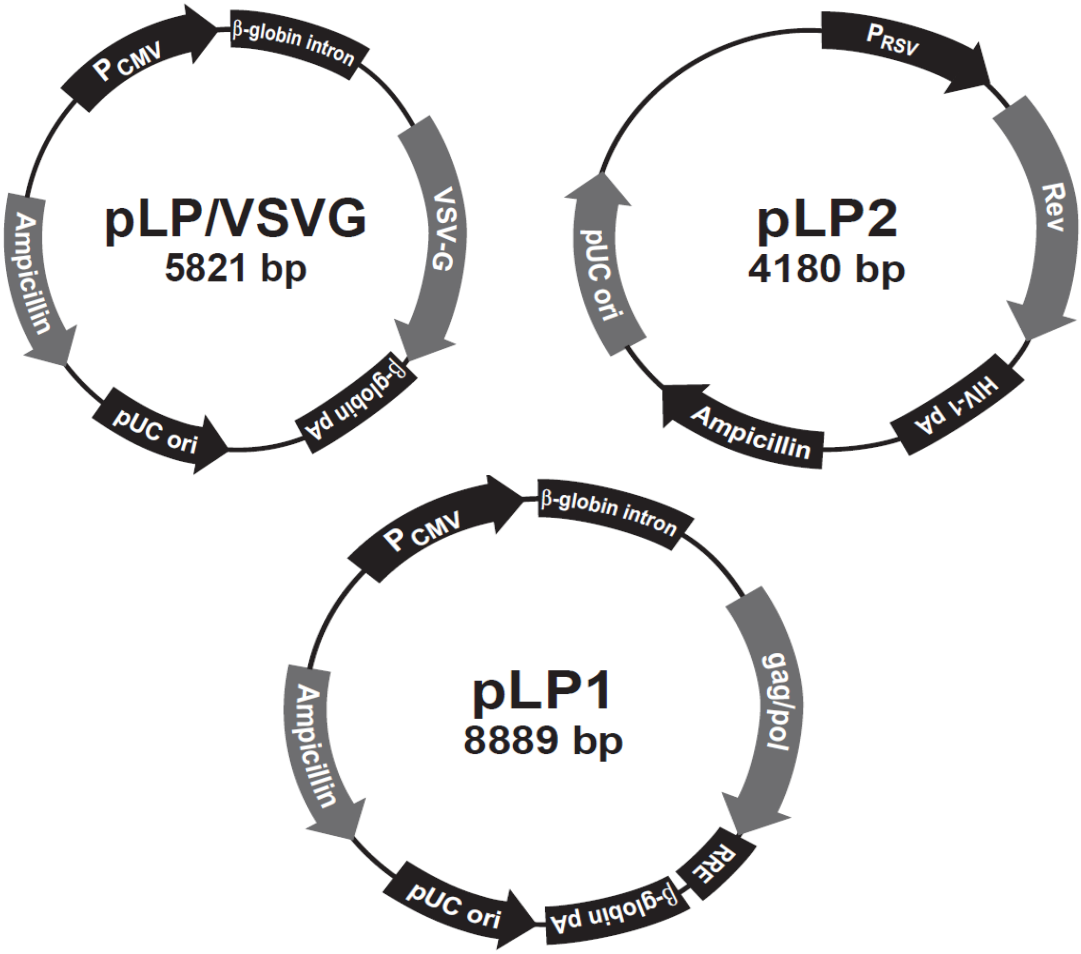


Fig. 29: Helper plasmids for lentivirus production.

pLP/VSVG carries envelop proteins, pLP2 the reverse transcriptase and pLP1 the gag/pol genes for Lentiviral functional assembly in packaging cells. They were cotransfected with FUCRW vector, in which WT Sox2 and K247R Sox2 coding sequences were cloned, to produce functional lentiviruses.

PRIMERS.

The following table 4 collects all oligonuclotide probes we used for (q)RT-PCR or Real Time PCR (A) and for cloning, sequencing and site- directed mutagenesis (B).

Oligonucleotide name	Oligonucleotide sequence 5'-3'
mSox2cds DIR	GGAGTGGAAACTTTTGTC
mSox2cds REV	GGAAGCGTGTACTTATCC
mβCatenin DIR	GAAGCGGCTTTCAGTCGAGC
mβCatenin REV	TCAGGCAGCCCATCAACTGG
mMtm1 DIR	GTCATACTCAGGTTGAAGGAA
mMtm1 REV	GCCATAGCTCATCCATTCA
mGng2-ps1 DIR	CTCAATCCTCTTCCACATAG
mGng2-ps1 REV	ATGCTGGTGACAATGATG
mFam110c DIR	GCATCTGGAGGACTGAAC
mFam110c REV	GTGTGACTGGTGGCTCTA
mCd200 DIR	GTTCTTAGGTCCGCCAGTG
mCd200 REV	TTACAGTTGCCAATGTTACTTCAC
mIl6 DIR	ACAAAGCCAGAGTCCTTCAGAGAG
mIl6 REV	TGGATGGTCTTGGTCCTTAGCC
Vcam1 FWD	GGTCCAAGTCCGTTCTGACC
Vcam1 REV	GCCAAACACTTGACCGTGAC
Tnfrsf12a FWD	GCTTCTTGTCCAGCGCGA
Tnfrsf12a REV	CAGTCTCCTCTATGGGGGTAGT
Parm1 FWD	ATCGCGAGCCCTCTTTTGTT
Parm1 REV	CCCACCTCATGGTATCTGGC

Tab . 4 A: Primers used in RealTime PCR and semiquantitative RT-PCR.

Oligonucleotide Name	Oligonucleotide sequence 5'-3'
K44R DIR	CGTCAGGAGGCCCATGAACGCCTTC
K44R REV	GGCCTCCTGACGCGGTCCGGGCTG
K89R DIR	ACCGAGAGGCGGCOGTTCATCGACGAG
K89R REV	CGGCCGCCTCTCGGTCTCGGACAAAA
K123R DIR	CTCATGAGGAAGGATAAGTACACGCTTCC
K123R REV	ATCCTTCCTCATGAGCGTCTTGGTTT
K247R DIR	GGTCAGGTCCGAGGCCAGCTCCAGC
K247R REV	CGGACCTGACCACAGAGCCCATGGAGCC
BamSox2 DIR	CGGGATCCTATAACATGATGGAGACGGAGC
EcoSox2 REV	GGAATTCTCACATGTGCGACAGG
Sp6	GATTTAGGTGACACTATAGAATA
pRK7_REV	GGACAAACCACAACTAGAATGC
Mus-2000NF2 DIR	CAAGGACAGCAAAACAAGACACCCCTC
Mus+537NF2 REV	CTAGGCCCGCGTTCTGGGTCTCTTAG
NheI-NF2 DIR	CTAGCTAGCCACAGGACCTATCTG
BglII-NF2 REV	GAAGATCTCACCTTTAATCCTCAG
EcoRI Sox2cds DIR	GGAATTCATGTATAACATGATGGAGACG
EcoRI Sox2cds REV	GGAATTCTCACATGTGCGACAG
XbaI Sox2cds DIR	GCTCTAGAATGTATAACATGATGGAGACG
XbaI Sox2cds REV	GCTCTAGATCACATGTGCGACAG

Tab . 4 B: Primers used for plasmid and lentivirus cloning and site specific mutagenesis.

BIOINFORMATIC ANALYSIS.

We used a computational approach to search for putative Sox2 Sumoylation sites, to scan promoter elements on clusters of co-regulated genes and to try functional annotation of interesting genes from our expression analysis.

In order to search for new Sumoylation sites in Sox2 we analyzed the Sox2 protein sequence (NP_035573.3) by using three different softwares i.e., SUMOplot (http://www.abgent.com/doc/Sumoplot/), SUMOsp 2.0 (http://Sumosp.biocuckoo.org; (Xue et al., 2006), SUMOfinder (http://cbg.garvan.unsw.edu.au/Sumofi). The SUMOplot score system is based on two criteria: direct amino acid match to the SUMO consensus site observed and shown to bind Ubc9 and substitution of the consensus amino acid residues with amino acid residues exhibiting similar hydrophobicity. SUMOsp 2.0 predicts out canonical and not canonical SUMO consensus sites, adding a database from literature of 279 verified non redundant Sumoylation sites from 177 different proteins. SUMOfinder software complete the search of putative SUMO sites focusing on PDSM (Hietakangas et al., 2006) and NDSM (Yang et al., 2006) in the protein of interest. All three programs performed with default or high stringency score and cut-off threshold parameters.

PSCAN (http://www.beaconlab.it/pscan) (Zambelli et al., 2009) is a useful free online software for a preliminary scan of core promoter regions between -950 nt and +50 nt far from transcriptional starting site (TSS) in a set of co-regulated genes. The bioinformatic tool lists over-represented or under-represented DNA binding motifs of transcription factors that could be common regulators of the genes of interest, combining ChIP-seq data, TRANSFAC and JASPAR databases information. We performed a first analysis to discriminate gene promoters containing from not containing the Sox2 putative binding site; then we tried to focus on common putative transcription factors with a high score for binding in the same 1000 bp region along with Sox2.

SITE-DIRECTED MUTAGENESIS BY PCR.

Lysines potentially bound by SUMO polypeptides have been changed into arginines, amino acids with the same chemical properties as Lysine. The protocol is based on two-step PCR (Fig. 30). The table 4B shows the primers used in the PCR reactions, while in Fig. a theoretic scheme for the reaction is reported.

In first step PCR reactions we used as a template the pRK7-HASox2 plasmid. For amplification was used the following reaction mixture. PCR products of the first step PCR , molecules of double-stranded DNA of a variable number of pairs of bases starting with the 5 ' or 3 ' of the sequence of the wild type protein and terminate at the oligo sequence mutated K DIR or K REV respectively.

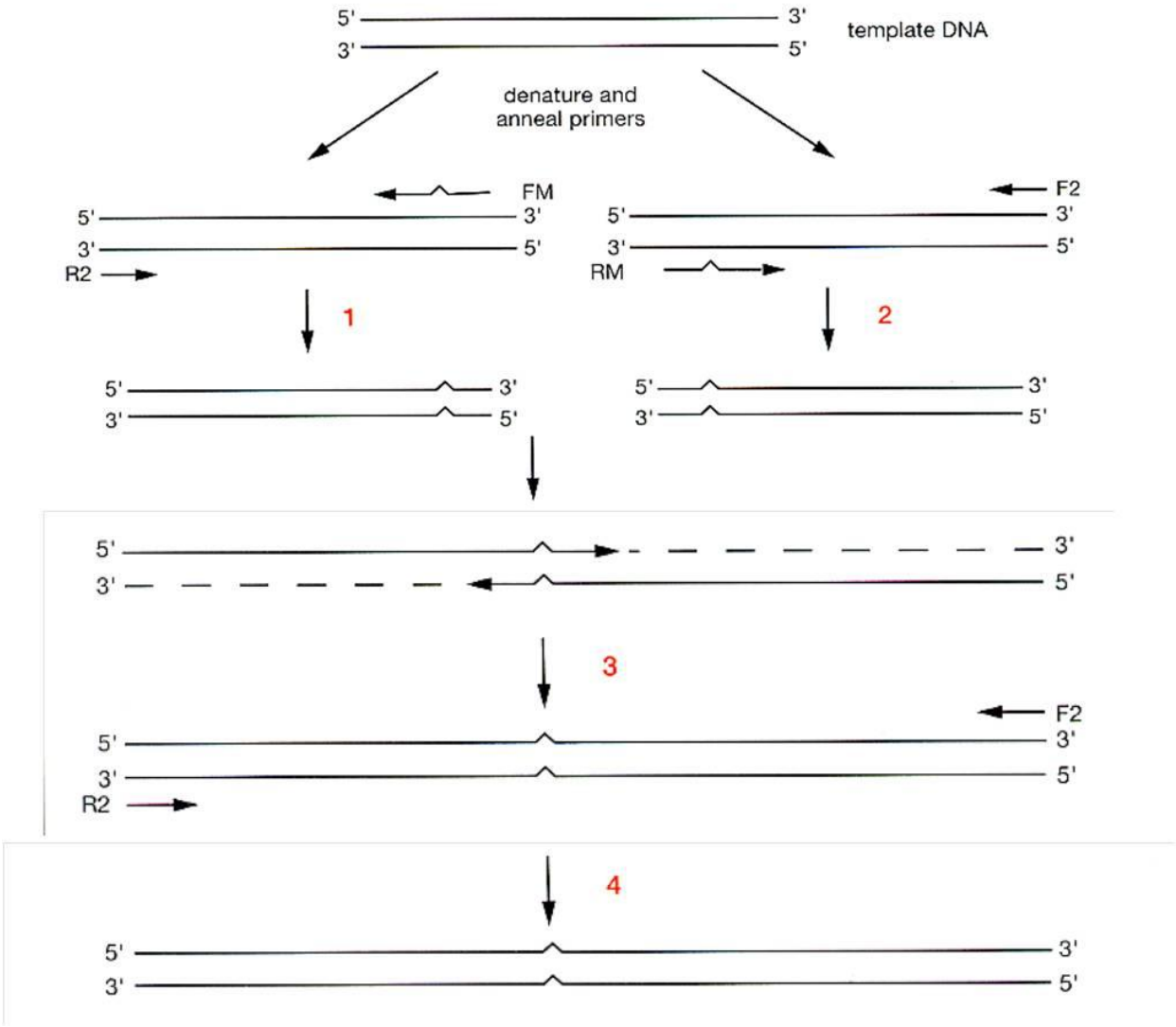


Fig. 30: Site-directed mutagenesis. The first step provides for two independent PCR reactions (1 and 2) , in which , respectively , the primer K DIR is associated with a primer REV (R2 in the figure) that anneals to the 3 'end of the template DNA and the primer K REV and ' associated with a primer DIR (F2 in the figure) to the 5 ' of the mold. The product of the first step (3) is a molecule of double-stranded DNA of a number of base pairs variable that starts with the 5 ' or 3 ' of the sequence of the wild type protein and ends at the mutated sequence of the oligo , DIR K REV K or respectively . The second step involves the combination of the two products resulting from each of two primers constructed for each mutant . These are appaieranno in correspondence of the region that contains the triplet mutated and the extension of the entire sequence proceeds the 3' -OH of each strand paired . After a few cycles is possible to amplify the entire cDNA of Sox2 with the primers 3 ' and 5 ' united previously (4) . In our experiments the DIR and REV primers that flank the cDNA sequence of Sox2 on the vector used as a template in PCR , respectively , Sp6 and pRK7_Rev .

DNA SUB-CLONING BY PCR AND ENZYMATIC DIGESTION.

Vector preparation for both Lentivirus or Transient Mammalian Expression plasmids relies on:

Digestion of at least 5 µg of Vector with the proper Restriction Enzyme (NEB or Promega) 37°C in high enzyme eccess or O/N; add Bovine Serum Albumin (BSA, Promega) and set reaction volume up to 40-50 µl.

Gel extraction: remove enzyme and its buffer without previous inactivation; remove any residual not digested vector; note that you may have only half of starting vector amount at this point! Used the Gel Extraction Kit (QIAGEN) by manufacturer protocol.

De-phosphorylation: use Antarctic Phosphatase (NEB) following the protocol suggested by manufacturer (at least 30-45 min reaction at 37°C). Heat inactivation is not required because of next step.

Enzyme and buffer removal by QIAquick PCR Purification Kit (QIAGEN): we need to clean as much as possible from Zn^{2+} ions useful for Phosphatase but not for following Ligase reaction and have your vector clean and resuspended in H_2O.

Ligation and Transformation of proper bacterial strain to control complete de-phosphorylation (with DNA Ligase) and complete digestion (without DNA Ligase) of orginal un-digested plasmid

The Insert preparation occurs by following general steps:

PCR to amplify cds of interest. Use a high fidelity Taq Polymerase, such as AccuPrime Pfx DNA Polymerase (Invitrogen). Primers with proper "extra"-nucleotide(s) for restriction enzyme site(s) 5'-flanking cds. Provide 5'-primer of Kozak consensus (if not in vector or not in ATG codon rich beginning cds). For site specific mutagenesis the required steps are detailed above and in Fig.

Gel extraction by QIAquick Kit (QIAGEN) to remove all PCR reagents and template

DIGESTION with proper Restriction Enzyme(s) (NEB or Promega)

Enzyme(s) and buffer removal by QIAquick PCR Purification Kit (QIAGEN), so Insert(s) is (are) clean and resuspended in H_2O for following Ligation

Ligation and transformation to control insert for complete removal of original template plasmid, with or without DNA Ligase.

Then we proceed to Vector and Insert(s) DNA agarose gel running or NanoDrop to check amount and purity before DNA Ligation, by the protocol provided by Promega. The next day we set up Bacterial Transformation by heat shock protocol of the proper strain based on our plasmid

type: One Shot Stb3 (Invitrogen) Chemically competent are used for virus preparation; DH5α competent for all the other plasmids. We used a complex medium, the Luria - Bertani (LB; Tryptone 5 g, Yeast extract 2.5 g, NaCl 5 g, Water to final volume of 500 ml) and we added 7.5 g of Bacto-Agar to prepare the bacterial culture plates. The selection of transformant colonies occurs with 100 µg/ml ampicillin.

Colony screening by PCR or/ and by minipreps: it is simple and rapid method to verify the presence of an insert in the plasmid from the all incubated colonies. We pick up a suitable number of for each mutant of Sox2 (in this case) and for colonies as a control into l0 µl H_2O each . After 5 minuntes of cell lysis at 100 ° C, we centrifuged and harvested 1/10 or 1/5 of Volume to be used in PCR screen.

When we find positive colonies, we perform a small scale plasmid DNA extraction (Qiagen MiniPrep kit), so we can quantify the DNA and further verify the sequence by the Big Dye Terminator Sequencing VI.I (AB PRISM) kit, with the recommended protocol. The reading of precipitated DNA is up to a facility Sequencer 3730 with 48 capillaries of Applied Biosystem .

At this point we can use a Plasmid Maxi kit (QIAGEN) to extract a significant amount of plasmid DNA for in vivo Sumoylation assay in HeLa cells.

TRANSFECTION INTO EUKARYOTIC CELLS.

Transfection is a technique that allows to introduce molecules of exogenous DNA within eukaryotic cells. For our experiments we performed all transfections by Lipofectamine 2000 reagent (Life Technologies), except for the Calcium/ Phosphate method used for packaging 293T cells (see Making lentivirus protocol).

Briefly, Lipofectamine 2000 protocol, according to manufacturer suggestions, require cells at least 70 % - 80 % confluent the day of transfection (plate them properly the day before, like 300-400.000 HeLa cells / well in a 12-well plate). Before transfection we have to replace normal culture medium with one NOT containing any antibiotics: these molecules would disrupt lipofectamine structure. We then prepare up to 1.5 µg of total DNA/Well of the hypothetical 12-well plate, diluting plasmids in 100 µl Opti-MEM: be careful to use the same amount of DNA among different transfections and the same ratio among strong or weaker transfected promoters. After 5 min RT incubation, we can add a previously prepared mix of 2-3 µl Lipofectamine 2000/well diluted in final 100 µl Opti-MEM. Gently mix and incubate 30 minutes at RT. Finally add drop-wise the transfection solution on the cells. Incubate the transfected cells at 37 ° C for

24-48 hours and then do the planned experiment: selection for stable cell lines or transgene expression analysis.
Protocol of transfections is detailed in Results section.

TOTAL PROTEIN EXTRACTION, SDS-PAGE, WESTERN BLOT.

The analysis of total protein extracts from cells transfected consists of four main steps: sample preparation; SDS-PAGE (Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis); Transfer of proteins on nitrocellulose or PVDF filter (Western boltting) ; immunodetection .
Total proteins extraction requires, after removal of the culture medium and two washes with 1x PBS, lysis in RIPA Buffer (150 mM NaCl, NaDoc 0.5 % , 1 % NP40 , 0.1 % SDS , Tris-HCl pH 7.5 50 mM , Protein Inhbitor 1x cocktail , 1 mM PMSF, 1x Phosphatase Inhibitors 2 and 3 SIGMA, 10 mM NEM for Sumoylation assays). Cell disruption is obtained by sonication and ice incubation. The concentration of total protein was determined with the Protein Assay Reagent (BioRad), compared with a standard curve based on increasing amount of 1mg/ml BSA.
Electrophoresis on 8% - 12% polyacrylamide gel in the presence of sodium dodecyl sulfate allows the separation of proteins, surrounded by the same negative charge, only according to their molecular weight. The electrophoretic migration is carried out at RT, 30mA constant.
The proteins were transferred " in a sandwich" on a nitrocellulose or a PVDF filter by the application of a constant electric field at 30 V overnight at 4 ° C or 2h at 100 V.
After staining filters with Ponceau Red (0.5% Ponceau S , 1 % glacial acetic acid) for 1 minute at RT, we blocked the nonspecific binding sites, by incubation for one hour at room temperature in 1x TBS (Tris buffered saline) , 5 % milk , 0.2 % Tween20 .
After three washes in fast 1x TBS + 0.2 % Tween20 , the filter was incubated for 1h at RT or overnight at 4°C with primary antibody diluted in 1x TBS , 3 % BSA , 0.02% Sodium Azide .
We used a polyclonal antibody against Sox2 , produced in rabbit (AB5603 , Millipore) diluted 1:1500 ; a monoclonal antibody against the HA epitope , produced in mouse (Santa Cruz, sc-7392) diluted 1:4000; monoclonal antiGAPDH (Santa Cruz, sc-25778) used 1:10000; polyclonal Merlin antibody #9186 (Cell Signaling); anti SMAD1 monoclonal rabbit antibody #6944 (Cell Signaling); anti pSMAD1/5 rabbit monoclonal antibody #9516 (Cell Signaling); anti Sox2 polyclonal antibody #2748 (Cell Signaling).

After 3 other washes the filter was incubated at room temperature for 45 minutes in secondary antibody conjugated to HRP (horse radish peroxidase) antibodies against rabbit or mouse (BioRad), depending on the used primary , diluted 1:5000 in both cases in 1x TBS and 0, 3 % Tween20.

After a quick wash in 1x TBS + 0.3% Tween20 and three others for 15 minutes each, the signal was detected with HRP reagent ECL or ECL Prime (GE Healthcare), according to the protocol provided by the manufacturer. The results were imprinted on autoradiographic for different exposure times.

THE T-REX INDUCIBLE CELL SYSTEM.

The strategy to get specific protein induction consists of two main elements. The first plasmid has a CMV promoter driven tandem couple of Tetracycline Operators (Tet Operator, $TetO_2$): they are two 19 nucleotide long sequences, with a 2 nucleotide spacer, each harboring two binding sites for Tet repressor molecule; a multiple cloning site (MCS) region downstream the $TetO_2$ makes an eventually in frame coding sequence of interest regulated by the presence of Tetracycline or a homologous molecule, such as Doxycycline. The second plasmid is called pcDNA6/TR and it drives constitutive expression of TetR gene (Tetracycline Repressor gene) through the CMV strong promoter. The absence of Tetracycline or Doxycycline, allows TetR expression, dimerization and high affinity binding to $TetO_2$ and, as a consequence, the inhibition of transcription of the coding sequence of interest. Tetracycline or Doxycycline added in cell culture medium triggers conformational changes in TetR molecule and dissociation from $TetO_2$ region; so it starts expression of gene of interest.

The molecule of election for the experiments is Doxycycline, that belongs to the same family of Tetracycline antibiotics, but it has much longer half-life span. Isolation of OB1 clones requires:

- Lipofectamine 2000 transfection of pcDNA6-TR plasmid;
- Selection of transfected cells by adding blasticidin antibiotic to cell culture medium
- Clonal expansion of 10 clones stably expressing TetR plasmid to be tested the inducible expression of increasing amount (40 ng, 400 ng and 4 µg) of a control gene, the luciferase, carried by pcDNA-TO-Luc plasmid.
- In parallel the inducible clones are tested for osteogenic differentiation.

- The better luciferase induced and more differentiated clones undergo to a new Lipofectamine transfection for the CMV-TO-HA/Sox2 plasmid and subsequent selection by zeocyn.
- The We finally select properties for our inducible Sox2 system and check for their eventual alteration in known biological effects because they express a HA/Sox2 fusion protein.

cDNA MICROARRAY EXPERIMENT ON INDUCIBLE SYSTEM.

We performed three replicate experiments for each set of conditions: we treated with 0.5 µg/ml doxycycline OB1TR#4HA/Sox2#1 cells for 6h, 12h, 24h, 48h. Then we obtained cDNA from samples in triplicate of each time point and performed cDNA Microarray hybridization following Affymetrix protocol. From row data, the preliminary statistical analysis removed experimental points and chips with intrinsic random high variability, reasonably not a result of gene expression change. To be noticed one set of untreated samples at 6h we had not to consider at all, so their induced values data are normalized to untreated 12h for both doxycicline cells after 6h or 12h of experiment. Then the identification of differentially expressed genes is based on the following criteria:

- Absolute reading of fluorescence from hybridized Affymetrix chips must be higher than 70, meaning the gene of interest is expressed in osteoblasts at a quite high detectable level;
- Statistical significative variability of gene expression among the triplicate samples and inside single repeats of each triplicate;
- More than 1.5 Fold change in expression of the gene of interest, measured as ratio between Sox2 induction after a time of doxycycline treatment and basal endogenous expression level of Sox2 in cells untreated for the same time.

RNA EXPRESSION ANALYSIS.

Actually Sox2 induction is proofed at mRNA level in subsequent experiment of Microarray validation through Real-Time RT-PCR on cDNA obtained from purified total RNA, extracted from cultured OB1TR#4HA/Sox2#1 cells grown in 0.1 µg/ml Doxycycline for 0, 12, 24 and 48 hours. Fold change of mRNA level is ratio between mRNA levels in Doxycycline treated vs untreated cells, compared to housekeeping mRNA level of β-catenin gene (Mansukhani et al.,

2005), following the ΔΔCt2 method (Pfaffl, 2001). RNA extraction was performed by TRIZOL reagent (Invitrogen). RNA purification followed the QIAGEN protocol of RNeasy Kit. The RT-PCR was performed using the Super ScriptIII First strand cDNA Synthesis System (Invitrogen), according to manufacturer protocol for random hexamer oligonucleotides. Real Time PCR is performed by SYBRGreen SUperMIx protocol (BioRad) on an iCycler iQ5 (BioRad).
A similar experiment was performed starting from cultured primary osteoblasts, following Cre-GFP adenovirus infection; they are immortalized primary osteoblast cell line from conditional Knock Out Sox2 $^{flox/-}$ or Sox2 $^{flox/flox}$ murine calvaria (Basu-Roy et al., 2010). Indeed in gene expression profile study of the Cre- infected Sox2 $^{flox/-}$ or Sox2 $^{flox/flox}$ cells (Seo et al., 2011).

LUCIFERASE ASSAYS.

All transcriptional assays in the Thesis are performed with a Promega Dual Luciferase Assay System or Luciferase Assay System, following the suggested protocol by the manufacturer.
In HeLa and 293T cells two independent experiments of transient transfection were performed in duplicate each; firefly luciferase data were normalized by protein amount and by cotransfected renilla luciferase plasmid activity.
For C3H10T1/2-BRE cells infected by lentiviruses two independent experiments were conducted in triplicate each; normalization of relative luciferase activity occurred on protein amount.

MAKING LENTIVIRUS PROTOCOL.

Day 0: Plate 6 293FT cell 10-cm plates per virus type, with around 5 x 10^6 cells/plate.
Day 1: The next day, when 293FT cells are 50-70% confluent, transfect with viral plasmids by Calcium Phosphate method:
Plasmid composition**:** (per plate of 293FT cells)

- 6.0 µg of your lentiviral plasmid
- 4.5 µg of PLP1 (*gag/pol*)
- 4.5 µg of PLP2 (*Rev*)
- 3.0 µg of VSVG (*capsid protein*).

Making virus preparation mixture: (per plate of 293FT cells)

- Sol. A: plasmid + water = 450 µl (or = 376 µl)
  2.5 M $CaCl_2$ = 50 µl (or 1M $CaCl_2$ = 124 µl)
- Sol. B: 2x HBS = 500 µl

Viral transduction:

a) Mix Sol. A to Sol. B and incubate 5 min at RT
b) While incubating the solution, aspirate medium from 293FT cell plates and add 9 ml of fresh medium to each plate
c) Add drop by drop 1 ml of mixture A+B to each plate and incubate O/N.

Day 2: Check on-going viral infection by Fluorescence microscope. Aspirate medium from 293FT cell plates, after incubation. Add 5-6 ml/plate of fresh medium and incubate O/N.

Day 3: Collect medium containing produced virus from each plate into a 50ml conical tube; carefully add 5-6 ml/plate of fresh medium to each 293FT cell plate and incubate O/N.Centrifuge the tubes 2000 rpm 10 min at RT to purify the virus. Transfer supernatant to a new 50ml conical tube and store at 4°C O/N.

Day 4: Collect and centrifuge the 293FT medium containing virus (see steps 5-7): combine the newly purified medium with the same one collected on Day 3. Filter the combined medium under sterile hood to further purify virus: use a Syringe and a 0.45 µm Low Protein Binding Filter to push 1 virus containing medium into 1 sterile glass bottle. Transfer filtered viral solution to ultracentrifuge tube (max vol = 35 ml). Weigh samples inside their steel containers to obtain balance by adding culture medium to the max tube weight. Ultracentrifuge the virus at 19400 rpm for 2 h at RT on a SW28 swingout rotor using appropriate Beckman tubes. Go back under sterile hood and carefully remove the ultracentrifuge tubes from the steel containers and discard the supernatant. Let tubes bottom-to-top turned on a paper towel to quickly dry pellet. Add into each virus ultracentrifuge tube 150 µl sterile 1X HBSS (Invitrogen): 5 min incubation RT, then resuspend by gently pipetting. Aliquot virus 50 µl / 1.5ml eppendorf tube and store at -80°C.

CHIP-PCR FOR VCAM1, PARM1 AND TNFRSF12A IN OB1 CELLS UPON SOX2 INFECTION.

A ChIP-Seq experiment specifically performed in OB1 cells (Basu Roy, personal communication). Briefly, 10^7 immortalized osteoprogenitors OB1 or OB1 cells transduced with a Sox2 lentivirus were cross-linked with 1% formaldehyde and quenched by 1M glycine. Cells were lysed in lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-Hcl (pH8.0), protease inhibitors) followed by sonication with a Misonix 3000 Sonicator to shear chromatin. Thirty mg of chromatin fragments were immunoprecipitated with 5ug of Sox2 antibody purchased from R&D Systems (AF2018) or IgG control overnight. The next day, protein G-agarose beads were added to the chromatin – antibody solution. Elution and reverse-crosslinking were performed as described in (Seo et al., 2011). Construction of DNA library for ChIP-Seq and GA-IIX Illumina sequencing were performed at Genome Technology Center at the NYU School of Medicine. Each sequencing experiment yielded 40 million raw reads 3nt long; the reads were mapped onto the mouse genome UCSC mm9 using bwa, obtaining 23-25 million high-confidence mapped reads. High-resolution genome-wide maps were derived and visualized in UCSC Genome Browser. Finally, the Macs Peak Calling package software was used to identify Sox2 enriched regions. Sequencing reads from IgG control were used as a negative control. OB1 cells were infected with a FUCRW lentivirus carrying Sox2 WT coding sequence and conducted immunoprecipitation of chromatin for Sox2 antibody against IgG antibody as a negative control, according to a previous protocol (Nelson et al., 2006). Data analysis reports 2416 Sox2 peaks above 3.9 Fold enrichment of binding compared to the ones resulting from OB1 cells transduced with empty FUCRW lentiviral vector; the enriched peaks rise in correspondence of promoter region of 2010 different genes in mouse osteoblasts.

PCR for Vcam1, Parm1 and Tnfrsf12a has been conducted as positive control on Input chromatin fragments not immunoprecipitated; no amplification signals in IgG ChIP samples works out as experimental negative control.

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