

VIMENTIN, A NOVEL REGULATOR OF THE TRANSCRIPTION FACTOR ATF4 AND
OSTEOBLAST DIFFERENTIATION

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To my parents

And

To my husband and daughter

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CHAPTER I

INTRODUCTION

The Skeleton

The functions of bones range from providing mechanical support to soft tissues to maintaining mineral homeostasis. These functions are accomplished by continuous tissue renewal, or bone remodeling, which involves the removal of old or damaged bone by osteoclasts (bone resorption) and the subsequent replacement of new bone formed by osteoblasts (bone formation). In normal bone remodeling, a balance between bone resorption and formation is tightly regulated to ensure a relatively constant bone mass and mechanical strength in mature healthy bone. Nonetheless, under certain pathological conditions an imbalance between bone resorption and formation may occur, leading to the development of bone disorders. One common disorder of bone remodeling, osteoporosis, is characterized by low bone mass; it causes bone fragility and an increased fracture risk (2001). At present, most treatments for osteoporosis are symptomatic in the form of inhibiting bone resorption, rather than stimulating bone

formation, due to the limited availability of anabolic approaches (Karsenty 2003). Further understanding of osteoblast differentiation may shed light on new and more potent treatments of osteoporosis.

Osteoblast Differentiation

Osteoblasts are the bone forming cells that originate from mesenchymal stem cells, the pluripotent cells residing in the bone marrow, muscles, and fat (Caplan and Bruder 2001; Jiang, Jahagirdar et al. 2002). The differentiation of osteoblasts is an adaptive process, modulated by mechanical loads, circulating calcium level, sex steroids, Bone Morphogenetic Proteins (BMPs), Parathyroid hormone (PTH), Transforming Growth Factor beta (TGF β) and other factors (Harada and Rodan 2003). These extracellular signals are integrated by intracellular signaling molecules and transcription factors, which are subsequently turned on or off in a tissue-specific manner. They selectively transactivate osteoblast related and/or specific genes, triggering and maintaining the osteoblast differentiation process. The differentiation program can be divided into several stages, including proliferation, extracellular matrix deposition, and mineralization.

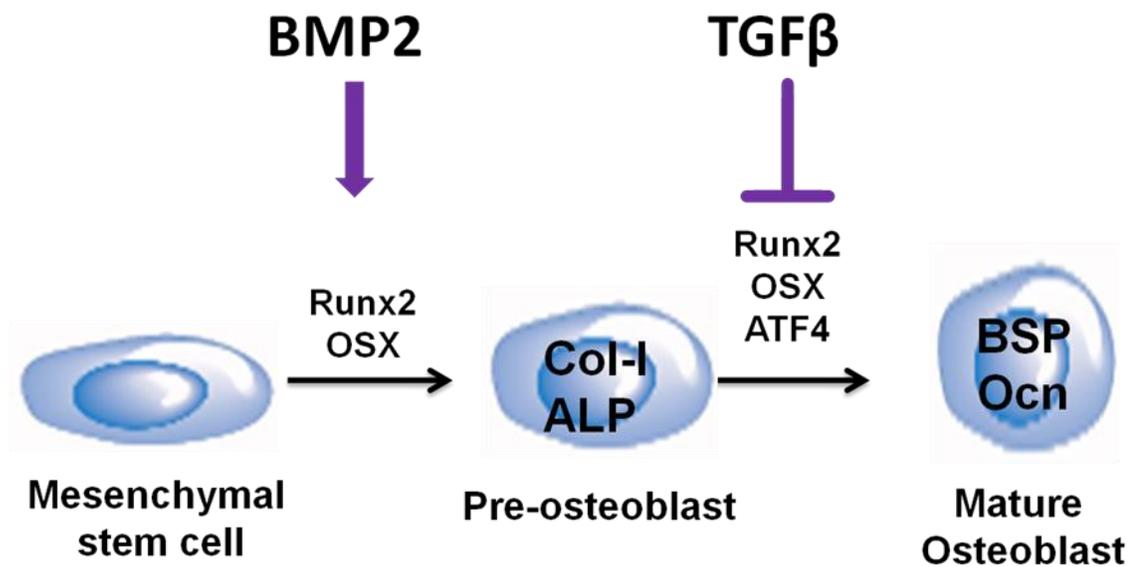


Figure 1. Osteoblast differentiation. Osteoblasts arise from pluripotent mesenchymal stem cells under the control of transcription factors including Runx2, Osterix (OSX), and activating transcription factor 4 (ATF4). Along differentiation, markers genes such as Collagen type I (Col-I) and alkaline phosphatase (ALP) in early osteoblasts and bone sialoprotein (BSP) and osteocalcin (Ocn) in mature osteoblasts will be gradually turned on. Osteoblastogenesis is regulated by several extracellular signals including bone morphogenic protein 2 (BMP2) and transforming growth factor β (TGF β).

During the early stages, alkaline phosphatase (ALP) and type I collagen (Col1 α 1) are first expressed, followed by the expression of late, and more specific, osteoblast genes such as bone sialoprotein (BSP), osteopontin (OPN) and osteocalcin (OCN). The expression levels of these genes are frequently used as indicators for the level of differentiation and activity of osteoblasts. After maturation, some osteoblasts become osteocytes (Dallas and Bonewald 2010), whereas some become lining cells and some undergo apoptosis (Manolagas 2000). In order to maintain the bone homeostasis, this differentiation program is intimately coupled to local areas of bone resorption and therefore replenishes osteoblasts and bone throughout life.

Transcriptional Control of Osteoblast Differentiation

Multiple transcription factors contribute to the regulation of osteoblast differentiation and function. To date, three such factors function in an osteoblast-predominant manner: Runx2/Cbfa1 (Ducy, Zhang et al. 1997), Osterix (Osx) (Nakashima, Zhou et al. 2002), and Activating Transcription Factor 4 (ATF4)(Yang and Karsenty 2004; Yang, Matsuda et al. 2004).

Runx2 is a member of the Runt domain family of transcription factors. It is known to be the earliest and most powerful molecular determinant of osteoblast differentiation (Karsenty 2003). Runx2 is involved in the transcriptional activation of many promoters including those of *Col1a1* and *Osteocalcin* (Banerjee, McCabe et al. 1997; Kern, Shen et al. 2001). Remarkably, Runx2-deficient mice have a cartilaginous skeleton without any osteoblasts (Komori, Yagi et al. 1997). Moreover, Runx2 is sufficient to induce osteoblast differentiation, and forced expression of Runx2 in skin fibroblasts leads to ectopic expression of *osteocalcin*, an osteoblast-specific gene. Similarly, the overexpression of Runx2 in odontoblasts induces their transdifferentiation into osteoblasts, forming a bone structure (Miyazaki, Kanatani et al. 2008). However, it is notable that overexpression of Runx2 under the control of a 2.3-kb mouse *Col1a1* promoter, which directs gene expression to immature and mature osteoblasts, does not increase bone mass, but rather induces osteopenia with multiple fractures. This phenotype is at least partially attributable to Runx2's inhibition of osteoblast maturation and function, as demonstrated by the severe reduction of osteocalcin expression from both the bones and calvarial osteoblasts of the Runx2-overexpressing mice, and diminished number of terminally differentiated osteoblasts as well as osteocytes (Liu, Toyosawa et al. 2001;

Geoffroy, Kneissel et al. 2002; Kanatani, Fujita et al. 2006). Thus, Runx2 is required to direct multipotent mesenchymal cells to the osteoblast lineage and trigger the expression of major bone matrix proteins, leading to an increase in immature osteoblasts. However, Runx2 exerts inhibitory effects on osteoblast maturation.

Osx is a zinc finger-containing transcription factor that is specifically expressed in osteoblasts. It is another transcription factor that is absolutely required for osteoblast differentiation, since inactivation of Osx also results in a complete absence of bone formation. The fact that Runx2 is normally expressed in Osx-null mice and that Osx is not expressed in Runx2-null mice indicates that Osx acts downstream of Runx2 (Nakashima, Zhou et al. 2002).

Activating Transcription Factor 4

Activating transcription factor 4 (ATF4/CREB2) belongs to the ATF/CREB family of basic region-leucine zipper (bZip) transcription factors, and it activates gene transcription by forming homo/hetero-dimers and subsequently binding to the consensus cAMP responsive element (CRE) binding site (Hai and Curran 1991; Ameri and Harris 2008). The mouse ATF4 has 349 amino acids. It contains two leucine zipper motifs, zipper I

(aa304-339) and zipper II (aa90-125), which mediate interactions within ATF4s or with other partners. It also contains a basic region (aa270-303), responsible for DNA-binding activity (Fig. 2A) (Liang and Hai 1997).

As an osteoblast specific transcription factor, ATF4 is important for osteoblast differentiation, osteogenesis, and terminal bone formation. ATF4-deficient embryos display delayed skeletal development (Fig. 3A) and mice lacking ATF4 develop a severe low-bone-mass phenotype caused by decreased bone formation (Fig. 3B). ATF4's function is at least partially transcriptional, since it binds directly to osteocalcin's promoter at the OSE1 site and transactivate its expression (Fig. 4A & 4B) (Yang, Matsuda et al. 2004). As a result, bones from ATF4-null mice have lower expression of *osteocalcin* (Fig. 4C). Conversely, ectopic expression of ATF4 in non-osteoblastic cells leads to overexpression of this osteoblast-specific gene (Fig. 4D) (Yang and Karsenty 2004), and in vivo, transgenic mice overexpressing ATF4 in osteoblasts display a high-bone-mass phenotype (Fig. 3C) (Elefteriou, Benson et al. 2006).

Although no mutations in ATF4 have been directly reported in human skeletal dysplasia, the skeletal defects of two human genetic diseases, Coffin-Lowry syndrome (caused by inactivating mutation of *Rsk2* gene) and a genetic disease resulting in cleft

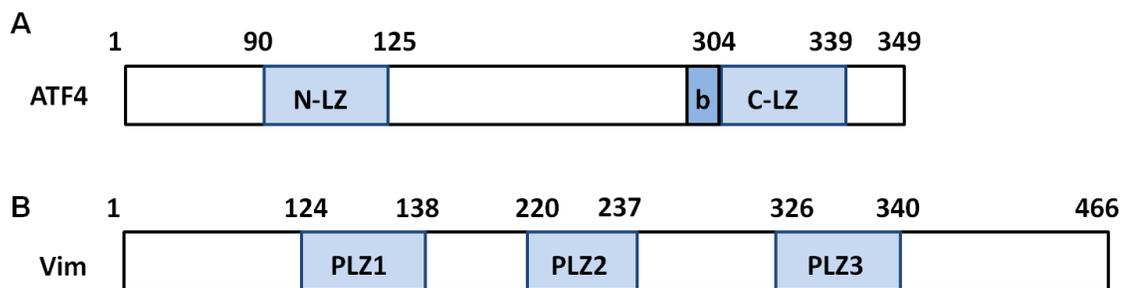


Figure 2. Schematic illustration of ATF4 and Vimentin primary structures. A. Schematic illustration of ATF4 primary structure showing two leucine zipper domains at N and C termini (N-LZ and C-LZ) . Letter b represents basic amino acid-rich DNA binding domain. **B.** Schematic presentation of vimentin primary structure showing three putative leucine zippers (PLZ1-3) of vimentin.

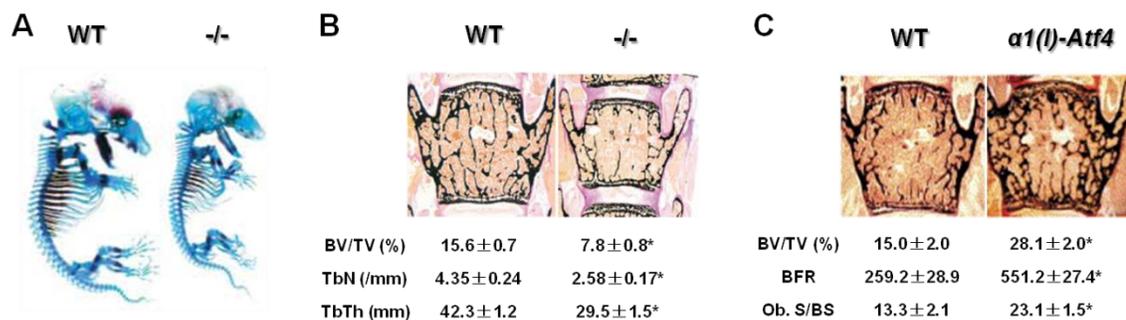


Figure 3. Skeletal phenotypes in the absence of ATF4 or when ATF4 is overexpressed in osteoblasts. A. Alcian blue/alizarin red staining of E14 wt and *Aff4*-deficient skeletons showing delayed skeletal development in absence of ATF4. **B.** Histomorphometric analysis of two-month-old wt and *Aff4*-deficient vertebrae, demonstrating the decrease in bone volume (BV/TV), trabeculae number (TbN), and trabecular thickness (TbTh) in *Aff4*-deficient mice. **C.** Histomorphometric analysis of two-month-old wt and $\alpha 1(I)$ collagen-*Aff4* vertebrae, demonstrating the increase in bone volume (BV/TV), bone formation rate (BFR), and osteoblast surface (Ob.S/BS) in $\alpha 1(I)$ collagen-*Aff4* bones.

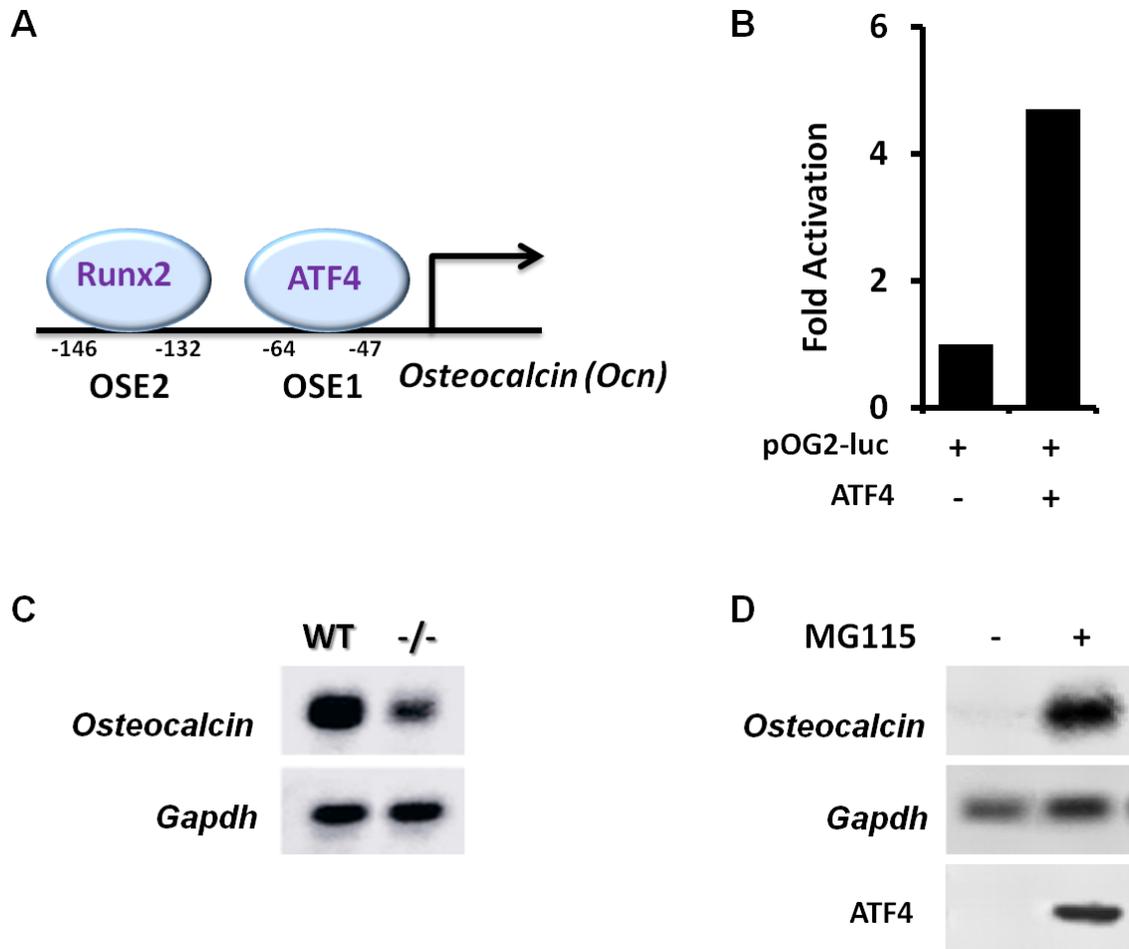


Figure 4. *Osteocalcin* Is a Target Gene of ATF4. **A.** *Osteocalcin* promoter (OG2) showing osteoblast specific element 2 (OSE2, Runx2 binding site) and osteoblast specific element 1 (OSE1, ATF4 binding site) **B.** DNA cotransfection using pOG2-luc reporter and expression vectors of ATF4 in COS cells showing that ATF4 transactivates the *Osteocalcin* gene. **C.** Decreased *Osteocalcin* expression in *Atf4*-deficient bones. **D.** ATF4 accumulation by proteasome inhibitor MG115 induces endogenous *Osteocalcin* expression in NIH3T3 non-osteoblasts.

palate and low bone mass (caused by inactivating mutation of *Satb2* gene), appear to be explained by a decrease in ATF4's activity (Yang, Matsuda et al. 2004; Dobreva, Chahrour et al. 2006). In another disease, neurofibromatosis type I, the high-bone-mass phenotype is associated with an increase of ATF4's activity (Elefteriou, Benson et al. 2006). Together with the severe bone phenotype observed in *Atf4*-deficient mice, these studies illustrate the importance of ATF4 in bone biology.

Regulation of ATF4 in the Context of Osteoblast Differentiation

Given the importance of ATF4 during osteoblastogenesis, it is therefore not surprising that it should be regulated through several mechanisms. The expression and function of ATF4 are regulated mainly at a post-transcriptional level. This concept is best demonstrated by the observation that although *Atf4* mRNA is ubiquitously expressed among different cell types and organs, its protein is restricted to osteoblasts, chondrocytes, and the eye (Yang and Karsenty 2004; Wang, Lian et al. 2009). In osteoblasts, this tissue-specific pattern was explained by a selective accumulation of the ATF4 protein, due to the lack of proteosomal degradation. Strikingly, ectopic expression of ATF4 protein in nonosteoblastic cells has been achieved with MG115, a proteasome

inhibitor, and functionally leads to *Osteocalcin* expression in these cells (Yang and Karsenty 2004).

In addition to regulation of the proper abundance of ATF4 protein, its activity has also been shown to be subject to phosphorylation-based activation. As previously described, abnormal activity of ATF4 appears to be the cause of Coffin-Lowry syndrome (CLS). The molecule responsible for CLS, RSK2, is a growth factor-regulated kinase. It interacts with ATF4 directly and phosphorylates mouse ATF4 at serine 251, which subsequently activates ATF4. As a result, phosphorylation of ATF4 at serine 251 is not detectable in Rsk2-deficient cells, and similar to *Atf4*-deficient mice, both Rsk2-deficient and *Rsk2*^{-/-};*Atf4*^{+/-} mice display low bone mass phenotypes (Yang, Matsuda et al. 2004). In addition, RSK2-independent phosphorylation and activation of ATF4 by protein kinase A (PKA) has also been observed at the serine 254 site in osteoblasts, favoring RANKL expression and subsequent osteoclast differentiation. Consequently, in *Nf1*^{-/-} osteoblasts, where PKA activity is higher and ATF4 is more highly phosphorylated at serine 254, a PKA inhibitor H89 blunts the increase in RANKL expression (Eleftheriou, Benson et al. 2006).

Inhibitory factors of ATF4's transactivation ability also exist in osteoblasts. One such factor is Factor Inhibiting ATF4-mediated Transcription (FIAT). This leucine zipper nuclear molecule lacks a basic domain for DNA binding but interacts with ATF4 to repress its transcriptional activity. In vivo, overexpression of FIAT in osteoblasts leads to low *osteocalcin* expression and osteopenia, similar to the phenotype of *Atf4*-deficient mice (Yu, Ambartsoumian et al. 2005). These reports demonstrate that ATF4-mediated transcriptional control of osteoblast activity is a tightly regulated phenomenon and that protein-protein interactions play a critical role in the precise regulation of gene expression in bone-forming cells.

Transforming Growth Factor β

Transforming growth factor β (TGF β) are cytokines that play important roles in several cellular functions such as proliferation, apoptosis, angiogenesis, wound healing, and bone formation (Massague 1998; Janssens, ten Dijke et al. 2005; Korpai and Kang 2010). The TGF β family contains three closely related isoforms in mammals - TGF β 1, TGF β 2, and TGF β 3. TGF β proteins, which are produced as latent dimers, can be activated by protease-mediated cleavage. Bioactive TGF- β then binds to a cell surface

receptor complex consisting of a pair of transmembrane serine/threonine kinases, TGF β receptor type I (T β RI) and TGF β receptor type II (T β RII), resulting in the phosphorylation and activation of T β RI kinase and consequent hierarchical phosphorylation events.

In the canonical pathway, the signal is further propagated through phosphorylation of receptor-regulated Smad (Smad2/3) transcription factors at the SSXS motif in the C-terminal tail by the T β RI kinase (Abdollah, Macias-Silva et al. 1997; Souchelnytskyi, Tamaki et al. 1997). Once phosphorylated, Smad2/3 forms a heterodimeric complex with Smad4 (Co-Smad) and associates with additional DNA binding cofactors to turn on target genes (Massague 1998).

Although the smads are critical mediators in the TGF β signaling pathway, a substantial body of evidence illustrates the existence of additional, smad-independent pathways, known as non-canonical pathways. These pathways include various branches of MAP kinase (MAPK) pathways, Rho-like GTPase signaling pathways, and phosphatidylinositol-3-kinase (PI3K)/AKT pathways (for review, see (Zhang 2009)). PI3K is a heterodimer with a p85 regulatory subunit and p110 catalytic subunit. The p85 subunit has two SH2 domains that bind to phospho-tyrosine with the consensus motif

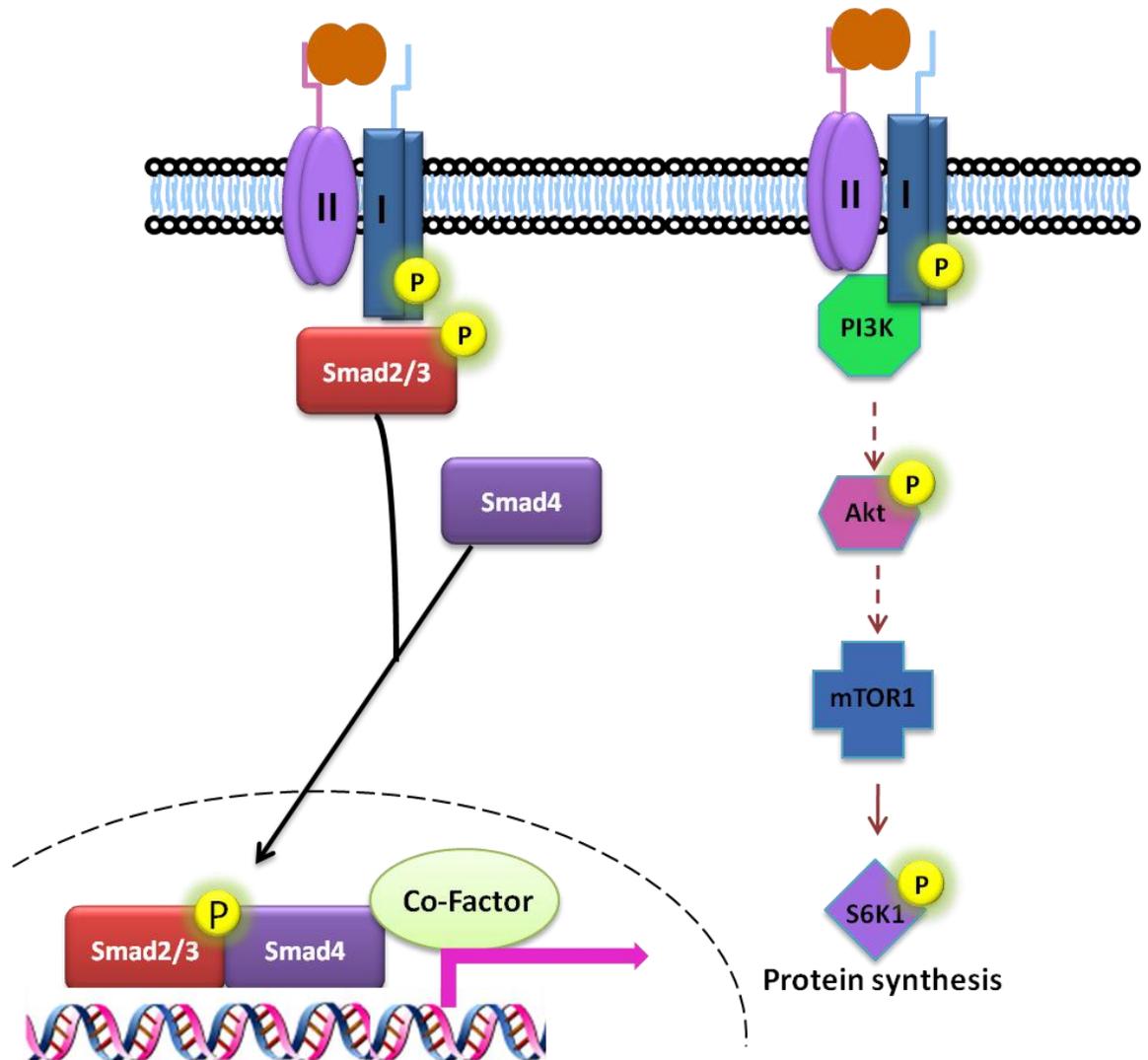


Figure 5. Simplified schematic representation of TGF β signaling pathways. In the canonical pathway (left), TGF β binds to the receptor T β RII (II), which recruits and phosphorylates T β RI (I), leading to activation of Smad2 and Smad3 by phosphorylation (P). Activated Smad2 and Smad3 form heterodimers with Smad4 and translocate to the nucleus. Together with co-activators, co-repressors and other transcription factors, the Smad complex regulates gene expression. Independent of the Smad2/3 activation, non-canonical signalings also exist. As an example in the right, TGF β can activate PI3K, possibly by inducing a physical interaction of its regulatory subunit. The activated PI3K then indirectly induces phosphorylation and activation of Akt, which in turn positively modulates mTOR function, resulting in the phosphorylation of S6K1 and consequent enhancement of cap-dependent translation.

(YXXM). This motif exists in many of the receptor tyrosine kinases and their substrates.

Although the mechanism of T β Rs-stimulated PI3K activity is still elusive, in a classical view of PI3K activation, the docking of PI3K in the vicinity of the plasma membrane through p85 recognition of the receptor phosphorylated YXXM activates the PI3K pathway. Once activated, PI3K converts PI-4,5-P₂ (PIP₂) into PI-3,4,5-P₃ (PIP₃), which localizes Akt to the membrane where it can be phosphorylated and activated by kinases such as phosphoinositide-dependent kinase-1 and 2 (PDK1 and PDK2) at two distinct residues, Thr-308 and Ser-473 (Rameh and Cantley 1999). The PI3K-dependent activation of Akt results in the phosphorylation and inhibition of the tumor suppressor proteins tuberous sclerosis 2 (TSC2), and the negative regulator of Ras-homolog enriched in brain (Rheb). The activation of Rheb therefore positively modulates mTOR function (Manning and Cantley 2003). mTOR in turn propagates the signals by phosphorylating two major targets: 4E-BP1 (first Thr37 and Thr46, then Thr70 and last Ser65) (Gingras, Gygi et al. 1999; Gingras, Raught et al. 2001) and S6K1 (Thr389), and eventually leads to dissociation of 4E-BP1 from eIF4E and S6K1 from eIF3, respectively.

Together these culminate in enhanced cap-dependent translation, particularly for many highly structured mRNAs, which are implicated in cell growth, proliferation and survival

(Mamane, Petroulakis et al. 2004), corresponding for protein synthesis induced by TGF β (Lamouille and Derynck 2007).

TGF β Signaling in Bone Biology

The TGF β family contains three closely related isoforms in mammals- TGF β 1, TGF β 2, and TGF β 3, and all three forms are detected in bone, with TGF β 1 being the most abundant (Hering, Isken et al. 2001). Numerous data from in vitro experiments have demonstrated the role of TGF β 1 in bone formation. Despite contrary results, most data agree with the following model. TGF β recruits osteoblast progenitors (Lucas 1989; Pfeilschifter, Wolf et al. 1990) and stimulates their proliferation (Robey, Young et al. 1987; Jian, Shen et al. 2006), thus expanding the pool of committed osteoblasts. On the other hand, it blocks the later stages of osteoblast differentiation, mineralization (Alliston, Choy et al. 2001; Maeda, Hayashi et al. 2004), apoptosis and transdifferentiation into osteocytes (Jilka, Weinstein et al. 1998; Karsdal, Larsen et al. 2002). The effect of TGF β on osteoblast differentiation can be monitored by the expression of osteoblast differentiation markers, e.g., ALP and osteocalcin, both of which have been shown to be inhibited (Harris, Bonewald et al. 1994).

In the past decade, some of the molecular mechanisms underlying TGF β actions in osteoblastogenesis have been elucidated at a transcriptional level. As previously mentioned, Runx2/Cbfa1 is a master regulator of osteoblast differentiation, and Runx2 binding sites are found in the promoters of several bone formation markers including collagen 1, ALP, and Osteocalcin (Stein, Lian et al. 2004). TGF β 1 targets Runx2 through the phosphorylation of Smad3, which in turn physically interacts with Runx2 at Runx2-responsive elements, resulting in repression of Runx2 and other osteogenic genes' expression (Alliston, Choy et al. 2001). Another molecule targeted by TGF β , Fra-2, a Fos-related protein of the AP-1 family, is a novel positive regulator of bone and matrix formation, which binds to and transactivates the osteocalcin gene and collagen 1 α 2 (Bozec, Bakiri et al. 2010). Treatment with TGF β 1 inhibits Fra-2's activation of the osteocalcin gene via phosphorylation of Fra-2 (Banerjee, Stein et al. 1996).

In addition, non-canonical TGF β signaling has been suggested to play a role in regulating osteoblast differentiation. For example, one signaling pathway activated in response to TGF β is the PI3K/Akt/mTOR pathway (Lamouille and Derynck 2007; Zhang 2009). Suppression of PI3K and mTOR signaling using inhibitors such as wortmannin, rapamycin, and NVP-BEZ235 has been shown to promote osteogenesis in vitro, as

measured by an increase in mineral formation or bone nodule formation and an upregulation of genes involved in osteogenesis including *osteocalcin* (Ogawa, Tokuda et al. 1998; Kratchmarova, Blagoev et al. 2005; Fitter, Dewar et al. 2008; Lee, Yook et al. 2010; Martin, Fitter et al. 2010). These observations suggest that TGF β also regulates osteoblastogenesis in a Smad-independent manner.

Information about the function of TGF β in bone biology has also been gained from the study of mouse models, in which the levels of TGF β signaling are manipulated when important players in this pathway are either eliminated/mutated by gene targeting or over-expressed in a tissue-specific manner. Given the broad application of the TGF β signaling, it is not unexpected that deficiency of several intermediates of the pathway causes embryonic death, precluding any study of their effect on bone development. This includes T β RI, T β RII, Smad4, and Smad2 (Nomura and Li 1998; Sirard, de la Pompa et al. 1998; Weinstein, Yang et al. 1998; Larsson, Goumans et al. 2001). In accordance with the role of TGF β in recruiting and stimulating the proliferation of osteoprogenitors, knocking out TGF β 1 results in decreased mineral content, accompanied by the absence of osteoblasts in trabecular bone (Geiser, Zeng et al. 1998; Atti, Gomez et al. 2002). TGF β is believed to block the later stages of osteoblast differentiation, mineralization

(Alliston, Choy et al. 2001; Maeda, Hayashi et al. 2004), apoptosis and transdifferentiation into osteocytes (Jilka, Weinstein et al. 1998; Karsdal, Larsen et al. 2002). Supporting this notion, loss of Smad3 was shown to result in osteopenia due to the inability of TGF β to inhibit osteoblast differentiation, thereby increasing the osteocyte accumulation (Borton, Frederick et al. 2001). Furthermore, overexpression of TGF β 2 under the osteocalcin promoter targeting mature osteoblasts leads to a dramatic loss of bone, together with increased osteoprogenitor cell numbers and impaired osteoblast differentiation (Erlebacher and Derynck 1996). Vice versa, a truncated T β RII driven by the osteocalcin promoter, which inhibits TGF β signaling in mature but not early osteoblasts, results in an increase in trabecular bone mass (Filvaroff, Erlebacher et al. 1999).

TGF β as a Therapeutic Target

Although TGF β appears to induce both positive and negative effects on bone cells, suppression of TGF β signaling post-natally tends to generate similar anabolic effects. Several preclinical and clinical strategies for disrupting TGF β signalling have been developed. Strategies such as preventing TGF β ligand interaction with receptors

through the use of ligand traps, including neutralizing antibody and soluble T β RII/III receptors, reducing TGF β translation by antisense-mediated technology, and reducing phosphorylation and activation of Smad proteins through TGF β receptor kinase inhibitors have been applied in a preclinical and clinical settings. Edwards et al. have shown that 4-week-treatment with 1D11, a TGF β -neutralizing antibody blocking all three forms of TGF β , increased BMD, trabecular thickness and Bone volume/Tissue volume, accompanied by elevated osteoblast numbers (Edwards, Nyman et al. 2010). Similarly, 6-week-treatment with SD-208, a T β RI kinase inhibitor, increased bone mass, trabecular bone volume, and mineral concentration of bone matrix, accompanied by increased osteoblast numbers and formation rate (Mohammad, Chen et al. 2009). These experimental outcomes indicate that therapies that reduce TGF β signaling may have significant clinical benefit in the treatment of low-bone-mass disease.

Vimentin

Intermediate filaments, along with Microtubules and Microfilaments are the major components of the cytoskeleton. The name 'intermediate filaments' comes from their diameter (10-12 nm) being intermediate between that of Microtubules (25 nm) and

Microfilaments (7-10 nm). The integrated network by the three filament systems is responsible for the mechanical integrity of the cell and is critically involved in such processes as cell division, motility and plasticity. Other than the difference in size, intermediate filaments exhibit several unique features: firstly, intermediate filaments appear to be the most dynamic of the three filament types. In particular, reversible dissociation-association of intermediate filament dimers can occur along the entire filament length and not just at the two ends as in microtubules and microfilaments (Strelkov, Herrmann et al. 2003). Secondly, they are expressed in a highly regulated manner in terms of tissue and developmental stage specificity (Toivola, Tao et al. 2005). Thirdly, intermediate filament proteins such as lamins are also found in the cell nucleus, forming a meshwork of filaments on the inside of the nuclear membrane.

Vimentin belongs to the type III intermediate filaments. It appears to regulate cell migration in many cell types. For example, vimentin deficient cells display reduced mechanical stability and motility in vitro (Eckes, Dogic et al. 1998), and vimentin knockout mice show an impaired ability to heal wounds in vivo (Eckes, Colucci-Guyon et al. 2000).

Vimentin is normally expressed in cells of mesenchymal origin, and particularly, in undifferentiated cells (Steinert and Roop 1988). In addition, vimentin can also be expressed in epithelial cells undergoing EMT, and is a widely recognized marker of the EMT-like phenotype. Furthermore, several studies have reported overexpression of vimentin in breast cancer (Kokkinos, Wafai et al. 2007). In particular, vimentin has been identified as a marker of basal-like breast cancer cells (Neve, Chin et al. 2006). Several factors have been documented as regulators for vimentin expression. These include platelet-derived growth factor (PDGF) and TGF β which up-regulates vimentin mRNA levels (Ferrari, Battini et al. 1986; Wu, Zhang et al. 2007), and a transcriptional repressor, ZBP-89, which down-regulates vimentin mRNA (Wieczorek, Lin et al. 2000). In addition, administration of PTH decreased the de novo biosynthesis of vimentin (Lomri and Marie 1990). However, it is not known whether, and if yes, how, the gene expression pattern of vimentin during either differentiation or EMT functionally contributes to the corresponding cellular events. In vitro studies have suggested high binding affinity of vimentin to a variety of synthetic and natural nucleic acids (Hartig, Huang et al. 1997), and this DNA-binding mediates translocation of vimentin into the nucleus of cultured cells (Hartig, Shoeman et al. 1998). These experimental observations led to the

hypothesis that vimentin not only fulfills general cytoskeletal functions in the cytoplasm but also participates in DNA-based events taking place in the nuclear interior. Interestingly, upon computational analysis, Capetanaki et al. predicted molecular interactions between vimentin and several CREB transcription factor family members based on their structural similarities localized to the important leucine zipper domains capable of coiled-coil interactions (Capetanaki, Kuisk et al. 1990). However, these hypotheses need further investigation and confirmation in mammalian cells.

CHAPTER II

VIMENTIN INHIBITS ATF4-MEDIATED *OSTEOCALCIN* TRANSCRIPTION AND OSTEOBLAST DIFFERENTIATION

The work in this chapter was published in the Journal of Biological Chemistry in September, 2009 (Lian, Wang et al. 2009). The paper was a collaborative effort between the laboratories of Xiangli Yang and Florent Elefteriou.

Introduction

Activating transcription factor 4 (ATF4) is an osteoblast-enriched transcription factor that regulates *osteocalcin* transcription and osteoblast terminal differentiation. To identify functional partners of ATF4, we applied ROS17/2.8 osteoblast nuclear extracts and purified recombinant His-ATF4 onto a Ni⁺ affinity matrix chromatography column. Vimentin was identified by liquid chromatography-mass spectrometry. Coimmunoprecipitation and pulldown assays revealed that vimentin interacted with ATF4 with its first leucine zipper domain. DNA cotransfection and gel retardation

demonstrated that vimentin inhibited the transactivation activity of ATF4 on *osteocalcin* by preventing it to bind OSE1, the ATF4 binding site on the *osteocalcin* promoter. Northern hybridization revealed that *vimentin* was expressed at a high level in immature osteoblasts and a low level in fully differentiated osteoblasts. Down-regulation of *vimentin* by small interfering RNA induced endogenous *osteocalcin* transcription in immature osteoblasts. Conversely, ectopic overexpression of vimentin in osteoblasts inhibited osteoblast differentiation as shown by lower alkaline phosphatase activity, delayed mineralization, and decreased expression of osteoblast marker genes such as *bone sialoprotein* and *osteocalcin*. Together, our data uncover a novel mechanism whereby a cytoskeletal protein, vimentin, acts as a brake on differentiation in immature osteoblasts by interacting with ATF4.

Osteoblasts, the bone-forming cells, originate from mesenchymal stem cells. The differentiation of mesenchymal stem cells to osteoblasts is regulated by several osteoblast-specific transcription factors, including Runx2 (Banerjee, McCabe et al. 1997; Ducy, Zhang et al. 1997; Komori, Yagi et al. 1997; Mundlos, Otto et al. 1997), osterix (Nakashima, Zhou et al. 2002), and ATF4 (Yang and Karsenty 2004; Yang, Matsuda et al. 2004). ATF4 belongs to the CREB leucine zipper (LZ)-containing protein family and is

known to regulate gene transcription by binding specifically to the ATF consensus core sequence, TGACGTCA (Hai and Hartman 2001). In osteoblasts, ATF4 binds to osteoblast-specific element 1 (OSE1), found in *osteocalcin gene 2* (Ducy and Karsenty 1995; Schinke and Karsenty 1999). The importance of ATF4 in skeletal biology is demonstrated by the fact that deletion of *Atf4* from the mouse genome results in a severe runt phenotype in addition to very low bone mass due to a failure of osteoblast terminal differentiation and synthesis of type I collagen, the main constituent of bone matrix proteins (Yang, Matsuda et al. 2004). ATF4 transcriptional activity can be enhanced by posttranslational modifications such as phosphorylation by RSK2 and suppressed by interacting with other proteins such as FIAT (Yang, Matsuda et al. 2004; Yu, Ambartsoumian et al. 2005).

Vimentin is a type III intermediate filament (IF) protein. IFs along with microtubules and actin microfilaments make up the dynamic cytoskeleton that maintains cell shape, enables intracellular transport, and supports cell division (Steinert and Liem 1990; Herrmann and Aebi 2004; Ivaska, Pallari et al. 2007). Similar to other IF proteins, vimentin is expressed in a cell type- and developmental stage-specific manner. Vimentin is often expressed in undifferentiated and proliferative cells of mesenchymal origin and is

eventually replaced with cell type-specific IF subunits upon differentiation (Tapscott, Bennett et al. 1981; Capetanaki, Ngai et al. 1984). Studies using *ras*-transformed cells (Olson and Capetanaki 1989) and transgenic mouse models (Capetanaki, Smith et al. 1989) have shown that vimentin regulates cell growth and differentiation. Recent studies using *vimentin*-deficient (*Vim*^{-/-}) mice have revealed that loss of vimentin leads to failures in vascular adaptation resulting in pathological conditions, such as reduction of renal mass (Terzi, Henrion et al. 1997), malformation of glia cells (Colucci-Guyon, Gimenez et al. 1999), impairment of wound healing (Eckes, Colucci-Guyon et al. 2000), reduced resistance of arteries to shear stress (Henrion, Terzi et al. 1997), and disturbance of leukocytes homing to lymph nodes (Nieminen, Henttinen et al. 2006). However, the molecular mechanisms whereby vimentin regulates cell differentiation remain elusive.

Here, we show evidence that vimentin interacts directly with ATF4 and prevents its transcriptional activity, thereby inhibiting its function in osteoblast differentiation. The inverse correlation between the expression patterns of *vimentin* and *osteocalcin*, a downstream target of ATF4, during osteoblast differentiation suggests that a decline in vimentin expression may be a prerequisite to the process of osteoblast differentiation.

Methods

Materials

Tissue culture medium and fetal bovine serum were purchased from Invitrogen. Restriction endonucleases and other DNA-modifying enzymes were purchased from either New England BioLabs or Promega. Radiolabeled nucleotides were obtained from PerkinElmer Life Sciences. Monoclonal anti-vimentin and antibodies against ATF4 (C20) were from Santa Cruz Biotechnology. All chemicals except for the indicated ones were from Sigma.

Cell Culture

COS1 monkey kidney cell and mouse osteoblastic MC3T3-E1 cell lines were cultured in Dulbecco's modified Eagle's medium and α -minimal essential medium, respectively. ROS17/2.8 rat osteoblastic cells were grown in Dulbecco's modified Eagle's medium/F-12 medium. All media were supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

His-tagged ATF4 and GST-Vimentin Protein Purification

Bacterially expressed His-tagged ATF4 protein was purified according to the method developed by Novagen. GST-vimentin protein was purified as described (Yang, Ji et al. 2000).

Pulldown Experiment

Nuclear extracts (NEs, 1 mg) isolated from ROS17/2.8 rat osteoblastic cells were incubated with or without His-ATF4 (10 µg) for 30 min at 4 °C and were loaded onto Ni²⁺-chelating Sepharose columns. After washing, proteins were eluted and resolved in 4–20% gradient SDS-PAGE and visualized by Coomassie Blue staining. Individual proteins of interest were excised and subjected to trypsin digestion and identified by liquid chromatography and mass spectrometry (LC-MS).

For mapping ATF4 binding domains of vimentin, 1 µg of purified GST-vimentin fusion protein, its variants, or GST (negative control) was incubated with glutathione-Sepharose beads in phosphate-buffered saline buffer (PBS, pH 7.4) at 4 °C with rotation for 1 h and washed three times with PBS buffer, pH 7.4. The beads were then incubated with His-ATF4 at 4 °C for 2 h followed by three washes with PBS, pH 7.4. Bound

proteins were eluted by boiling for 5 min in 2× SDS sample buffer, separated by SDS-PAGE, and stained with Coomassie Brilliant Blue.

Similar methods were used to map vimentin binding domains of ATF4, except 1 µg of purified His-ATF4 or truncated variants was incubated with Ni-NTA-agarose (Qiagen) in PBS, pH 8.0, containing 10 mM imidazole at 4 °C with rotation for 1 h and then washed three times with PBS containing 15 mM imidazole. GST-vimentin was then added and incubated with rotation for 2 h at 4 °C after washing three times with PBS containing 50 mM imidazole.

NE Preparation

Nuclear extracts of osteoblastic cells were isolated essentially according to the methods described (Dignam, Lebovitz et al. 1983; Schinke and Karsenty 1999).

Vimentin Subcellular Localization

MC3T3-E1 cells were transiently transfected with vectors expressing GFP-vimentin or GFP alone. Cells were then fixed 48 h after transfection in 2% paraformaldehyde for 20 min at room temperature, permeabilized with 0.5% Triton X-

100 for 5 min, and stained with DAPI to visualize nuclei. Images were taken with an Olympus BX41 fluorescence microscope with filters for GFP and DAPI.

Immunoprecipitation

COS1 cells (85% confluent) in 10-cm plates were transfected with 8 µg of FLAG-ATF4, 6 µg of HA-vimentin, or both using Lipofectamine (Invitrogen). Cells were treated with 25 µM MG115 to stabilize ATF4 for 5 h before harvesting (Yang and Karsenty 2004). Isolated NEs (500 µg) were immunoprecipitated with 5 µl of anti-FLAG M2 beads (Sigma) or anti-HA beads (Abcam) for 8 h at 4 °C. After washing three times with ice-cold Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.4), immunocomplexes and NEs (50 µl) as input controls were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, and revealed by Western blotting using anti-FLAG M2 (Sigma) or anti-HA (Abcam) antibodies.

Plasmids

Mouse *vimentin* cDNA was amplified by reverse transcription-PCR using bone cDNA as template and cloned in pcDNA3.1+ mammalian expression vector (Invitrogen),

pEGFP-C1 expression vector (Clontech), or pGEX-4T1 bacterial expression vector (Amersham Biosciences). Three different truncation forms of mouse vimentin cDNA, named Vim1 (containing amino acids 1–155), Vim2 (containing amino acids 205–254), and Vim3 (containing amino acids 314–438), respectively, were PCR-amplified, flanked with cloning sites EcoRI and Sall, and inserted into mammalian expression vector pCMV-HA (Clontech) and bacterial expression vector pGEX-4T2. Mouse ATF4 cDNAs were subcloned into pCMV6CFLAG (Sigma) from a mammalian expression vector pCMV5/ATF4 (Yang, Matsuda et al. 2004). The integrity of all cDNA and production of the fusion protein were confirmed by DNA sequencing and Western blotting, respectively.

DNA Transfection and Luciferase Assay

COS1 cells were seeded at a density of 5×10^4 /well in 24-well plates and transfected with 0.2 μg of the reporter plasmid (6OSE1-Luc or 3AP1-Luc), 0.05 μg of β -galactosidase, 0.2 μg of transcription factor plasmid (FLAG-ATF4 or FosB) with or without 0.2 μg of pcDNA3.1-vimentin using Lipofectamine. Cells were lysed 24 h later, and the luciferase activity was normalized to the β -galactosidase activity.

Short Hairpin RNAs and Transfection

Short hairpin RNAs targeting mouse Vim construct were cloned by ligating annealed oligonucleotides 5'-ACCTCAAACGAGTACCGGAGACAGGTtcaagagACCTGTCTCCGGTACTCGTTTTT-3' into a BbsI/BbsI-digested psiRNA-hH1neo G2 vector (InvivoGen, San Diego, CA). 2T3 cells (2×10^5 cells/well in 6-well plates) were transfected with 1.5 μg of the reporter plasmid, 1.5 μg of siRNA-control or siRNA-Vim, and 0.2 μg of β -galactosidase plasmid). Cells were lysed 48 h later, and the luciferase activity was normalized to the β -galactosidase activity. Total RNA was collected 48 h after transfection for Northern blot analysis.

Electrophoretic Mobility Shift Assays (EMSAs)

ROS17/2.8 NEs or His-ATF4 and increasing amounts of GST-vimentin, its deletion variants, or GST were incubated with 5 pmol of a radiolabeled double-stranded OSE1 oligonucleotide (Ducy and Karsenty 1995) at room temperature for 10 min. EMSA was performed as described (Schinke and Karsenty 1999).

Establishment of Permanent Cell Lines

MC3T3-E1 cells (106 cells/10-cm dish) were transfected with 5 µg of pcDNA3.1 vector or pcDNA3.1-vimentin. Cells were trypsinized and replated in α -minimal essential medium containing 10% fetal bovine serum, and 400 µg/ml G418 was added 2 days after transfection followed by 2-week selection. The expression of *vimentin* was confirmed by Northern blot analysis using the bovine growth hormone poly(A)⁺ as a probe.

Northern Blot Analysis

Total RNA from different adult mouse tissues, MC3T3-E1 cells, primary calvarial osteoblasts, or bone marrow stromal cells was isolated using TRIzol (Invitrogen) according to the manufacturer's protocols. Total RNA (5 µg) was resolved in 1% agarose gel, transferred onto nylon membranes. The membrane was cross-linked by UV light and hybridized following standard protocols with the indicated cDNA probes described previously (Yang and Karsenty 2004; Yang, Matsuda et al. 2004) except for vimentin cDNA (nucleotides 792–1218), which was cloned in this study (see *Plasmids* above for cloning strategies).

Osteoblast Differentiation Assays

Confluent (d0) MC3T3-E1 in 12-well plates overexpressing vector (control) or vimentin were grown in G418-containing medium supplemented with 5 mM β -glycerophosphate and 100 μ g/ml ascorbic acid for 2, 4, 6, 12, or 20 days. Alkaline phosphatase activity was measured, and mineralization of osteoblasts was assayed by von Kossa staining as described (Yang, Ji et al. 2000).

Statistics

Data are expressed as mean \pm S.D. Statistical analysis was performed using an unpaired *t* test.

Results

Identification of Vimentin as a potential ATF4-interactor

To identify ATF4-interacting proteins in osteoblasts, we used a biochemical approach with nuclear extracts from ROS17/2.8 rat osteoblasts and His-tagged ATF4.

ATF4 was tagged with 6 histidines at its N terminus and expressed in bacteria to obtain large quantities of protein. Purified His-ATF4 was then incubated with nuclear extracts isolated from ROS17/2.8 rat osteoblasts, and the mixture was applied to a Ni-NTA column. After repeated washing, the ATF4-bound proteins were eluted, and elution fractions were resolved in a gradient SDS-PAGE. Slices containing a single protein band were subjected to trypsin digestion and LC-MS analysis. Among the specific ATF4-bound proteins identified in the LC-MS analysis, one 37 kDa band was revealed as vimentin, a type III intermediate filament protein (Fig.6 , A and B).

Vimentin is a cytoskeletal protein that has been well recognized as a protein localized in the cytoplasm and perinucleus, although it can be transported into the nucleus by DNA oligonucleotide mediation (26). The fact that it was pulled out from osteoblast nuclear extracts supported the nuclear localization of vimentin. To confirm this notion, we made GFP-vimentin fusion expression vector and introduced it into MC3T3-E1 cells. Our results showed that GFP-vimentin was present predominantly in the nucleus of a subset of GFP-positive cells, but GFP alone was distributed evenly in both nucleus and cytoplasm of all the GFP-positive cells (Fig. 6C).

Vimentin interacts with ATF4

Pulldown assays using either purified GST-vimentin or GST alone showed that ATF4 bound to GST-vimentin but not to the GST control (Fig. 7A). Conversely, vimentin bound to His-tagged ATF4 but not to Ni-NTA-agarose beads (Fig. 7B), confirming a direct interaction between vimentin and ATF4 *in vitro*. Reciprocal immunoprecipitation assays using nuclear extracts of COS1 cells transfected with HA-vimentin and/or FLAG-ATF4 expression vectors revealed that vimentin interacted with ATF4 in cells as well because FLAG-ATF4-bound agarose beads precipitated HA-vimentin, and conversely, HA-vimentin precipitated FLAG-ATF4 (Fig. 7C). These results indicate that the interaction between vimentin and ATF4 is direct and specific *in vitro* and in cells.

ATF4 has been predicted to form heterodimers preferentially through LZ domains (27), and it contains two LZ domains, namely N-LZ and C-LZ. The basic amino acid-rich DNA binding domain (*b*) is located close to C-LZ (Fig. 7D). Interestingly, we found three leucine-rich repeats within vimentin by visual examination of its primary structure and named them putative LZs (PLZ1 to PLZ3, Fig. 2E). To define which PLZ(s) mediated the interaction between vimentin and ATF4, we created three truncated variants of vimentin, Vim1, Vim2, and Vim3, which contained PLZ1, PLZ2, or PLZ3, respectively. We also

used three truncated variants of ATF4 described previously (Yang, Matsuda et al. 2004) that covered amino acids 1 to 151 (1–151, containing N-LZ), 110 to 221 (110–221), and 186 to 349 (186–349, containing C-LZ), respectively, in the following experiments.

Pulldown assays demonstrated that full-length vimentin or its truncated variant Vim1 bound strongly to full-length ATF4 (Fig. 7F). Full-length ATF4 and the two ATF4 truncated variants, 1–151 and 186–349, interacted with full-length vimentin (Fig. 7G). From these results, we inferred that Vim1 binds the C-LZ domain of ATF4. Together, these results establish a novel interaction between a nuclear transcription factor, ATF4, and a cytoskeleton protein, vimentin.

Vimentin Represses the Transactivation Activity of ATF4

Osteocalcin expression is a hallmark of osteoblast terminal differentiation (Aubin, Liu et al. 1995). ATF4 was originally identified as one of the two main regulators of *osteocalcin gene 2* transcription (Ducy and Karsenty 1995; Ducy, Zhang et al. 1997; Schinke, McKee et al. 1999; Yang, Matsuda et al. 2004). To understand the functional relevance of the vimentin-ATF4 interaction, we first tested whether vimentin affected the

transcriptional function of ATF4. Consistent with previous findings (Yang and Karsenty 2004), ATF4 activated the *p6OSE1-Luc* reporter containing six copies of OSE1 (Ducy and Karsenty 1995; Schinke and Karsenty 1999) by about 50-fold in COS1 cells. This ATF4-dependent activation was inhibited by 70% when a vimentin expression vector was cotransfected (Fig. 8A). Similarly, vimentin inhibited ATF4-dependent activation of p160-Luc, a reporter construct containing a 160-bp native *osteocalcin* promoter, by 70% (Fig. 8B). The inhibition of ATF4-induced luciferase activity by vimentin was specific to ATF4 because vimentin did not affect the ability of FosB, another LZ-containing transcription factor, to activate a chimeric reporter gene containing three copies of AP1 binding sites, *pAP1-Luc* (Fig. 8C). These data indicate that vimentin selectively represses the transactivation activity of ATF4.

Vimentin Inhibits ATF4 Binding to Its Cognate DNA

Two important observations prompted us to test whether vimentin inhibited ATF4-mediated transactivation of *osteocalcin* by interfering with the binding of ATF4 to its cognate DNA element. First, vimentin bound to the ATF4 C-LZ, the motif in proximity

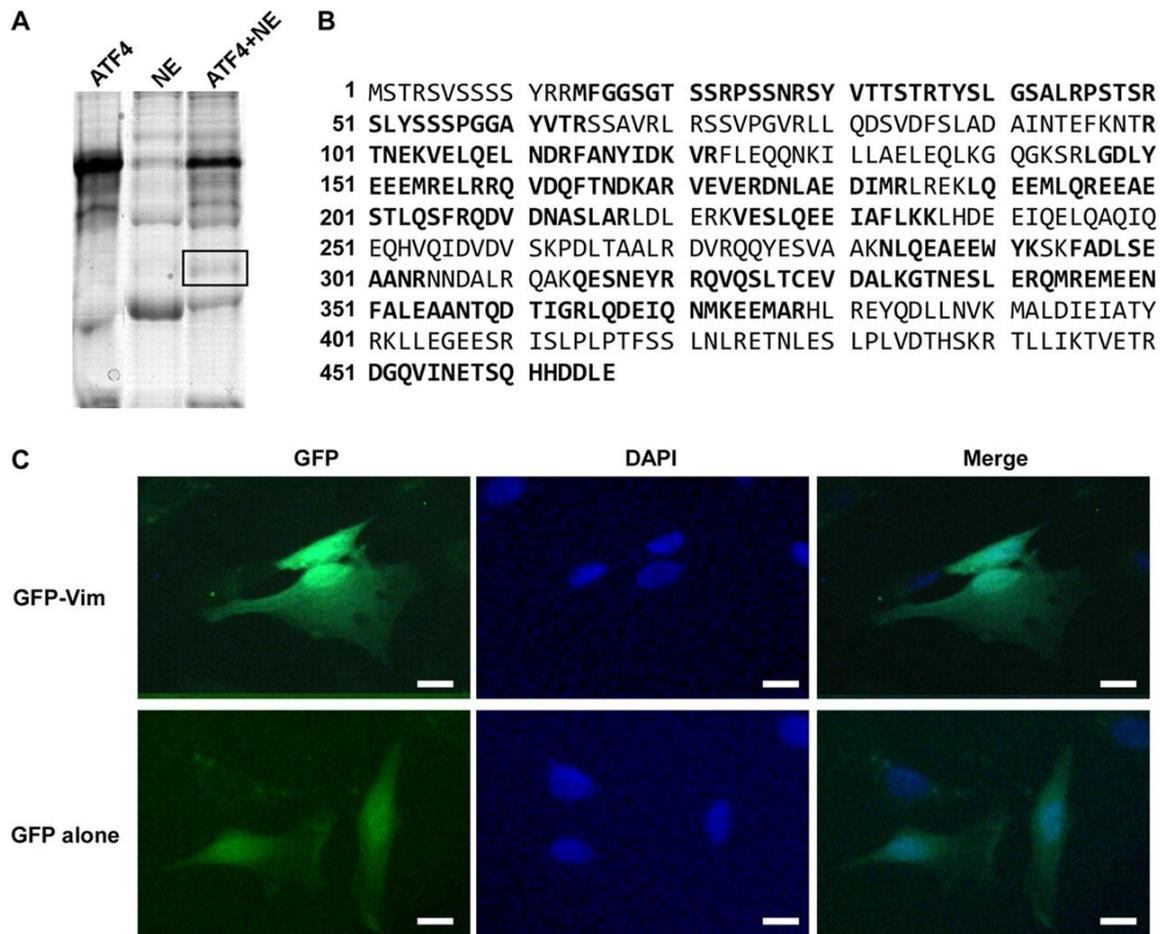
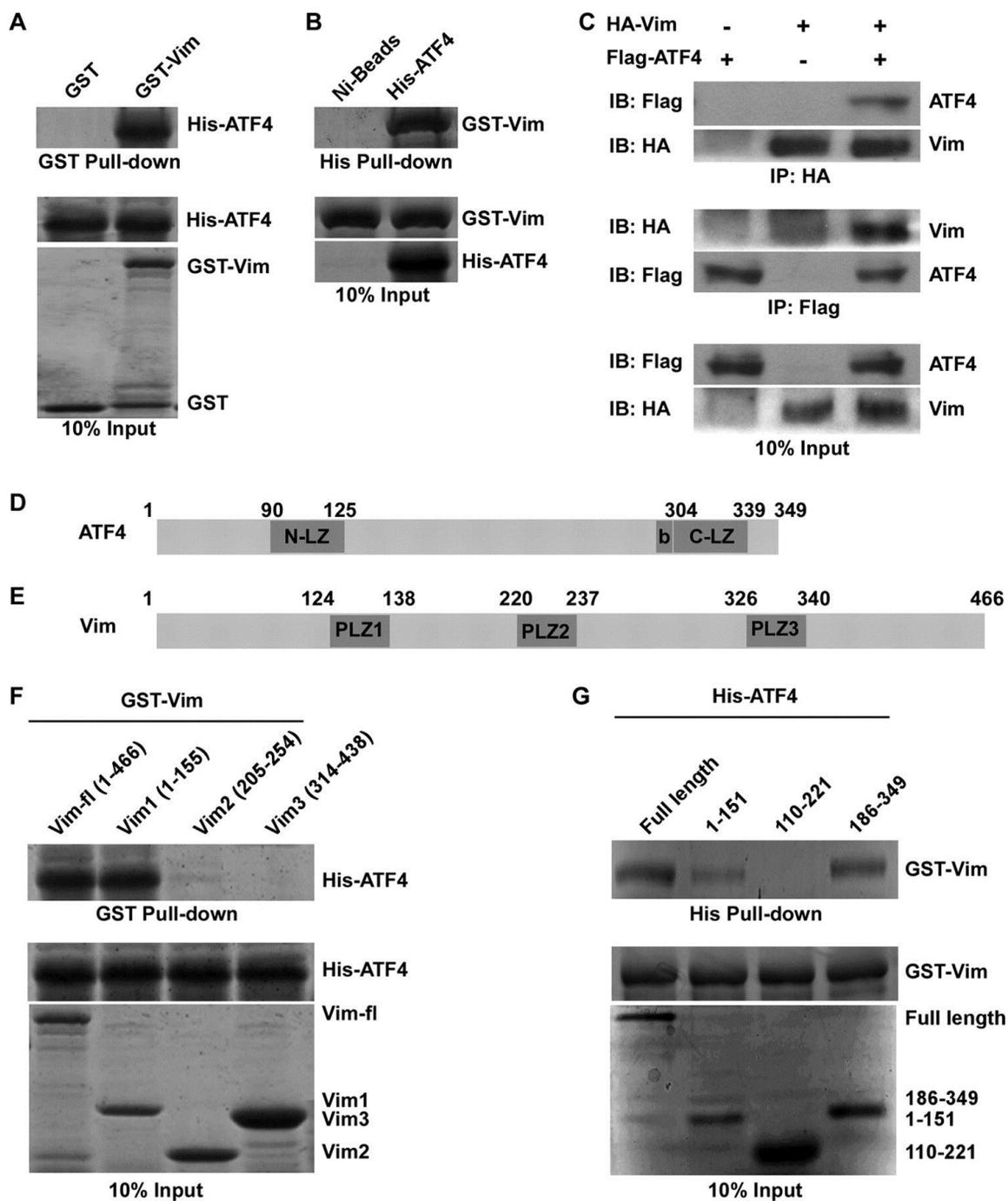


Figure 6. Identification of vimentin. A, pull-down assay using His-ATF4 (ATF4, lane 1) as bait and NEs from ROS17/2.8 osteoblastic cells (lane 2) as pool of proteins. Proteins were separated with a 4–20% gradient SDS-PAGE and stained with Coomassie Brilliant Blue. A 37 kDa band (boxed) was excised and analyzed by MS. B, amino acid sequence of vimentin. MS analysis revealed peptides (bold letters) that covered 54% of vimentin. C, GFP-tagged vimentin (upper panel) transfected into MC3T3-E1 osteoblastic cells and analyzed for subcellular localization in comparison with GFP vector (lower panel) alone. Nuclei were visualized using DAPI staining (middle panel). Scale bar, 10 μ m.

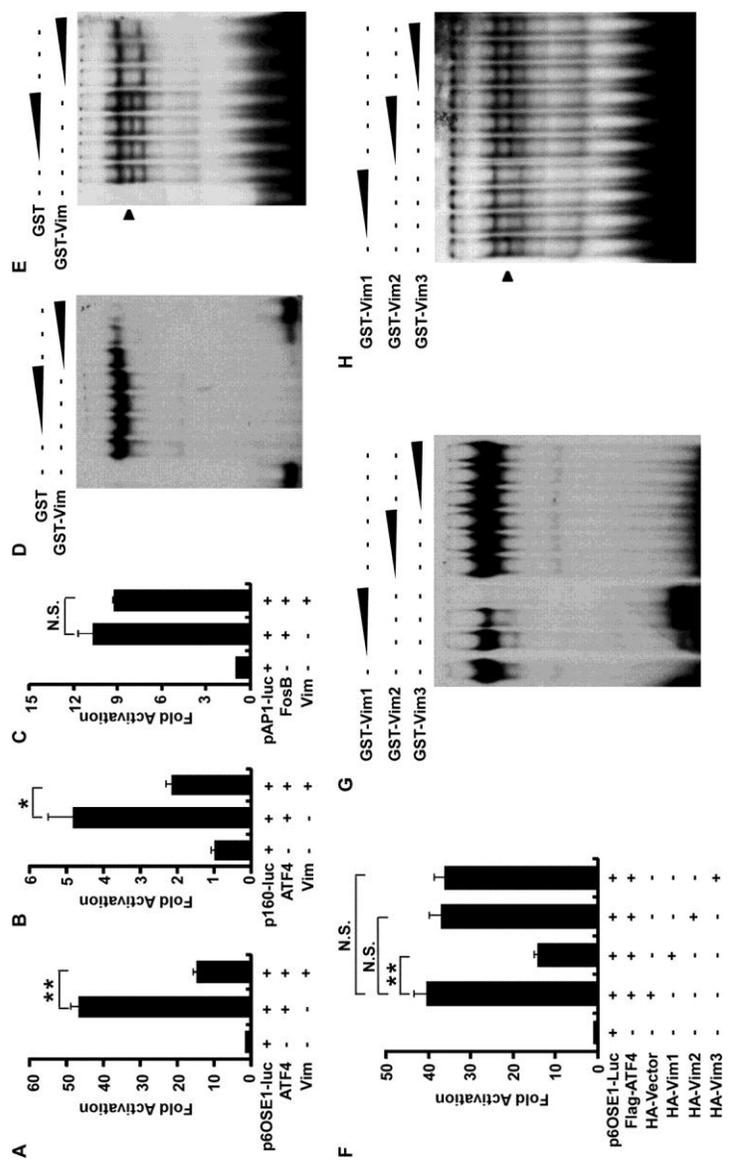
Figure 7. Vimentin (*Vim*) interacts with ATF4. *A*, pulldown assay showing that GST-vimentin binds directly to ATF4. *B*, pulldown assay using His-ATF4 and Ni-NTA resin column showing that ATF4 binds directly to vimentin. *C*, reciprocal coimmunoprecipitation. NEs of COS cells transfected with HA-vimentin and/or FLAG-ATF4 were immunoprecipitated with anti-HA or anti-FLAG antibody and visualized with anti-FLAG and anti-HA antibodies (*top two panels*). Total protein (10%) of each transfection served as loading control (*bottom panel*). *D*, schematic illustration of ATF4 primary structure showing two LZ domains at N and C termini. *Letter b* represents basic amino acid-rich DNA binding domain. *E*, schematic presentation of vimentin primary structure showing three PLZs of vimentin. *F*, PLZ1 of vimentin interacting with ATF4. Pulldown assay was carried out using purified GST-vimentin and its truncated variants GST-Vim1–3 and His-ATF4. Note that only full-length vimentin (*Vim-fl*) or its variant containing the first PLZ interacted with His-ATF4. *G*, LZs of ATF4 interacting with vimentin. Pulldown assay was performed using purified His-ATF4 and its three indicated deletional variants. Note that full-length ATF4 (*Full length*) and both of its LZ-containing variants, 1–151 and 186–349, bound vimentin.



to the basic DNA binding domain (Fig. 6, D and G), which may create a steric barrier for ATF4 to access the DNA. Second, vimentin can be detected in the nucleus of many cell types, including osteoblasts (Fig. 6C) and epithelial cells (Monteiro, Hicks et al. 1994; Rogers, Eckelt et al. 1995). ATF4 binds OSE1 in the *osteocalcin* promoter *in vitro* and *in vivo* as demonstrated by EMSA and by the use of transgenic mice (Ducy and Karsenty 1995; Schinke and Karsenty 1999; Yang and Karsenty 2004; Yang, Matsuda et al. 2004). Using a similar assay, we found that GST-vimentin inhibited His-ATF4 binding to OSE1 dose-dependently. As a negative control and consistent with the pulldown assay results (Fig. 7A), GST alone did not have any effect on His-ATF4 binding to the OSE1 (Fig. 8D). Accordingly, GST-vimentin fusion protein but not GST alone inhibited endogenous ATF4 binding to OSE1 in a dose-dependent manner (Fig. 8E).

Given the mapping data that indicated that Vim1 was sufficient to mediate the interaction between vimentin and ATF4 (Fig. 7F), we tested whether Vim1 affected the transactivation activity of ATF4. DNA cotransfection using a Vim1 expression plasmid showed an inhibition of ATF4-dependent activation of *6OSE1-Luc* reporter to an extent similar to that of full-length vimentin, whereas Vim2 or Vim3 had no effect (Fig. 8F).

Figure 8. Vimentin (*Vim*) inhibits ATF4-dependent transactivation of *osteocalcin* transcription by blocking its binding to OSE1. A–C, DNA cotransfections of COS1 cells with reporter construct containing six copies of ATF4 binding site OSE1, p6OSE1-Luc (A), a native *osteocalcin* promoter, *p160-Luc* (B), three copies of binding sites of AP1 family (C), and/or expression vectors of ATF4, FosB, and/or vimentin as indicated. Note that ATF4-induced but not FosB-induced luciferase activity was inhibited by more than 70% by vimentin. D, vimentin inhibiting the binding of ATF4 to OSE1. EMSA was performed using purified His-ATF4 and radiolabeled OSE1 as a probe. Note that vimentin inhibited ATF4 binding to OSE1 dose-dependently. E, EMSA showing that vimentin inhibits endogenous ATF4 (*middle bands* are ATF4·OSE1 complexes (9)) from ROS17/2.8 osteoblastic cells binding to OSE1. F, DNA cotransfection in COS1 cells with p6OSE1-Luc and expression vectors of FLAG-ATF4 and/or HA-Vim1, -Vim2, or -Vim3. G and H, EMSA. Note that GST-Vim1 inhibited His-ATF4 (G) or endogenous ATF4 (H) binding to OSE1 in a dose-dependent manner. The luciferase activities were normalized for the β -galactosidase activity and are presented as fold activation relative to the luciferase levels of the reporter construct alone. Values are the mean \pm S.D. of three independent experiments. *, $p < 0.01$; **, $p < 0.001$; N.S., not significant.



Consistently, Vim1 but not Vim2 or Vim3 inhibited His-ATF4 or endogenous ATF4 binding to OSE1 in a dose-dependent manner (Fig. 8, G and H). These data indicate that vimentin inhibits ATF4 transcriptional activity by affecting its binding to cognate DNA via the first PLZ domain of vimentin.

Down-regulation of vimentin induces endogenous osteocalcin transcription

To strengthen further our findings that vimentin inhibited the transcriptional activity of ATF4, we next tested whether knocking down endogenous *vimentin* in immature osteoblasts would conversely induce *osteocalcin* transcription. We thus constructed a vector expressing short hairpin RNA of *vimentin* (psiRNA-Vim) and introduced it into 2T3 mouse osteoblasts. Northern blot analysis demonstrated that endogenous *vimentin* mRNA was decreased in cells transfected with psiRNA-Vim. Supporting our hypothesis, endogenous *osteocalcin* mRNA was induced in cells depleted in vimentin by psiRNA-Vim (Fig. 9A). To confirm these expression data, we performed DNA cotransfection assays to determine whether vimentin depletion affected endogenous ATF4-dependent activation of luciferase activity driven by 6OSE1 (p6OSE1-Luc) or native 160-bp *osteocalcin* promoter (p160-Luc). Our results revealed that psiRNA-*Vim* but not the empty psiRNA vector enhanced ATF4-induced luciferase

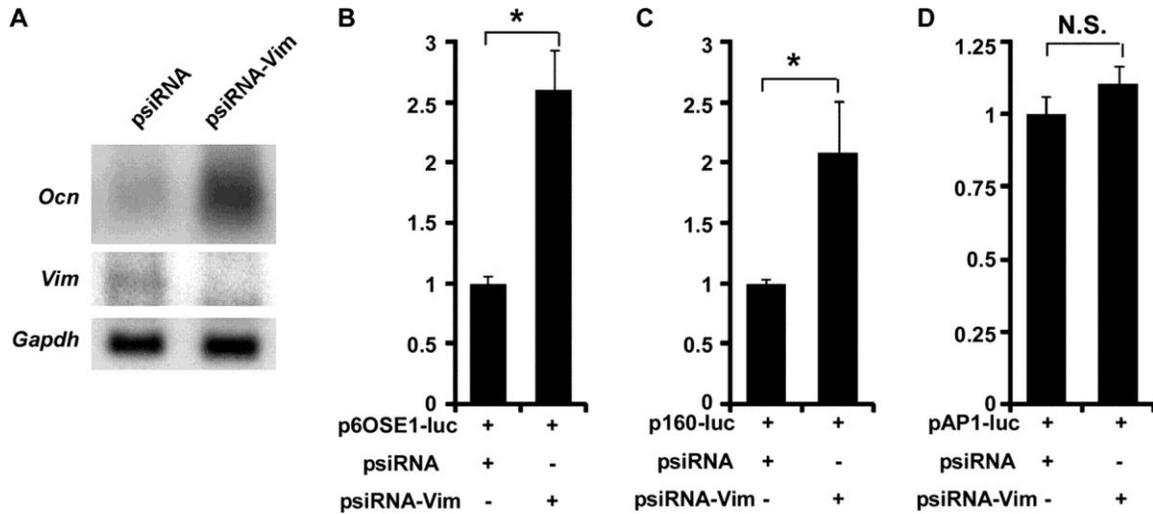


Figure 9. Down-regulation of vimentin by siRNA enhances *osteocalcin* expression. A–C, DNA cotransfections of 2T3 osteoblastic cells with reporter p6OSE1-Luc (A), p160-Luc (B), AP1-Luc (C), and vectors of control (*psiRNA*) or vimentin (*psiRNA-Vim*). Note that vimentin siRNA induced activation of *osteocalcin* promoter by >2-fold. Values are the mean \pm S.D. of three independent experiments. *, $p < 0.01$; N.S., not significant. D, Northern blot using total RNA from 2T3 osteoblasts transfected with control or siRNA against vimentin. Note that depletion of vimentin triggered *osteocalcin* expression. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA was used as a loading control.

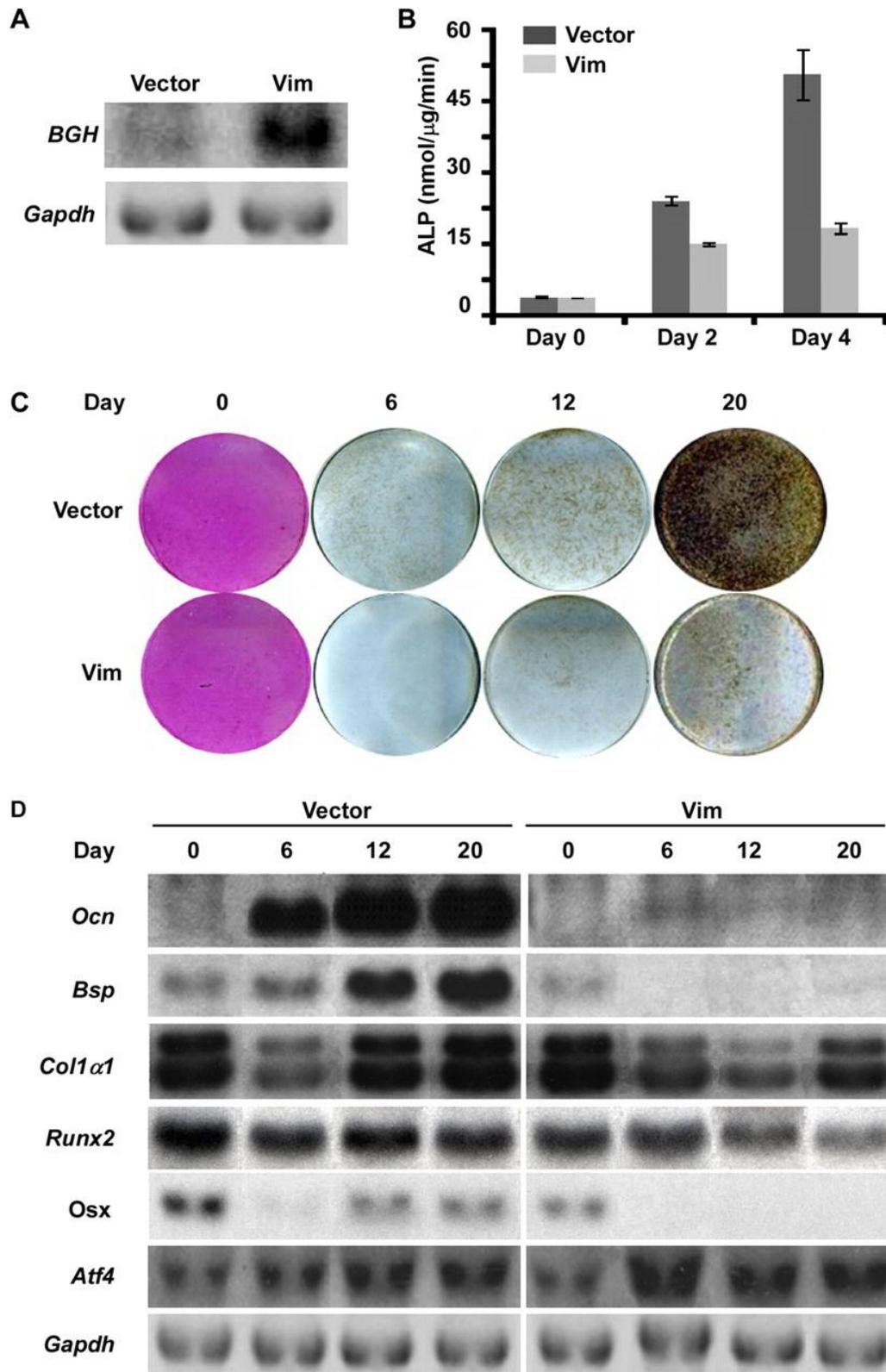
activity 2.5-fold in both 6OSE1-Luc and p160-Luc reporter constructs (Fig. 9, B and C).

This induction in luciferase activity was specific to endogenous ATF4 because psiRNA-*Vim* did not significantly enhance the AP1-induced luciferase activity (Fig. 9D). Therefore, we conclude that vimentin acts as an inhibitor on endogenous *osteocalcin* transcription and thus as a break on the transactivation activity of ATF4.

Vimentin delays osteoblast differentiation

To address whether the inhibitory action of vimentin on the transactivation activity of ATF4 had any biological relevance and specifically regulated osteoblast differentiation, we created MC3T3-E1 mouse osteoblastic cells that stably expressed *vimentin*. Stable expression of *vimentin* was confirmed by Northern blot analysis (Fig. 10A). The progression of osteoblast differentiation was monitored by alkaline phosphatase assay, von Kossa staining of mineralized nodules, and the temporal expression of immature osteoblastic differentiation markers, such as *type I collagen (Col1 α 1)*, and differentiated osteoblast markers, such as *osteocalcin* and *bone sialoprotein (Bsp)*. In addition, the expression of *Runx2* and *osterix*, another two important osteoblast differentiation factors, was also examined. MC3T3-E1 cells carrying empty vector responded to osteogenic

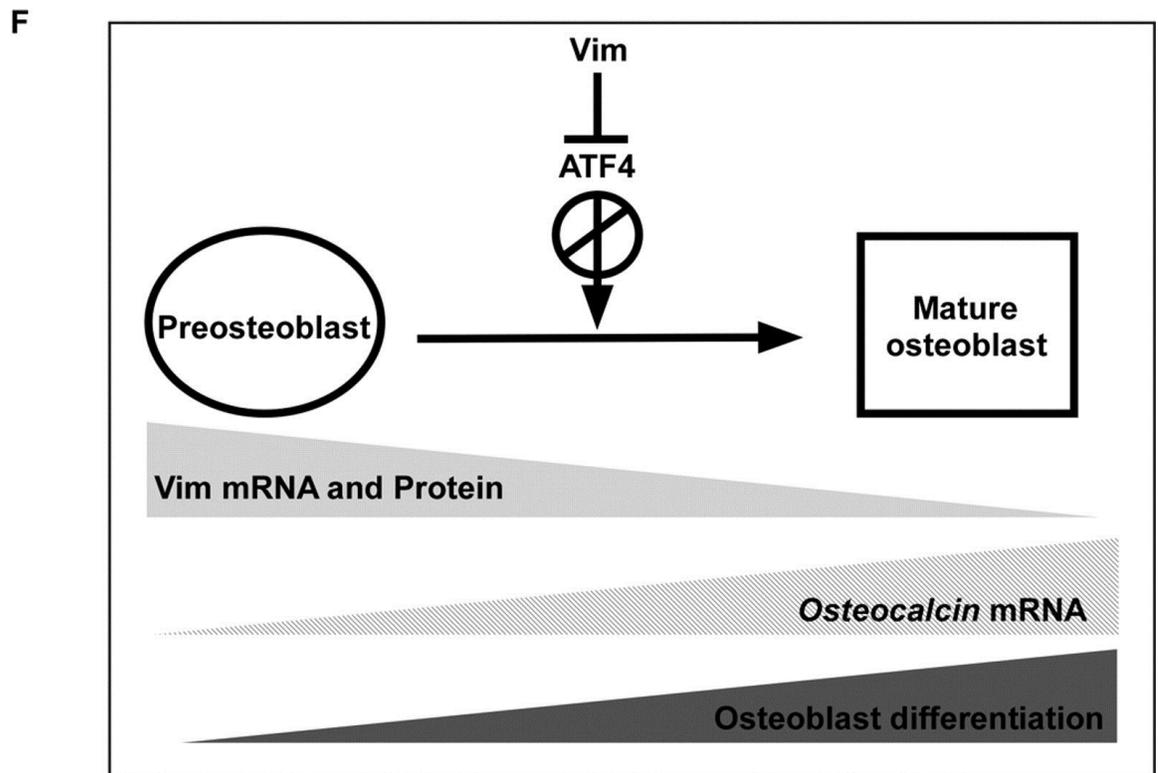
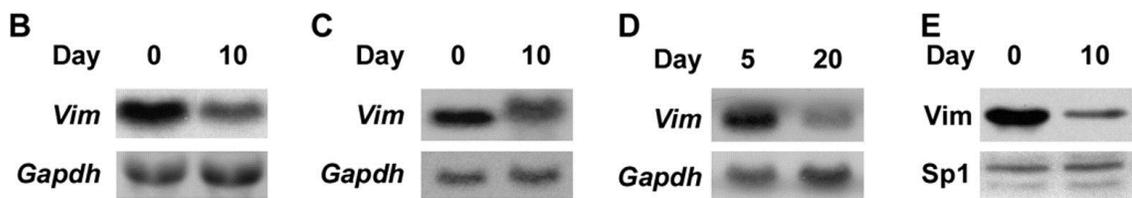
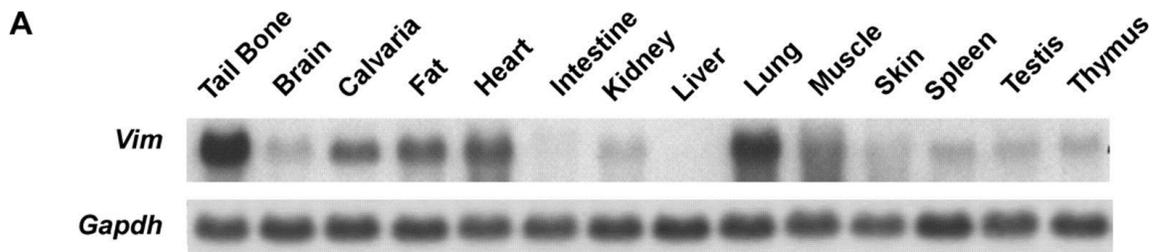
Figure 10. Vimentin delays osteoblast differentiation. *A*, overexpression of *vimentin* in MC3T3-E1 osteoblasts. Vimentin in pcDNA3.1 expression vector was stably expressed in the MC3T3-E1 osteoblastic cell line. Bovine growth hormone (*BGH*) was used to probe for exogenous vimentin. *B*, vimentin decreasing alkaline phosphatase (*ALP*) activity. Decreased alkaline phosphatase activity at 2 and 4 days after osteogenic induction was observed in MC3T3-E1 cells overexpressing vimentin compared with the controls. *C*, vimentin inhibiting mineralization of MC3T3-E1 cells. von Kossa staining was done at 6, 12, and 20 days after differentiation of control cells (*Vector*) and vimentin-overexpressing cells. Cells were counterstained with acid fuchsin on day 0 before osteogenic differentiation induction showing that the same amount of cells were plated. *D*, Northern blot analysis showing the expression of *osteocalcin* (*Ocn*) and *bone sialoprotein* (*Bsp*), two mature osteoblast marker genes, and *Col1a1*, an early osteoblast marker, together with three transcription factors that are important for osteoblast differentiation, *Runx2*, *osterix* (*Osx*), and *ATF4* (*Atf4*). Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA was used as a loading control.



induction as indicated by increasing alkaline phosphatase activity over time. This response was inhibited in cells overexpressing vimentin 2 and 4 days upon induction (Fig. 10B). As expected, 6 days after differentiation induction, mineralized nodules started to form in MC3T3-E1 cells that carried control empty vector. In contrast, no mineralized nodules were observed in day 6 cultures of MC3T3-E1 cells that overexpressed vimentin. The onset of mineralized nodule formation in vimentin-expressing cells was only detected after 12 days of differentiation, and the number and size of mineralized nodules were still severely less after 20 days of differentiation compared with control cells (Fig. 10C).

Consistent with the mineralization results, two marker genes for mature osteoblast, *osteocalcin* and *Bsp*, were expressed at day 6 after differentiation induction and increased over time in control MC3T3-E1 cells carrying empty vector. However, in cells that overexpressed vimentin, the mRNA levels of *osteocalcin*, *Bsp*, and *osterix* were not readily detectable at all-time points examined, including days 6, 12, and 20 after differentiation induction, whereas the expression of *Atf4*, *Col1 α 1*, and *Runx2* was not overly changed, especially if one takes into account of loading controls (Fig. 10D).

Figure 11. Expression pattern of vimentin (Vim) mRNA and protein. A, Northern blot analysis using total RNA from indicated mouse tissues showing that *vimentin* mRNA is highly expressed in bone. B–D. Northern blot using total RNA from MC3T3-E1 (B), primary calvarial osteoblasts (C), and primary bone marrow stromal cells (D) at the indicated times after differentiation induction. Note that *vimentin* mRNA is down-regulated at day 10 or day 20 after osteoblast differentiation induction. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA was used as a loading control. E, Western blot analysis using nuclear extracts of rat primary calvarial osteoblasts. Note that vimentin protein decreased during osteoblast differentiation. These results are representatives of three independent experiments. F, schematic illustration of the role of vimentin in osteoblast differentiation. In preosteoblasts, vimentin is highly expressed, and it inhibits *osteocalcin* transcription and premature osteoblast differentiation by binding to ATF4. Along with the progression of osteoblast differentiation, the vimentin level decreases, and thus the inhibition of ATF4 is released, *osteocalcin* transcription is then initiated, and osteoblast terminal differentiation is achieved.



From these results, we conclude that vimentin inhibits (i) endogenous *osteocalcin* transcription and (ii) osteoblast differentiation. The observation that vimentin inhibited the expression of mature osteoblast marker genes more dramatically than that of immature osteoblast marker genes was strikingly similar to what was seen in the *Atf4*^{-/-} mice (Yang, Matsuda et al. 2004). This similarity strengthens our hypothesis that vimentin may execute its function in osteoblasts by affecting the function of ATF4.

Vimentin expression pattern in the osteoblastic lineage

To understand the physiological role of vimentin during osteoblast differentiation, we examined the expression pattern of *vimentin* in various mouse tissues, MC3T3-E1 osteoblasts, primary calvarial cells, and bone marrow stromal osteoprogenitors that have the potential to differentiate into osteoblasts (Aubin 2001). Northern blot analysis showed that *vimentin* was highly expressed in vertebral bones, and it was the most abundant message RNA detected among all of the tissues examined (Fig. 11A). Interestingly, we found that the level of *vimentin* mRNA in MC3T3-E1 cells and primary calvarial osteoblasts decreased over time during the course of differentiation: high at the initiation of differentiation (day 0, Fig. 11, B and C), and low at the end of differentiation (day 10,

Fig. 11, *B* and *C*). Similarly, in alkaline phosphatase-positive mouse bone marrow stromal osteoprogenitors, *vimentin* mRNA was high at day 5 after differentiation induction, but low at day 20 after differentiation induction (Fig. 11*D*). Consistently, vimentin protein showed the same expression pattern as its mRNA in rat primary osteoblasts (Fig. 11*E*). Collectively, these results suggest that down-regulation of vimentin during osteoblast differentiation may be a required mechanism to relieve its inhibition of ATF4 transcriptional activity and to promote osteoblast terminal differentiation (Fig. 11*F*).

Discussion

In summary, this study identified a novel interaction between vimentin and ATF4. To our knowledge, our work revealed for the first time a direct interaction between an intermediate filament protein and a transcription factor. The experimental data establish that through its interaction with ATF4, vimentin prevents the binding of ATF4 to its target DNA and thus leads to delayed osteoblast differentiation. Consistent with its inhibitory role, vimentin expression is progressively decreased at both mRNA and protein levels during the course of osteoblast differentiation, whereas the expression of *osteocalcin*, a

transcriptional target gene of ATF4, progressively increases. This piece of evidence suggests that at an early stage of osteoblast differentiation, the transcriptional activity of ATF4 is repressed by vimentin, which explains why *osteocalcin* transcription does not occur in these cells. This hypothesis is supported by the observation that ATF4 binds to *osteocalcin* promoter at day 8 but not at day 0 of differentiation in primary osteoblasts (Yu, Akhouayri et al. 2009). Therefore, removal of vimentin-mediated inhibition is a prerequisite for preosteoblasts to differentiate into terminal osteoblasts.

Our study has also defined that the PLZ domains of vimentin mediate the interaction between vimentin and ATF4. The sequence and structural similarities between the leucine-rich repeats of vimentin and the LZ motif of several other LZ-containing nuclear regulators belonging to the CREB family, such as CREB, Fos, and Jun, have been described previously. Based on computational analysis, the authors predicted the existence of molecular interactions between vimentin and these nuclear regulators through their similar leucine-rich amphipathic helices (Capetanaki, Kuisk et al. 1990). Our mapping data have now provided experimental evidence supporting these predictions, although only one of such putative LZ motifs in vimentin was shown to be responsible for this interaction. The biological significance of this association is

supported by evidence demonstrating that the PLZ1 of vimentin behaves identically to the full-length protein to inhibit ATF4 binding and activation of the *osteocalcin* promoter. Therefore, this provides insights into the possibility that small molecules could be screened for their ability to block the binding of vimentin to ATF4 and to stimulate osteoblast terminal differentiation and function.

It is not understood at present whether the interaction between vimentin and ATF4 takes place in the nucleus or the cytoplasm. Several observations strongly suggest that vimentin, despite its well-known cytoplasmic location, interacts with ATF4 within the nucleus. First, vimentin inhibits ATF4 to bind its cognate DNA. Second, vimentin was pulled out from ROS28/1.7 osteoblast nuclear extracts and recognized by an anti-vimentin antibody in Western blotting in the nuclear extracts of primary calvarial osteoblasts. Third, GST-vimentin was detected in the nucleus of MC3T3-E1 osteoblasts (Fig. 6C). Supporting this, vimentin was observed independently in the nucleus of nonosteoblastic cells (Rogers, Eckelt et al. 1995) and osteoblasts (Monteiro, Hicks et al. 1994). Interestingly, vimentin can be modified by phosphorylation, leading to the generation of a 50-kDa truncated vimentin (Singh and Arlinghaus 1989), the same molecular mass as vimentin observed in rat osteoblasts (Fig. 11E). It is unknown

whether ATF4 binds to monomeric or polymeric forms of vimentin, which of these forms is present within the nucleus, and whether such interactions dictate the inhibitory and nuclear function of vimentin. Further studies are needed to elucidate these mechanisms.

Although less dramatically, *vimentin* overexpression also caused a decrease in the expression of some marker genes for immature osteoblasts, particularly *osterix* and *Runx2*, two important osteoblast differentiation factors (Ducy, Zhang et al. 1997; Komori, Yagi et al. 1997; Mundlos, Otto et al. 1997; Nakashima, Zhou et al. 2002). This result is interesting because it suggests that vimentin may also inhibit the function of *osterix* and *Runx2* in the regulation of osteoblast differentiation. However, whether this effect is via a direct interaction between vimentin and *Runx2* or *osterix* through domains other than the leucine-rich repeats, or via other indirect actions remains to be determined.

Based on our data, we speculate that the disappearance of vimentin in osteoblasts allows the progression of immature osteoblasts to fully differentiated and functional osteoblasts. One may wonder why there is such a need for several inhibitors of differentiation in the osteoblasts. One plausible explanation is that such inhibitors are important for the prevention of premature differentiation of osteoblasts, allowing for a sufficient pool of preosteoblasts with the proliferative potential to be maintained at the

site of bone formation, in response to extracellular cues. Interestingly, some extracellular cues, such as parathyroid hormone (Lomri and Marie 1988) and transforming growth factor (Lomri and Marie 1990), regulate *de novo* synthesis of vimentin in osteoblasts. However, it is unknown whether this regulation is temporal or dependent on differentiation progression. Further studies are needed to identify molecules involved in the regulation of vimentin expression in osteoblasts.

CHAPTER III

TGF-BETA-INDUCED REPRESSION OF ATF4 BY VIMENTIN DECREASES OSTEOCALCIN EXPRESSION AND INHIBITS OSTEOBLAST DIFFERENTIATION.

Introduction

Maintenance of osteoblast progenitors and their differentiation are highly synchronized events that are controlled by extracellular clues. TGF β is a bone-enriched extracellular growth hormone that plays important roles in bone, and it functions through supporting osteoblast progenitors (James, Levine et al. 2005; Vallier, Alexander et al. 2005; Watabe and Miyazono 2009) while inhibiting terminal differentiation (Centrella, Horowitz et al. 1994; Maeda, Hayashi et al. 2004).

Extracellular signaling factors can exert their functions through regulating transcriptional factors which directly control the on and off of tissue/cell-specific genes (Alliston, Choy et al. 2001; Liu, Black et al. 2001; Liu, Kang et al. 2004). Several osteoblast specific transcription factors have been identified in the past decades, including Runx2, osterix, and ATF4, the absence of which leads to poor or even failed

osteoblast differentiation and bone formation (Ducy, Zhang et al. 1997; Komori, Yagi et al. 1997; Otto, Thornell et al. 1997; Nakashima, Zhou et al. 2002; Yang, Matsuda et al. 2004). As an osteoblast specific transcription factor, ATF4 is important for osteoblast differentiation, osteogenesis, and terminal bone formation. Mutation of ATF4 leads to low bone mass, which is accompanied by low expression of osteoblast-specific genes, including osteocalcin. ATF4's function is at least partially transcriptional, since it binds directly to osteocalcin's promoter at the OSE1 site and transactivates its expression (Yang, Matsuda et al. 2004). In addition, ectopic expression of ATF4 in non-osteoblastic cells leads to overexpression of this osteoblast-specific gene's expression (Yang and Karsenty 2004).

Direct regulation of ATF4 has been demonstrated through many ATF4 interactors, such as RSK2, PKA, FIAT, STAB2, and Vimentin, and several of them have been shown to further have an effect on osteoblast differentiation and/or bone formation (Yang, Matsuda et al. 2004; Yu, Ambartsoumian et al. 2005; Dobрева, Chahrour et al. 2006; Elefteriou, Benson et al. 2006; Lian, Wang et al. 2009) . We previously demonstrated the interaction between vimentin and ATF4. Through binding with ATF4 at its leucine zipper domain, vimentin inhibits ATF4's transcriptional activity on osteocalcin gene expression.

Overexpression of vimentin leads to a delay in osteoblast differentiation and low expression of endogenous level of osteocalcin. Conversely, down-regulation of vimentin leads to up-regulation of endogenous osteocalcin gene expression (Lian, Wang et al. 2009).

Vimentin has been shown to be up-regulated by TGF β in several biological conditions such as EMT and cancer progression, with the functional relevance not clear to date (Margetts, Bonniaud et al. 2005). In this paper we show for the first time that TGF β up-regulates vimentin in osteoblasts through a non-canonical TGF β pathway resulting in vimentin protein synthesis, which mediates the inhibitory effect of TGF β on osteoblast differentiation. TGF β inhibits the endogenous as well as the ATF4-mediated *osteocalcin* expression, and these inhibitions were abolished when vimentin was targeted by siRNA knockdown. ATF4 is an important target of TGF β since osteoblasts lacking ATF4 are less or even un-responsive to TGF β signaling as compared to wild-type osteoblasts in terms of ALP activity, osteocalcin expression, and bone nodule mineralization. Although blocking TGF β signaling in vivo with 2G7 antibody can elevate bone mass, this effect was not observed in *Atf4*-deficient mice. Our findings revealed a new mechanism whereby TGF β , through a non-canonical pathway, upregulates vimentin,

which in turn inhibits ATF4's transcriptional activity as well as osteoblast differentiation and bone formation.

Methods

Materials

Tissue culture medium and fetal bovine serum were purchased from Invitrogen. Anti-Vim antibodies were from Santa Cruz Biotechnology (V9, against rat Vim) and Biovision (#3634, against mouse Vim). Antibodies against ATF4 (C20) and Sp1 (PEP2) were from Santa Cruz Biotechnology, against HA-tag from Abcam (ab91110), against Flag from Sigma (M2), and against phospho-Smad2 (#3101) from Cell Signaling. Recombinant human TGF- β 1 was from R&D systems. All chemicals except for the indicated ones were from Sigma.

Northern blot analysis and real time RT-PCR

Total RNA from adult mouse long bones, primary calvarial osteoblasts, 2T3 cells, or bone marrow stromal cells was isolated using TRIzol (Invitrogen) according to the manufacturer's protocols. 5 μ g total RNA was resolved in 1% agarose gel and

transferred onto nylon membranes. The membrane was cross-linked by UV light and hybridized following standard protocols with the indicated cDNA probes described previously (Lian, Wang et al. 2009). Real time RT-PCR was performed using a standard TaqMan or SYBR Green PCR Kit protocol on an Applied Biosystems 7300 machine. After treatment with DNase I, total RNA (2 µg) was reverse-transcribed with reverse transcriptase (Invitrogen) using 100 µM random hexamer primers. Specific oligonucleotide primers were either synthesized for SYBR Green (*Ocn*, forward CTACAAACGCATCTACGGTATCAC and reverse AGA GAGGACAGGGAGGATCAAG) or from Applied Biosystems for TaqMan system (*Gapdh*, 4352339E, RefSeq: NM_008084.2).

Immunofluorescence

ROS17/2.8 cells were grown on 4-well cultureslides for 16 h until reaching sub-confluence, washed with PBS, and fixed with Methanol for 20 min at -20 °C. Cells were then permeabilised and blocked with 5%BSA with 0.2% Triton X-100 in PBS for 30 min, and incubated with the respective primary antibody in 2% BSA overnight at 4°C. ATF4 proteins were stained with 1 µg/ml polyclonal anti-ATF4 antibody (C-20, Santa Cruz) and

Vim proteins were stained with 0.5 µg/ml monoclonal anti-Vim antibody (V9, Santa Cruz). After PBS-washing, cells were incubated with cy3-conjugated sheep anti-mouse or FITC-conjugated goat-anti-rabbit secondary antibody for 1 h at RT. After washing, cells were counter-stained with DAPI, washed briefly, and mounted in Aqua-Poly Mounting solution (Polysciences).

Pharmacological treatments

ROS17/2.8 cells or primary calvarial osteoblasts were grown on 60mm plates until 90% confluent and then incubated in serum free medium overnight. For TGFβ1 treatment, DMSO, SB505124 (Sigma), cycloheximide (Sigma), Wortmannin (EMD4Biosciences), or Rapamycin (EMD4Biosciences) were pre-incubated with cells for 30-60 min before addition of TGFβ1 or vehicle (4mM HCl with 1 mg/ml BSA). For whole cell lysate (WCL) and nuclear extracts (NE) collection, cells were collected 45 min or 4 hrs following TGFβ1 treatment. For luciferase assay, cells were lysed 6 hours post TGFβ1 treatment.

Transfection and Luciferase assay

Cells were seeded at a density of $2.5 \times 10^4/\text{cm}^2$ for 20 hrs and transfected with 0.6 $\mu\text{g}/\text{ml}$ of each indicated reporter plasmid (p6xOSE1-Luc, p6xmOSE1-Luc, or p3xAP1-Luc) together with 0.06 $\mu\text{g}/\text{ml}$ of β -galactosidase, mammalian expression plasmid (FLAG-ATF4, FosB, or HA-Vim), or psiRNA plasmids (psiRNA vector or psiRNA-Vim) using Lipofectamine. Cells were lysed 24 hrs later, and the luciferase activity was measured and normalized to the β -galactosidase activity as described (Lian, Wang et al. 2009).

Microcomputed tomography (μCT) analysis

Femur samples were collected and fixed overnight in 4% PFA (pH 7.4) and then 70% ethanol. Trabecular bone were evaluated using a μCT imaging system (Scanco μCT 40; Scanco Medical, Bassersdorf, Switzerland). The femur was fit into the specimen tube aligned with scanning axis. Tomographic images were acquired at 55kV and 145 mA with an isotropic voxel size of $12\mu\text{m}$, and at an integration time of 250 ms with 500 projections collected per 180 degree rotation. For 100 slices distal to the growth plate, contours were fit to the inner layer of the cortical bone. Applying the threshold of 300 mg

hydroxyapatite/cm³ to each femur, volume of trabecular bone tissue (TV), trabecular bone volume (BV) as well as volumetric density (in mgHA/cm³) of the mineralized tissue (BMD) were measured as fraction of mineralized tissue within the trabecular bone.

Osteoblast differentiation, ALP assay, von Kossa assay

Confluent (D0) primary calvarial osteoblasts in 24-well plates were grown in α MEM supplemented with 5 mM β -glycerophosphate and 100 μ g/ml ascorbic acid for 2 days followed by Alkaline phosphatase staining, 12 days subject to von Kossa staining and RNA isolation as described (Lian, Wang et al. 2009).

TGF β neutralizing antibody treatment regimen

10-week-old WT and *Atf4*^{-/-} mice (n>5) were treated with 10mg/kg of 2G7 or control IgG 3 times per week for 4 weeks by sterile intra-peritoneal injection.

Statistics

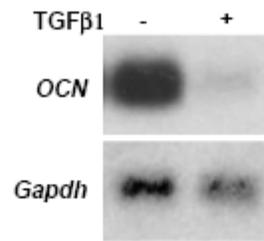
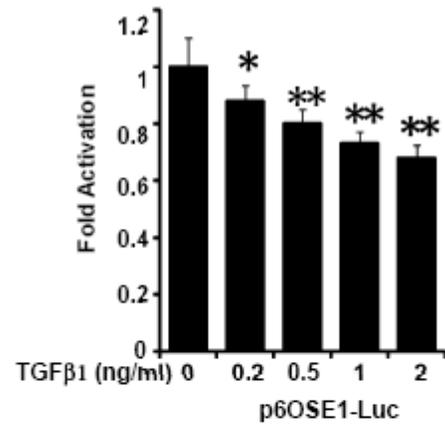
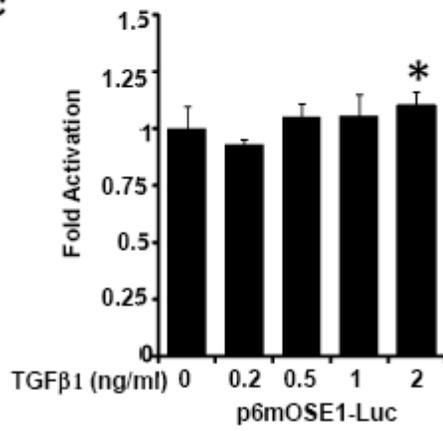
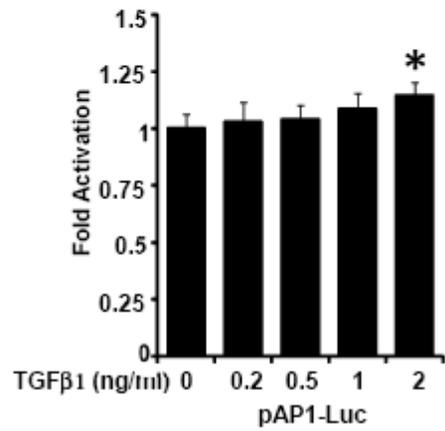
Data are expressed as mean \pm S.D. Statistical analysis was performed using an unpaired *t* test except for the 2G7 study, where paired *t* test was used.

Results

TGF β inhibits ATF4-mediated *Osteocalcin* expression.

As an inhibitor of osteoblast terminal differentiation, TGF β prevents expression of osteoblast-specific genes such as *osteocalcin* ((Centrella, Horowitz et al. 1994), Fig.12A). The suppression of *Osteocalcin* expression by TGF β is similar to what is observed in *Atf4*^{-/-} bone, and furthermore, ATF4 has been shown to be one of the transcription factors activating *osteocalcin* expression (Yang, Matsuda et al. 2004). These observations open the possibility that TGF β may inhibit osteoblast differentiation through suppressing ATF4. A luciferase construct driven by 6 copies of OSE1 (p6xOSE1-luc) has been shown to be specifically activated by ATF4 (Yang, Matsuda et al. 2004); this approach allowed us to test specifically if TGF β targets ATF4. We therefore transfected p6xOSE1-luc into ROS17/2.8 cells as a specific indicator of ATF4's transcriptional activity and treated these cells with increasing amount of TGF β 1 ranging from 0.2 to 2 ng/ml. TGF β 1 dose-dependently inhibited ATF4's transcriptional activity (Fig. 12B), and these effects were not observed or even reversed at a high dose of TGF β 1 when p6xmOSE1-luc (which ATF4 fails to bind due to point mutations

Figure 12. TGF β inhibited ATF4-mediated *Osteocalcin* expression. **A.** TGF β suppressed *Osteocalcin* expression in primary calvarial osteoblasts. Northern blot analysis on *Ocn* from total RNA of osteoblasts treated with TGF β 1 (2 ng/ml) or vehicle for 10 days upon differentiation. *Gapdh*, loading control. **B-D.** TGF β dose-dependently inhibits ATF4-mediated *Ocn* transactivation. ROS cells transfected with a construct containing 6 copies of ATF4 binding site of the *Ocn* promoter (p6XOSE1-Luc, **B**), mutant OSE1 that ATF4 fails to bind (p6xmOSE1-Luc, **C**), or 3 repeats of AP1 binding site (negative control, **D**).

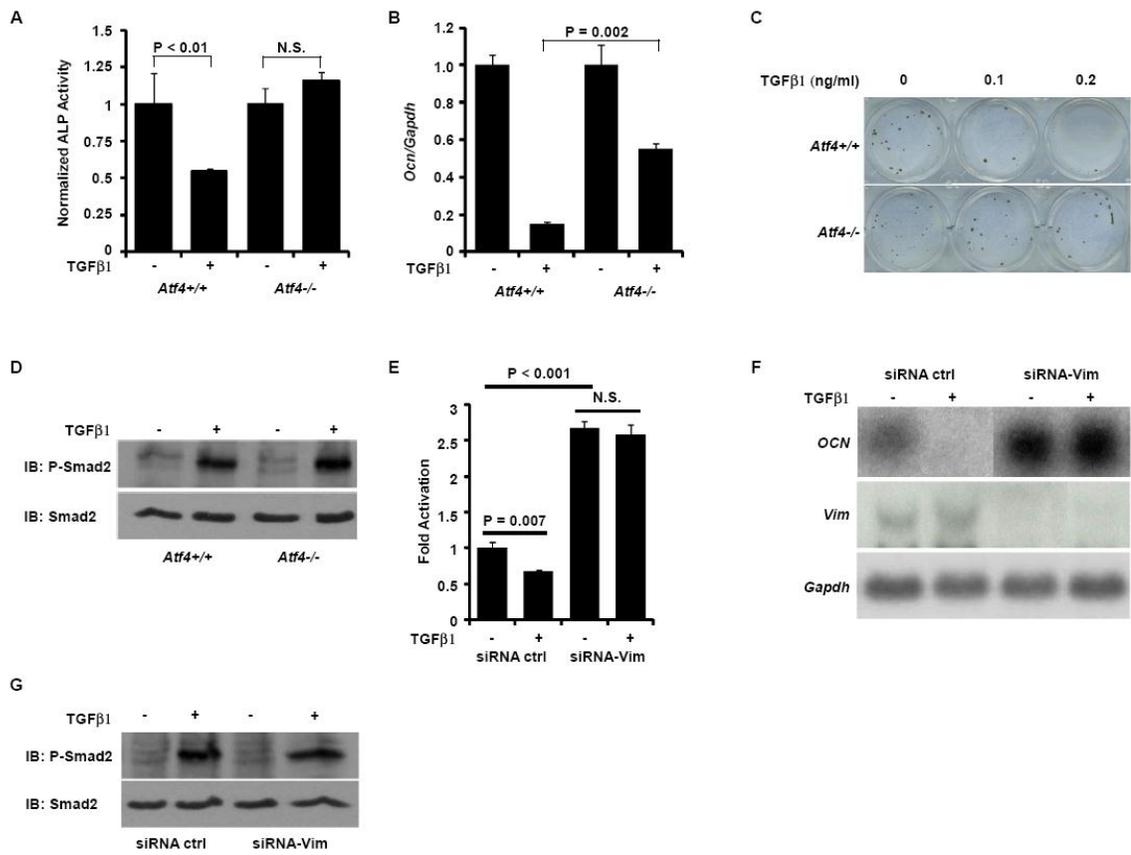
A**B****C****D**

(Yang, Matsuda et al. 2004)) or pAP1-luc (an unrelated promoter for another leucine zipper transcription factor), were transfected, suggesting that ATF4 is a specific target of the TGF β signaling pathway (Figs. 12 C&D).

ATF4 is required for TGF β -mediated inhibition on primary calvarial osteoblast differentiation and vimentin mediated the effect of TGF β on ATF4.

During osteoblast differentiation, osteoblastic genes, such as alkaline phosphatase (ALP) and *osteocalcin* (*Ocn*), will be turned on, and as a subsequent event, bone nodules will be formed. The expression level of ALP and *Ocn* is thus an indicator of the maturation of osteoblasts. Inhibition of osteoblast differentiation by TGF β is accompanied by decrease in ALP activity, suppressed osteocalcin expression, as well as a reduced number of bone nodules formed (Centrella, Horowitz et al. 1994) and WT in Figs. 13A, B&C). Since TGF β 1 dose-dependently inhibits ATF4's transactivation ability, we next test if this inhibition is crucial in mediating TGF β 's effect on osteoblast differentiation by treating WT and *Atf4*^{-/-} primary osteoblasts with TGF β 1. In the absence of ATF4, TGF β 's inhibition on ALP activity was totally blocked, the ratio of *osteocalcin* downregulation was partially restored and similar amount of bone nodules were formed

Figure 13. TGF β -mediated inhibition on primary calvarial osteoblast differentiation was blocked in ATF4-deficient cells, and vimentin mediated the effect of TGF β on ATF4. A-C. WT and *Atf4*^{-/-} calvarial osteoblasts were treated with TGF β 1 (0.2 ng/ml) or vehicle for 2 days for ALP activity assay (**A**); and 10 days for Real-time RT PCR analysis on *osteocalcin* expression (**B**); and von Kossa assays of mineralized nodules (**C**). **D.** Western blot analysis on whole cell lysate of WT or *Atf4*^{-/-} bone marrow stromal cells (upper 2 panels) and calvarial osteoblasts (lower 2 panels) upon TGF β 1 (0.5 ng/ml) or vehicle treatment for 45 min. Phospho-smad2 was blotted as indicator of intact canonical TGF β signaling. Total smad2, loading control. **E.** TGF β 1 (0.5 ng/ml) or vehicle treated 2T3 cells transfected with p6xOSE1-luc reporter and *siRNA-Vim* or control. **F.** 2T3 osteoblastic cells were treated with TGF β 1 (0.5 ng/ml) or vehicle in the presence and absence of siRNA against *Vim*. Total RNA were collected 2 days following TGF β 1 treatment. Northern blot probing *Ocn*, *Vim*, and *Gapdh* RNA. **G.** 2T3 osteoblastic cells were treated with TGF β 1 (0.5 ng/ml) or vehicle in the presence and absence of siRNA against *Vim*. Whole cell lysate were collected 45 min following TGF β 1 treatment. Western blot detecting phosphor-smad2 and total smad2 were shown.



in the presence of TGF β 1, indicating that TGF β inhibits osteoblast differentiation in part through targeting ATF4 (Figs. 13A, B&C). The abolished effect of TGF β was not due to disrupted TGF β receptors, as regular TGF β signaling was intact as shown by normally phosphorylated smad2 (Fig. 13D) and smad3 (data not shown) in *Atf4*^{-/-} osteoblasts following TGF β 1 treatment. Taken together, these results suggest that ATF4 serves as a crucial target of TGF β signaling.

We have previously shown that vimentin inhibits *osteocalcin* expression through suppressing ATF4's transcriptional activity and thus osteoblast differentiation (Lian, Wang et al. 2009). To test directly if TGF β inhibits ATF4-mediated *osteocalcin* expression via up-regulation of vimentin, we transfected p6xOSE1-luc into 2T3 osteoblastic cells while silencing vimentin with shRNA against it and then treated the cells with TGF β 1 or vehicle. Consistent with Fig. 12B, TGF β 1 inhibits ATF4-mediated *osteocalcin* expression; however this inhibition was abolished when vimentin was knocked down (Fig. 13E). As reported, TGF β 1 inhibits endogenous *osteocalcin* expression ((Centrella, Horowitz et al. 1994), Fig.2F compare 1st with 2nd lanes), and the endogenous osteocalcin level in 2T3 cells was elevated when vimentin was knocked down ((Lian, Wang et al. 2009), Fig.2F compare 1st and 3rd lanes). Similar to the ATF4-

mediated *osteocalcin* expression as determined by p6xOSE1-luc, TGF β 's inhibition of endogenous osteocalcin transcription was also blocked when vimentin was turned down, indicating vimentin is required for mediating this effect (Fig. 13F). To rule out the possibility that main players upstream in the TGF β signaling pathway were defective when Vimentin was turned down, we again tested the level of Smad2 phosphorylation following TGF β treatment, and the same extent of phosphorylation was observed in control and vimentin-knockdown cells (Fig. 13G), confirming it was the absence of vimentin that abolished TGF β 's effect. These data collectively imply that ATF4 is required for TGF β -mediated inhibition on primary calvarial osteoblast differentiation, and vimentin mediates the effect of TGF β on ATF4.

TGF β -neutralizing antibody does not increase bone volume in ATF4-deficient mice

TGF β is becoming a therapeutic target in several clinical and preclinical studies (Korpal and Kang 2010). Monoclonal TGF β -neutralizing antibodies, such as 2G7 and 1D11, have been shown to suppress lung metastases and increase bone mass through binding and reducing the biological activity of all three forms of TGF β (Arteaga, Hurd et al. 1993; Pinkas and Teicher 2006; Edwards, Nyman et al. 2010). If ATF4 is an

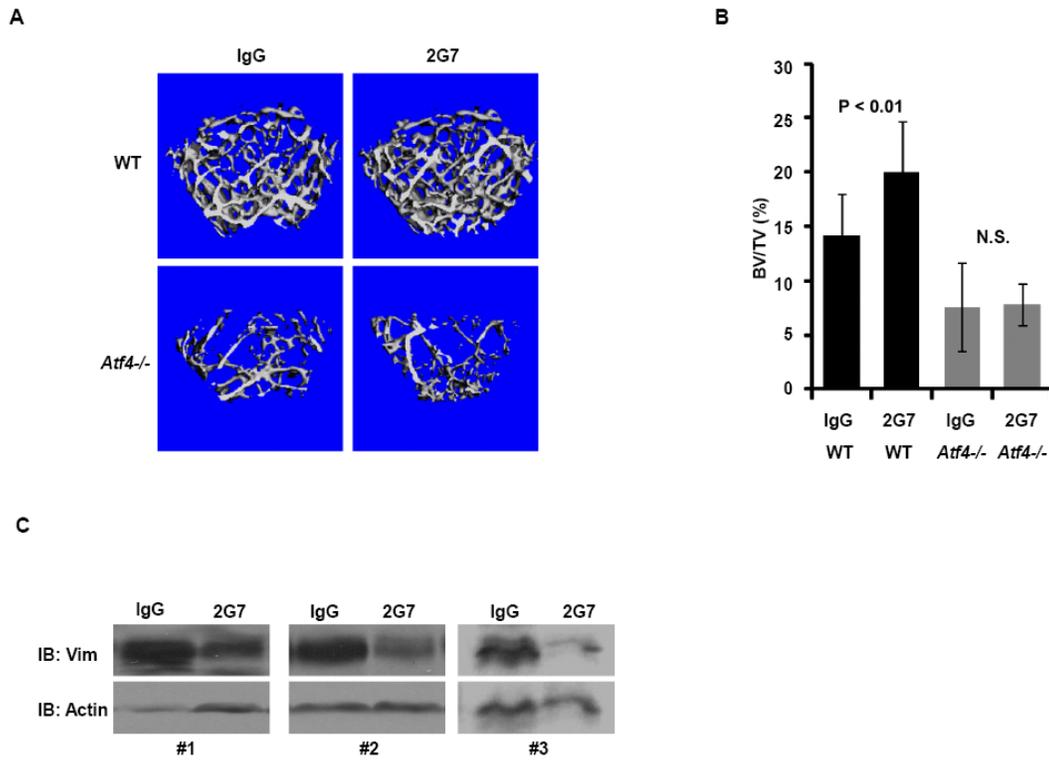
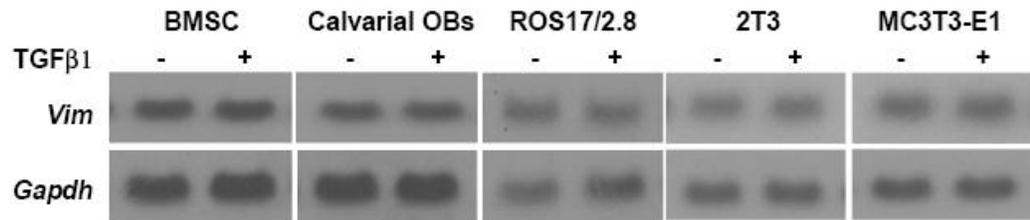
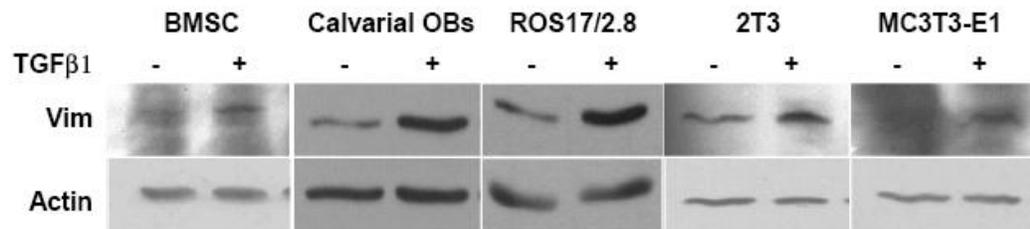
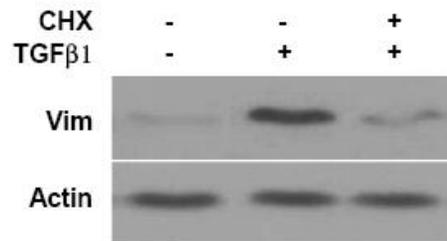
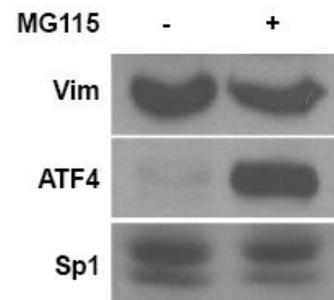


Figure 14. *Atf4*^{-/-} femur bone mass does not increase upon 2G7 treatment. A & B. μ CT analysis of trabecular bones from WT and *Atf4*^{-/-} femurs treated with 2G7 or IgG control. N=4 for WT group. N=6 for *Atf4*^{-/-} group. **B.** Quantification of Figure A. **C.** Western blot analysis of 3 independent pairs of WT long bones demonstrating an inhibited expression of vimentin protein in the presence of 2G7.

important target of TGF β , further inhibiting TGF β signaling with 2G7 antibody would not restore bone mass in the absence of ATF4. This scenario is worth testing not only as a proof of concept approach, but also would be practical in the clinic, given the human diseases associated with insufficient ATF4 activity (Yang, Matsuda et al. 2004; Dobreva, Chahrour et al. 2006). To test this hypothesis, we injected 2G7 intraperitoneally into wild-type and *Atf4*^{-/-} mice for 4 weeks. Similar to reports from the 1D11 monoclonal antibody study, 2G7 increased bone mass in wild-type mice by more than 30%. However, it fails to further increase the bone mass in *Atf4*-deficient mice (Fig. 14A & B). The resistance to 2G7 treatment observed in *Atf4*-deficient bones suggests that ATF4 is an important molecule downstream of the TGF β -mediated bone remodeling event. Since depletion of vimentin via RNA silencing abolished the TGF β -mediated inhibition on ATF4, we next investigate if TGF β exerts its effect through stimulating vimentin expression in vivo by comparing WT bones treated with IgG control with those treated with 2G7 TGF β -neutralizing antibodies. As shown in Fig. 14C, all three pairs of long bones examined displayed a decrease in endogenous level of vimentin when the TGF β signaling was blocked with the 2G7 antibody, indicating that TGF β induces endogenous vimentin expression in bones.

Figure 15. TGF β stimulated Vim expression in osteoblast-related cells. **A.** Northern blot analysis of indicated cells treated with TGF β 1 (0.2 ng/ml) or vehicle for 4 hours. **B.** Whole cell lysate (WCL) from indicated cells administered with TGF β 1 (0.2 ng/ml) or vehicle for four hours. **C.** Effect of protein synthesis inhibitor cycloheximide (CHX) on TGF β -induced upregulation of Vim. **D.** Western blot analysis of COS1 cells treated with MG115 (proteasome inhibitor) or vehicle. Sp1, loading control.

A**B****C****D**

TGF β upregulates vimentin in osteoblasts

We then investigated whether vimentin is upregulated by TGF β specifically in osteoblasts. It has been reported in C2C12 myogenic cells that TGF β 1 elevated Vimentin mRNA levels (Wu, Zhang et al. 2007). However, among all the osteoblastic cell lines tested, including bone marrow stromal cells (BMSCs), primary rat calvarial osteoblasts, rat osteoblast-like ROS17/2.8 cells, 2T3 cells, and MC3T3-E1 cells, none of them displayed increased vimentin RNA levels when treated with TGF β 1 (Fig.15A), whereas vimentin protein level was elevated dramatically (Fig.15B).

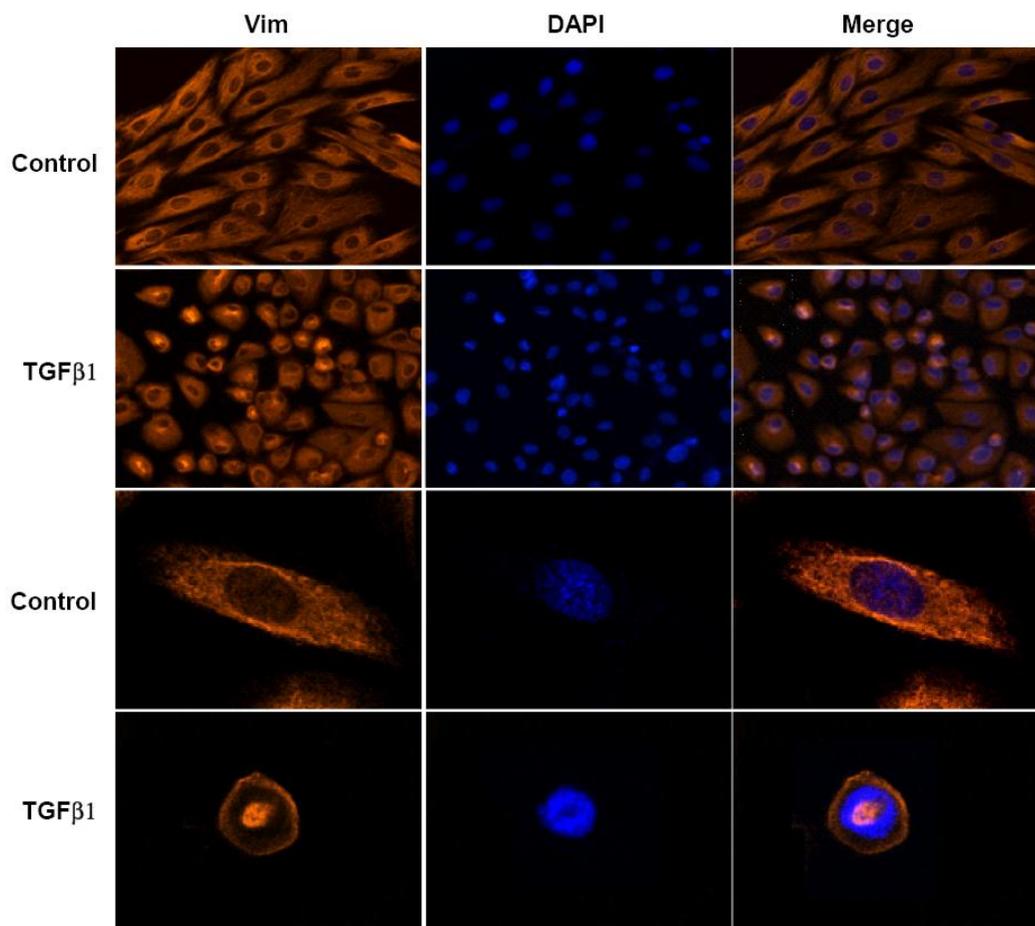
The upregulation of a protein can often be achieved through direct accumulation of its mRNA, induced protein synthesis, and/or aborted protein degradation. Since the level of *vimentin* mRNA was not changed upon TGF β treatment, we further investigated, at a post-transcriptional level, whether the translation or degradation of vimentin protein is affected following TGF β treatment. When treated with protein synthesis inhibitor cycloheximide, vimentin protein can no longer be elevated by TGF β , suggesting a protein synthesis pathway is involved (Fig. 15C). ATF4 protein is known to accumulate when the proteasome degradation machinery is inhibited by MG115 ((Yang and Karsenty 2004) and Fig. 15D). However, this effect was not observed for vimentin

protein (Fig.15D). These results collectively imply that the upregulation of vimentin induced by TGF β is mainly mediated by protein synthesis but not through inhibition of its protein degradation.

TGF β induces vimentin translocation in osteoblasts

As a transcription factor, ATF4 exerts its function through directly interacting with its cognate binding site within the nucleus. In a scenario where vimentin suppresses ATF4's transcriptional activity in osteoblasts, one would expect the presence of vimentin in the nucleus of osteoblasts. Consistent with our general understanding about vimentin as a cytoskeletal protein, under a normal cellular condition, vimentin was largely found outside of the nucleus in ROS17/2.8 cells (Fig. 16, control). I then tested whether under certain condition, for example, when treated with TGF β , vimentin would translocate into the nucleus of osteoblasts and therefore interact with ATF4 and inhibit its activity. Consistent with this hypothesis, when I treated ROS17/2.8 cells with TGF β , vimentin dramatically reorganized and concentrated within the nucleus, further supporting my previous observation that vimentin inhibit ATF4's binding to its cognate binding site, OSE1.

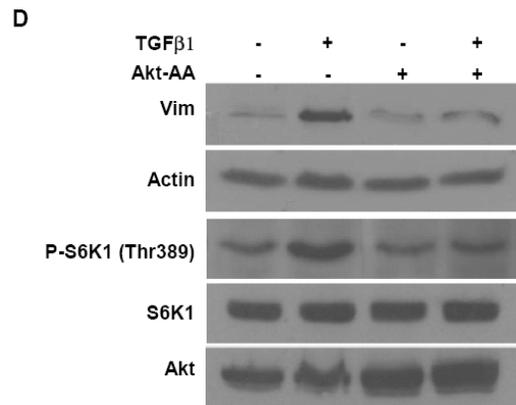
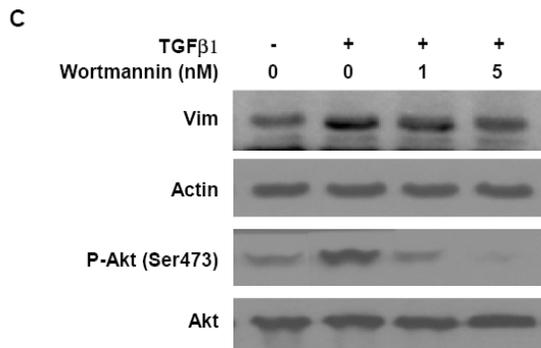
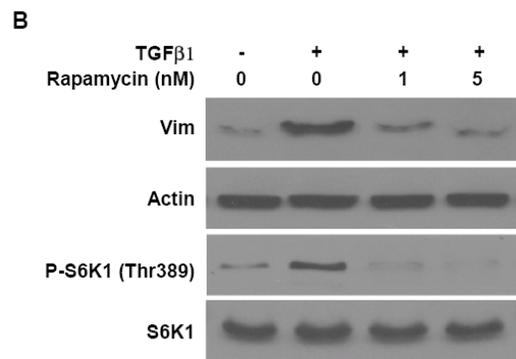
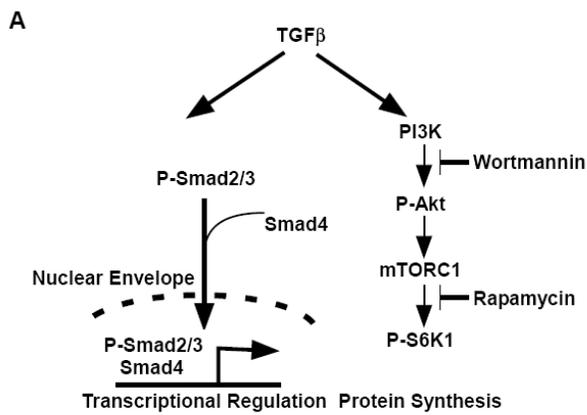
Figure 16. TGF β induces vimentin translocation in osteoblasts. Confocal immunofluorescent analysis of ROS17/2.8 cells, treated with TGF β (0.5ug/ml) or vehicle for 5 hours, using Vimentin mouse mAb V9 from Santa Cruz (orange color from Cy3-conjugated secondary antibody). Blue pseudocolor = TO-PRO $^{\text{®}}$ -3 (fluorescent DNA dye). 20x for upper two panels and 63x for lower two panels. Experiments were performed with Zeiss LSM 510 inverted confocal microscope from the VUMC Cell Imaging Shared Resource.



TGF β -mediated up-regulation of vimentin depends on PI3K-Akt-mTOR pathway

TGF β functions through both canonical and non-canonical pathways, and the phosphorylation of Smad2/3 is a key signaling event in the canonical pathway (Massague 1998). Once phosphorylated, Smad2/3 forms a heterodimeric complex with Smad4 (Co-Smad) and associates with additional DNA binding cofactors to turn on target genes, and thus modulates downstream events directly at the transcriptional level (Massague 1998). On the other hand, in a non-canonical manner, TGF β induces protein synthesis during epithelial to mesenchymal transition (EMT), and this translational regulation results from activation by TGF β of mammalian target of rapamycin (mTOR) through phosphatidylinositol 3-kinase (PI3K) and Akt, leading to the phosphorylation of S6 kinase 1 (S6K-1) and eukaryotic initiation factor 4E-binding protein 1, which are direct regulators of translation initiation (Lamouille and Derynck 2007). This PI3K/Akt/mTOR/S6K-1 pathway can be blocked by Wortmannin (a specific inhibitor of PI3K), Akt-AA (a dominant negative Akt construct), or Rapamycin (a specific inhibitor of

Figure 17. TGF β induces vimentin via PI3K-Akt-mTOR signaling pathway. A. Divergent TGF β signaling pathways induce translational regulation through smads and translational regulation through the PI3K/Akt/mTOR pathway. **B.** Western blot analysis of ROS17/2.8 cells treated with TGF β 1 (0.2 ng/ml) or vehicle and Rapamycin of indicated amount (nM) for 1 hr staining for phospho-S6K1 (Thr389) and 4 hrs staining for Vim. **C.** Western blot analysis of ROS17/2.8 cells treated with TGF β 1 (0.2 ng/ml) or vehicle and Wortmannin of indicated amount (nM) for 1 hr staining for phospho-Akt (Ser473) and 4 hrs staining for Vim. **D.** Western blot analysis of ROS17/2.8 cells transfected with dominant negative Akt construct (Akt-AA) blocked the TGF β -mediated vimentin up-regulation.



mTOR complex 1) (Arcaro and Wymann 1993; Stokoe, Stephens et al. 1997; Wullschleger, Loewith et al. 2006; Lamouille and Derynck 2007). Because vimentin protein synthesis is stimulated by TGF β 1, we next examined whether this TGF β -induced production of vimentin is blocked when this pathway is suppressed pharmacologically with Wortmannin or Rapamycin (Fig. 17A). The phosphorylation of S6K-1 induced by TGF β 1 was turned down when treated with 1-5 nM of Rapamycin (Fig. 17B). Similarly, at doses as low as 1-5 nM, Wortmannin blocked the phosphorylation of Akt induced by TGF β (Fig. 17C). At both conditions, TGF β failed to elevate vimentin protein level, suggesting a requirement of this pathway in mediating TGF β 's effect on vimentin (Fig. 17B & 17C). To rule out the off-target effect of the pharmacological manipulations, we then over-express a Akt mutant (Akt-AA) which has previously been shown to prevent Akt activation through dominant-negative interference (Stokoe, Stephens et al. 1997). Similar to the previous reports, this Akt-AA construct blocked the phosphorylation of S6K-1 ((Lamouille and Derynck 2007) and Fig. 17D). The observation that TGF β failed to up-regulate vimentin protein level in the presence of Akt-AA further support the notion that the PI3K/Akt/mTOR/S6K-1 pathway is mediating the TGF β -induced vimentin up-

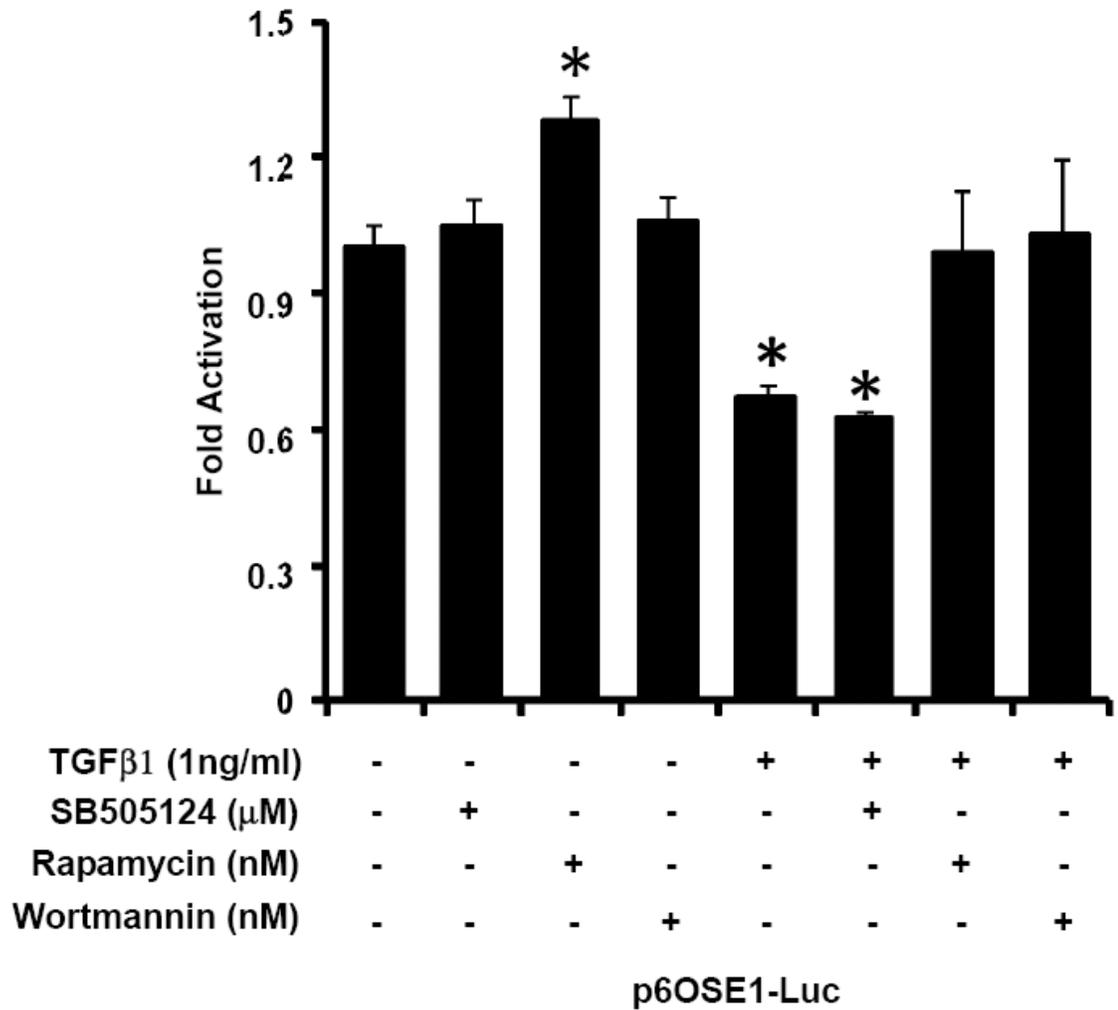


Figure 18. Inhibition of ATF4-mediated *Osteocalcin* expression by TGFβ was abolished when the PI3K-Akt-mTOR pathway was blocked. Luciferase assay of ROS17/2.8 cells transfected with p6OSE1-luc followed by 6 hours of the incubation of SB505124 (0.4 μM), Rapamycin (5nM), or Wortmannin (5nM), together with TGFβ1 (1ng/ml) or vehicle.

regulation in osteoblasts. Moreover, at the dose of interest, rapamycin abrogated TGF β -stimulated vimentin upregulation with the phosphorylation of smad2/3 intact provides another piece of evidence that TGF β 1 induces vimentin protein synthesis independent of the canonical pathway.

Inhibition on the PI3K-Akt-mTOR pathway prevents the ATF4-mediated *osteocalcin* expression from being inhibited by TGF β

We previously established vimentin as an inhibitor against osteoblast differentiation (Lian, Wang et al. 2009). Since the inhibition of the PI3K/Akt/mTOR pathway blocked the TGF β -induced vimentin up-regulation, these pharmacological treatments may also result in a release of the inhibition on osteoblast differentiation by TGF β . In effect, the treatments of rapamycin and wortmannin have both been demonstrated to promote the osteoblastic differentiation (Kratchmarova, Blagoev et al. 2005; Lee, Yook et al. 2010). To test if the inhibition on TGF β -mediated vimentin upregulation via targeting the PI3K/Akt/mTOR pathway has a specific effect on the ATF4-induced *osteocalcin* expression, we treated p6OSE1-luc-expressing ROS17/2.8 cells with rapamycin and wortmannin, with SB505124 as a negative control. In the

presence of TGF β , the ATF4-mediated *osteocalcin* expression was inhibited, which was rescued by rapamycin or wortmannin, but not by SB505124. This is in line with the observations that TGF β failed to upregulate vimentin when rapamycin or wortmannin was co-administered (Fig.18).

Decreased bone mass in vimentin-deficient mice

Based on TGF β and vimentin's inhibition on ATF4 and osteoblast differentiation, I suspected that vimentin-deficient mice would display high-bone-mass phenotype. I then collected and analyzed femur from 3-month-old vimentin-deficient mice. Nevertheless, μ CT analyses revealed decreased bone volume (BV/TV, Fig.19A), connective density (Conn. D, Fig.19B), trabecular numbers (Tb N, Fig.19C) and trabecular thickness (Tb Th, Fig.19D), and significantly increased trabecular spacing (Tb Sp, Fig.19E) in these vimentin-deficient femur. These observations suggested that absence of vimentin at a whole-body level results in abnormalities of bone homeostasis.

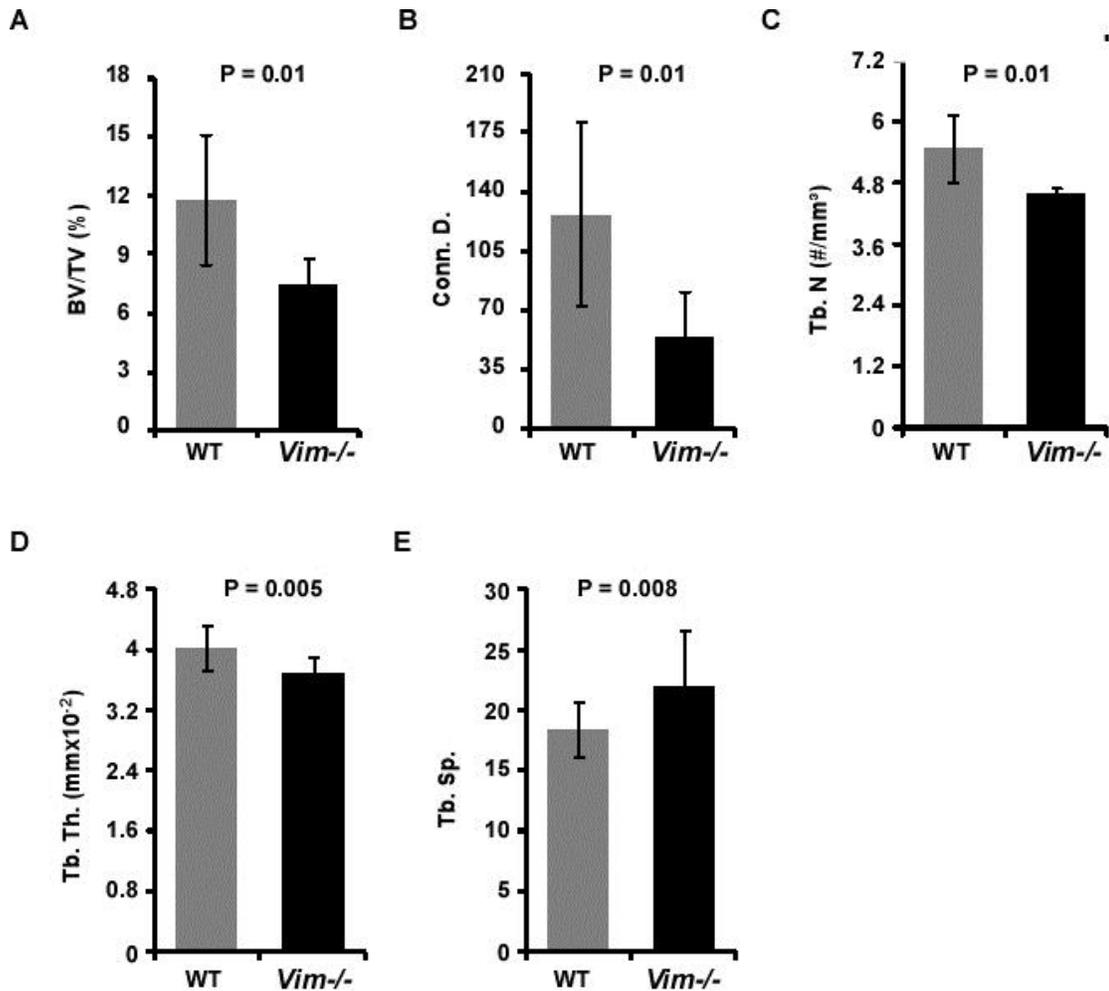


Figure 19. μ CT analysis showing that adult *Vim*^{-/-} femur bones display bone abnormalities. Significantly decreased bone volume (BV/TV, **A**), connective density (Conn. D, **B**), trabecular numbers (Tb N, **C**) and trabecular thickness (Tb Th, **D**), and significantly increased trabecular spacing (Tb Sp, **E**) in 3-month old *Vim*^{-/-} mice compared to their WT littermates. n=7.

Discussion

In this study, we show for the first time that TGF β up-regulates vimentin in osteoblasts through a non-canonical TGF β pathway resulting in vimentin protein synthesis, which mediates the inhibitory effect of TGF β on osteoblast differentiation. TGF β inhibits the endogenous as well as the ATF4-mediated *osteocalcin* expression, and these inhibitions were abolished when vimentin was targeted by knockdown. ATF4 is an important target of TGF β since osteoblasts lacking ATF4 are less or even not responsive to TGF β signaling as compared to wild-type osteoblasts. In line with this finding, although blocking of TGF β signaling in vivo with 2G7 antibody can elevate bone mass, this effect was not observed in ATF4-deficient mice. Our findings revealed a new mechanism whereby TGF β , through a non-canonical pathway, upregulates vimentin, which in turn functionally inhibits ATF4's transcriptional activity as well as osteoblast differentiation and bone formation.

ATF4 as a novel transcription factor targeted by the TGF β signaling during osteoblast differentiation

TGF β has been well known for inhibiting osteoblast differentiation in vitro. However, the mechanism underlying this is not well established. Runx2 has been identified as a main transcription factor targeted by TGF β through Smad3. Since Runx2 is believed to be the master regulator of osteoblast differentiation, and lies upstream of ATF4 with regard to the regulatory pathway, it opens the possibility that the inhibition of ATF4-mediated *osteocalcin* expression by TGF β is actually relayed by Runx2. However, since the inhibitory effect of TGF β on p6OSE1-luc was observed 4 hours post TGF β treatment, which was much earlier than what was observed on p6OSE2-luc (12 hours); this inhibition of ATF4 is likely to be independent of Runx2. Secondly, we were able to reverse the inhibition of TGF β on p6OSE1-luc when rapamycin or wortmannin was applied, which does not affect the phosphorylation and activation of Smad2/3. This further strengthens the hypothesis that the TGF β -mediated inhibition on ATF4 is distinct from effects on Runx2.

Although Runx2 is a well-accepted target of TGF β signaling, since Runx2^{-/-} cells can hardly differentiate into osteoblasts or form the bone, it is unlikely to prove that the

osteoblast differentiation program is not or less affected by TGF β in the absence of Runx2. However, this becomes possible with the ATF4 model since *Atf4*^{-/-} osteoblasts, although less differentiated, are still functional. The fact that *Atf4*^{-/-} osteoblasts are resistant to or less affected by TGF β signaling further suggested ATF4 as an important target of TGF β . However, our findings do not lessen the importance of Runx2 as a target of the TGF β signaling. Although *Atf4*^{-/-} osteoblasts responded much less to TGF β in terms of *osteocalcin* expression as compared to wildtype cells, they did have less transcription of *osteocalcin* in the presence of TGF β , suggesting that other targets of TGF β signaling, such as Runx2, are still present in the absence of ATF4.

Vimentin is a molecular relay of the TGF β signaling in osteoblast

Vimentin has frequently been reported to be upregulated by TGF β in non-osteoblast cells including C2C12 and be associated with several physiological or pathological conditions such as EMT and cancer, but the functional relevance of this is not fully understood. Here we report that although *vimentin* mRNA (this makes it distinctive to the C2C12 case) is not responsive to TGF β signaling in osteoblasts, vimentin protein is up-regulated by TGF β in osteoblasts through a non-canonical manner.

We previously reported that vimentin directly binds to and inhibits ATF4's transcriptional activity. The fact that TGF β inhibits ATF4's transcriptional activity and this inhibition was blocked when vimentin was knocked down with siRNA suggested that the up-regulation of vimentin by TGF β is functionally inhibiting the osteoblast differentiation program. Our study does not exclude the possibility that vimentin is mediating the effect of other signaling events. It has recently been reported that PTH, the intermittent administration of which increases bone mass, increases ATF4's expression and activity in osteoblasts (Yu, Franceschi et al. 2008). Interestingly, administration of PTH to human osteoblastic cells decreased the de novo biosynthesis of vimentin (Lomri and Marie 1990), giving the possibility that vimentin serves as a molecular switch transmitting extracellular clues to osteoblasts.

We previously reported that both vimentin mRNA and protein level were gradually turned down during osteoblast differentiation. However, TGF β was only able to control the vimentin protein levels. This indicates that other signaling pathways are involved in regulating its transcription and/or RNA stability.

Clinical implications of the TGF β -mediated inhibition of ATF4

TGF β is becoming a therapeutic target in several clinical and preclinical studies (Korpal and Kang 2010). Monoclonal TGF β -neutralising antibodies, such as 1D11, have been shown to increase bone mass through binding and reducing the biological activity of all three forms of TGF β (Edwards, Nyman et al. 2010). With the knowledge that ATF4 lies downstream of signaling and the observation that *Atf4*^{-/-} mice do not respond to TGF β neutralizing antibody 2G7, we may provide a molecular basis for clinical cases involving a small population of patients who do not respond to bone restoring therapies that involve inhibiting TGF β signaling events. For example, ATF4 has been suggested as a critical substrate of RSK2, the mutation of which leads to Coffin-lowry syndrome, a mental retardation condition associated with skeletal abnormalities. As a result, Coffin-lowry patients may not respond to the TGF β based treatment due to the low activity of ATF4, a scenario comparable to the *Atf4*^{-/-} model.

Phenotypes of vimentin-deficient mice

Given the inhibitory effect of vimentin observed in this study on ATF4 and osteoblast differentiation and the expression pattern of vimentin at both RNA and protein

level, the low-bone-mass phenotype of vimentin-null mice is unexpected, since all else being equal, knocking out of vimentin in osteoblast should result in a better osteoblast differentiation and subsequent high bone mass. Several factors may contribute to our observations: 1) the mouse model I relied on is a whole-body knockout, in which vimentin is mutated in both osteoblasts and osteoclasts. Osteoclasts may be hyper-activated in the absence of vimentin in a direct and/or indirect manner. Vimentin may serve as a direct inhibitor within osteoclasts to suppress their differentiation or activity, but this is not very likely due to a low expression level of vimentin in osteoclasts; alternatively, given that osteoblasts facilitate osteoclast differentiation through secreting factors such as RANKL, the inactivation of vimentin in osteoblasts may indirectly activate osteoclasts in vivo. 2) Vimentin may be required for inhibition of osteoblast differentiation in osteoprogenitor cells that give rise to a sufficient pool of preosteoblasts. Inactivation of vimentin may release this inhibition leading to a premature osteoblast differentiation. The net result in adult mice may be a decreased number of active bone matrix-secreting osteoblasts and less bone. 3) Inactivation of vimentin may systematically lead to unfavorable changes in circulating factors such as estrogen and vitamin D, which may negatively affect bone homeostasis.

CHAPTER IV

CONCLUSIONS, DISCUSSION AND FUTURE DIRECTIONS

ATF4 is an important transcription factor required for timely osteogenesis, accrual bone mass maintenance during adulthood, as well as proper and adequate osteoblastogenesis. As a result, it is of no surprise that ATF4 serves as a critical regulatory point tightly monitored under the osteoblast differentiation program. In particular, this molecule is highly regulated at a post-transcriptional level, where proteasome-mediated degradation, phosphorylation-based activation, and protein-protein interaction all affect the ultimate function of ATF4 in the context of osteoblast differentiation and tissue-specific gene expression. As a transcription factor, ATF4 is directly involved in the process of osteoblast differentiation in part by directly turning on the transcription of osteoblast-related genes. In physiological context, most of the tissue-specific differentiation is triggered initially by extracellular clues, such as Parathyroid Hormone (PTH) and Transforming Growth Factor beta (TGF β). It has recently been reported that PTH, the intermittent administration of which increases bone mass,

increases ATF4's expression and activity in osteoblast, and the anabolic actions of PTH in bone are severely impaired in mice lacking ATF4 (Yu, Franceschi et al. 2008). Our work provides the first piece of evidence that ATF4 is also the target of the TGF β signaling pathway. During osteoblast differentiation, the *osteocalcin* gene is gradually turned on, a hallmark of osteoblast maturation. TGF β inhibits the terminal differentiation of osteoblasts, accompanied by decreased bone nodule formation and suppressed elevation of osteocalcin expression. Since *osteocalcin* is identified as a target of ATF4 in osteoblasts by direct promoter binding assay (chromatin immunoprecipitation and gel shift assay) and reporter assay in vitro and a genetic model in vivo, we tested whether ATF4 is functionally regulated by TGF β by using reporter assay with a luciferase reporter under the control of 6 copies of Osteoblast Specific Element 1 (OSE1), named p6OSE1-luc, which was documented as the ATF4's binding site in the *osteocalcin* promoter. The activity of p6OSE1-luc, but not that of p6mOSE1-luc which ATF4 fails to activate, gradually decreased when increasing amount of TGF β 1 was given, with the protein level of ATF4 unchanged, suggesting that TGF β 1 dose-dependently inhibits ATF4's transcriptional activity.

Deficiency in ATF4 or inhibition of ATF4's transactivation ability, for example, by FIAT, a leucine zipper nuclear molecule lacking a basic domain for DNA binding that interacts with ATF4 to repress its transcriptional activity, has been shown to result in low osteocalcin expression and osteopenia. We reason TGF β may exert its inhibitory effect on osteoblast differentiation and bone mass partially through repressing ATF4, and if this hypothesis holds true, treatment of TGF β on *Atf4*-deficient osteoblasts should result in less inhibitory effect. To monitor the level of osteoblast differentiation, we measured ALP activity, the number of bone nodule formed, and the expression of *osteocalcin*, all of which have been shown to be inhibited by TGF β . Interestingly, although a comparatively low dose of TGF β 1 ranging from 0.1-0.2 ng/ml significantly down-regulated ALP activity, bone nodule formation, and expression of *osteocalcin* in wild-type calvarial osteoblasts as previously reported, these inhibitions were much more mild in the absence of ATF4. These in vitro experimental results established ATF4 as a functional target of TGF β signaling in these bone-forming cells. In vivo, several strategies for disrupting TGF β signaling have been developed, such as 1D11 and 2G7, the TGF β -neutralizing antibodies blocking all three forms of TGF β . Suppression of the TGF β signaling post-natally has been known to result in anabolic effects. For example, a 4-week treatment of

1D11 increased BMD, trabecular thickness and BV/TV, accompanied by elevated osteoblast numbers (Edwards, Nyman et al. 2010). Similarly, comparing the effect of 2G7 treatment on wild-type and *Atf4*-null mice will give insights to the role of ATF4 as an in vivo target of TGF β . While administration of 2G7 on wild-type mice results in similar phenotypes as 1D11 such as increased bone mass, this effect was impaired when ATF4 is mutated, confirming in vivo that ATF4 is involved in transmitting the effect of TGF β in the maintenance of bone mass.

Mutations of ATF4 have not yet been reported in any known human disease so far, and this may suggest how vital ATF4 can be as a multifunctional transcription factor functioning in many aspects of developmental biology. However, in the clinic, several pathological conditions associated with skeletal defects have been linked to the inadequate activity of ATF4, including Coffin-Lowry syndrome (caused by inactivating mutation of *Rsk2* gene) and a genetic disease resulting in cleft palate and low bone mass (caused by inactivating mutation of *Satb2* gene) (Yang, Matsuda et al. 2004; Dobrev, Chahrour et al. 2006). Although not directly tested, our finding of the resistance to TGF β -neutralizing antibody treatment observed in *Atf4*-deficient mice suggests a

potential limitation in the application of anti-TGF β therapy in these patients who may resemble an ATF4-null condition to some certain extent.

TGF β is known to exert its function through both canonical and non-canonical pathways, depending on whether the phosphorylation of Smad2/3 is required. A classic view of the TGF β -mediated inhibition of osteoblast differentiation mainly involves Runx2, the master regulator of osteoblast differentiation. TGF β represses Runx2's transactivation ability in a canonical manner, through the phosphorylation of Smad3, which in turn physically interacts with Runx2 at Runx2-responsive elements, resulting in repression of Runx2 and other osteogenic genes' expression (Alliston, Choy et al. 2001). This smad-mediated inhibition of Runx2 illustrates how the TGF β signaling directly targets the transcriptional machinery. However, other mechanisms beyond the canonical pathway also exist. The fact that SB505124, a small molecule inhibiting TGF β -induced Smad2/3 phosphorylation, is unable to rescue TGF β 's suppression on ATF4 suggests other pathways may also play a role. We therefore tested the effect of Rapamycin and Wortmannin, inhibitors specifically targeting a branch of the non-canonical TGF β signaling pathway, where TGF β induces the phosphorylation of PI3K, which in turn activates Akt, and subsequently mTOR (Lamouille and Derynck 2007; Zhang 2009).

Indeed, Rapamycin and Wortmannin, but not SB505124, reverse the inhibitory effect of TGF β on ATF4's transcriptional activity, implying that TGF β also employs the non-canonical pathway and targets gene expression indirectly in a more sophisticated way.

Both Runx2 and ATF4 are expressed in an osteoblast-predominant manner. Although the mechanism is not totally unveiled, these two proteins have been found to activate the promoter of several identical genes, including osteocalcin, Indian hedgehog, and RANKL. Our observation that, in addition to Runx2, ATF4 is another transcription factor regulated by the TGF β signaling pathway provides one more piece of evidence of how these two molecules are functionally related. Yet, three levels of differences distinguish ATF4 and Runx2 in the context of the TGF β modulation. First, the effect of TGF β 1 on Runx2 was not observed until 10 hours post treatment, while the ATF4-mediated transcription was inhibited by TGF β as early as 6 hours following treatment, suggesting that the machinery corresponding for the effect on ATF4 is comparatively early-responsive and independent of the suppression of Runx2. Second, although not directly tested, since TGF β 1 has been shown to inhibit Runx2's transactivation ability through phosphorylated Smad3, which can be blocked pharmacologically by SB505124. The application of SB505124 should prevent the suppression on Runx2. However, ATF4

is inhibited to the same extent by TGF β 1 in the presence or absence of SB505124. This discrepancy again suggests that distinctive mechanisms are involved in targeting ATF4 and Runx2. Thirdly, at a comparatively low dose (ranging from 0.1 to 0.2 ng/ml), TGF β 1 was not able to block several aspects of calvarial osteoblast differentiation in the absence of ATF4, as demonstrated by unchanged ALP activity and similar number of bone nodules formed when challenged by TGF β 1. This indicates that, under certain conditions, ATF4 can be the main target of the TGF β signaling event, independent of Runx2. However, I suspect that a high dose of TGF β 1 would also involve Runx2 as an important regulatory target and in that scenario, Atf4-deficient osteoblasts will also respond, perhaps less severely, to TGF β 1. Indeed, the observation that, although much less effective, osteocalcin gene expression is still subject to TGF β 1's regulation in Atf4-deficient cells suggests that Runx2 may be affected by TGF β 1 mildly in these cells.

Through a pull-down assay with ATF4 as the bait and nuclear extract from osteoblast-related ROS17/2.8 cells as the pool of prey, we discovered the novel interaction between the intermediate filament vimentin and our transcription factor of interest, ATF4. This finding was supported by co-immunoprecipitation assay in mammalian cells and further by pull-down assay with purified recombinant GST-tagged

vimentin and Histidine-tagged ATF4, and various truncated forms of each. These experimental observations for the first time provide direct evidence for the hypothesis proposed by Capetanaki et al., who, upon computational analysis, predicted molecular interactions between vimentin and several CREB transcription factor family members based on their structural similarities localized to the important leucine zipper domains capable of coiled-coil interactions (Capetanaki, Kuisk et al. 1990). In addition, in vitro studies have suggested high binding affinity of vimentin to a variety of synthetic and natural nucleic acids (Hartig, Huang et al. 1997), and this DNA-binding mediates transport of vimentin into the nucleus of cultured cells (Hartig, Shoeman et al. 1998). These experimental clues converge at the hypothesis that vimentin not only fulfills general cytoskeletal functions in the cytoplasm but also participates in DNA-based events taking place in the nuclear interior. We then tested the functional relevance of the ATF4-vimentin interaction. Ectopic expression of vimentin in COS1 cells suppressed the ATF4-mediated transcription of osteocalcin gene, and vice versa, depletion of vimentin through RNA interference elevated osteocalcin expression significantly. Furthermore, GST-tagged vimentin dose-dependently inhibit ATF4's DNA binding ability in a gel-shift assay. Further on a cellular level, we found that overexpression of vimentin in MC3T3-

E1 osteoblasts led to a delay in osteoblastogenesis. Consistent with the inhibitory effect of vimentin, both vimentin mRNA and protein are down-regulated during osteoblast differentiation. This makes biological sense in terms of paving the way for further differentiation and maturation. These data, from a functional point of view, suggest that vimentin does not simply provide structural support to cells, but also actively participates in transcriptional control in the progression of osteoblast differentiation. Further experiments could be done to confirm or test mechanistically the vimentin-mediated regulation of ATF4. For example, in addition to the gel-shift assay, we can use the Chromatin-Immunoprecipitation (CHIP) assay to directly test whether the overexpression of vimentin can actually inhibit the ATF4-DNA interaction in mammalian cells, and if so, whether this is due to the reduced amount of ATF4 reaching the nuclei or a less efficient binding ability of ATF4 due to interaction with vimentin. In the former scenario, I would expect to detect less ATF4 from the nuclear extracts when vimentin is over-expressed.

Several pieces of information led us to hypothesize that TGF β regulates the expression of vimentin in osteoblasts. Firstly, the presence of TGF β -neutralizing antibody, 2G7, blocked the expression of vimentin *in vivo*. Secondly, consistent with the observation that vimentin level is gradually turned down during osteoblast differentiation,

in a natural setting, a spontaneous decrease in T β RI and T β RII was observed as human bone marrow stromal cells (BMSCs) progress from osteoprogenitor cells to maturing osteoblasts and a decrease of TGF β /receptor interactions was reported during osteoblast differentiation (Centrella, Casinghino et al. 1995; Takeuchi, Nakayama et al. 1996; Walsh, Jefferiss et al. 2003). Thirdly, in C2C12 cells and during EMT transition, TGF β have been documented to up-regulate vimentin levels (Margetts, Bonniaud et al. 2005; Wu, Zhang et al. 2007). We therefore investigated specifically if TGF β up-regulates vimentin in osteoblasts. For all the osteoblast-related cells we have tested, including BMSCs, calvarial osteoblasts, 2T3 cells, MC3T3-E1 cells, and ROS17/2.8 cells, TGF β 1 induces vimentin protein expression, while leaving its mRNA level unchanged. In a canonical TGF β signaling event, the activation of T β RI leads to Smad2/3 mediated transcriptional regulation. The unaffected level of vimentin mRNA implies that TGF β elevates vimentin through a non-canonical pathway. Supporting this notion, the increase in vimentin protein level was abrogated by rapamycin and wortmannin, both of which hinder a branch of non-canonical pathways (mediated by PI3K-Akt-mTOR) which is implicated in the enhancement of the cap-dependent translation. Moreover, at the effective dose, neither rapamycin nor wortmannin affected the phosphorylation of

Smad2/3 induced by TGF β 1, further confirming that TGF β 1 upregulates vimentin in a smad-independent manner.

Suppression of PI3K and mTOR signaling using inhibitors such as wortmannin, rapamycin, and NVP-BEZ235 has been shown to promote osteogenesis in vitro, as measured by an increase in mineral formation or bone nodule formation and an upregulation of genes involved in osteogenesis including *osteocalcin* (Ogawa, Tokuda et al. 1998; Kratchmarova, Blagoev et al. 2005; Fitter, Dewar et al. 2008; Lee, Yook et al. 2010; Martin, Fitter et al. 2010). These findings have been explained by an increase in Runx2 levels following the pharmacological treatments. However, since the overexpression of Runx2 under the control of a 2.3-kb mouse *Col1a1* promoter inhibits mature osteoblast formation, as demonstrated by the severe reduction of osteocalcin expression from both the bones and calvarial osteoblasts of the Runx2-overexpressing mice, and diminished number of terminally differentiated osteoblasts (Liu, Toyosawa et al. 2001; Geoffroy, Kneissel et al. 2002; Kanatani, Fujita et al. 2006), other mechanisms may be considered. In effect, vimentin may be the candidate molecule, as suppression of the PI3K/Akt/mTOR pathway blunts the increase in vimentin level, and a decrease of

vimentin level has been associated with upregulation of osteocalcin expression or even maintainance of the level of osteocalcin in the presence of TGF β .

We also examined if there is a causal relationship between the up-regulation of vimentin and inhibition on ATF4's transactivation activity by TGF β . This is made possible by the availability of a luciferase reporter, p6OSE1-luc, which is specifically activated by ATF4. The level of ATF4-activated p6OSE1-luc is turned down upon TGF β treatment, whereas down-regulation of vimentin through RNA interference hinder this effect, suggesting a novel axis composed of TGF β -vimentin-ATF4 which serves as a brake against osteoblast terminal differentiation. Upon binding to its receptor, TGF β first induces the expression of vimentin through the PI3K-Akt-mTOR non-canonical pathway, and vimentin, through binding to ATF4, inhibits its transcriptional activity, resulting in repression of osteoblast terminal differentiation. Consistently, depletion of vimentin prevents ATF4 from being inhibited by TGF β , and the absence of ATF4 further abolishes the inhibition of TGF β on osteoblast differentiation.

To test the existence of the TGF β -vimentin-ATF4 axis in vivo, in addition to treating wild-type and *Atf4*-deficient mice with the TGF β neutralizing antibody, it will also be necessary to compare the effect of 2G7 on wild-type with that of vimentin-deficient

mice. A straightforward outcome would be an increase in BV/TV in wild-type mice, but a smaller or even no increase in vimentin-deficient mice, given that vimentin may be required to mediate TGF β 's inhibitory effect in osteoblasts. Although not likely, the bone mass may still be increased to a similar extent in vimentin knockout mice. This is a possibility because, first of all, although the TGF β -vimentin-ATF4 axis exists independently from the canonical signaling, impairment of Smad2/3 phosphorylation leads to a dramatic increase in the osteoblast differentiation. Although treatment of SB505124 does not increase ATF4-mediated p6OSE1 controlled luciferase expression, this does not exclude that in a physiological context, expression of other endogenous target of ATF4 may still be elevated by SB505124. Second of all, within non-canonical TGF β signaling, vimentin may not be the exclusive factor withholding ATF4's activity in vivo. In order to elucidate the relevance of vimentin in mediating TGF β 's impact on ATF4, we need to thoroughly examine all the known ATF4-targeted genes' expression in osteoblasts from vimentin-null mice after 2G7 administration.

We have shown in vitro that overexpression of vimentin in MC3T3-E1 osteoblasts has a strong effect in delaying osteoblast differentiation. Yet, this phenomenon has not been examined in vivo. Two genetic strategies can be used to illustrate the physiological

role of vimentin – to either over-express vimentin or knockout vimentin from the genome.

In order to test the role of vimentin in a spatial- and temporal-specific manner within osteoblasts, we can generate transgenic constructs of vimentin driven by either a 2.3kb Col1A1 promoter, which over-expresses vimentin in both immature and mature osteoblasts, or an OCN promoter, which specifically over-expresses vimentin only in mature osteoblasts. Because vimentin is progressively down-regulated during osteoblast differentiation, I would expect that over-expressing vimentin in late osteoblasts will cause a more dramatic effect than in early osteoblasts, in which a high level of vimentin is maintained and perhaps a plateau has already been reached. If this holds true, a similar, if not identical, low-bone-mass phenotype will likely be observed in Col1A1-vim and OCN-vim mice. Conversely, knocking out vimentin from the genome will allow us to eliminate its inhibitory action and result in a high-bone-mass phenotype under a simplified scenario. However, since bone mass is a net result of both bone formation by osteoblasts and bone resorption by osteoclasts, knocking out vimentin as a whole may lead to complicated consequences, especially if vimentin is also involved in osteoclastogenesis and subsequent function. Furthermore, because vimentin is required by several cell types during migration, disruption of vimentin in osteoprogenitors, which

need to migrate to relevant sites in a timely manner, will result in an accelerated osteoblastogenesis, but not necessarily better bone formation, due to uncoupled bone formation resulting from impaired migration of osteoprogenitors. Counting the number of osteoblasts successfully reaching bone surface in vimentin-deficient mice will provide clues of whether this is happening physiologically. Alternatively, we can avoid a complicated interpretation by utilizing the conditional knockout technology, with which vimentin is knocked out only in a later stage during osteoblast differentiation, after the arrival of osteoprogenitors on to the bone surface. This is technologically possible in the near future since the EUCOMM production center has generated a conditional-ready targeting vector of vimentin, where its critical exon is surrounded by loxP sites, and ES cells carrying this construct are planned to be made in this year. We could then specifically knock out vimentin during the later differentiation state by Cre recombinase under the control of osteocalcin promoter.

REFERENCES

- (2001). "Osteoporosis prevention, diagnosis, and therapy." JAMA **285**(6): 785-795.
- Abdollah, S., M. Macias-Silva, et al. (1997). "TbetaRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling." J Biol Chem **272**(44): 27678-27685.
- Alliston, T., L. Choy, et al. (2001). "TGF-beta-induced repression of CBFA1 by Smad3 decreases cbfa1 and osteocalcin expression and inhibits osteoblast differentiation." EMBO J **20**(9): 2254-2272.
- Ameri, K. and A. L. Harris (2008). "Activating transcription factor 4." Int J Biochem Cell Biol **40**(1): 14-21.
- Arcaro, A. and M. P. Wymann (1993). "Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses." Biochem J **296 (Pt 2)**: 297-301.
- Arteaga, C. L., S. D. Hurd, et al. (1993). "Anti-transforming growth factor (TGF)-beta antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-beta interactions in human breast cancer progression." J Clin Invest **92**(6): 2569-2576.
- Atti, E., S. Gomez, et al. (2002). "Effects of transforming growth factor-beta deficiency on bone development: a Fourier transform-infrared imaging analysis." Bone **31**(6): 675-684.
- Aubin, J. E. (2001). "Regulation of osteoblast formation and function." Rev Endocr Metab Disord **2**(1): 81-94.
- Aubin, J. E., F. Liu, et al. (1995). "Osteoblast and chondroblast differentiation." Bone **17**(2 Suppl): 77S-83S.

- Banerjee, C., L. R. McCabe, et al. (1997). "Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1 is a major component of a bone-specific complex." J Cell Biochem **66**(1): 1-8.
- Banerjee, C., J. L. Stein, et al. (1996). "Transforming growth factor-beta 1 responsiveness of the rat osteocalcin gene is mediated by an activator protein-1 binding site." Endocrinology **137**(5): 1991-2000.
- Borton, A. J., J. P. Frederick, et al. (2001). "The loss of Smad3 results in a lower rate of bone formation and osteopenia through dysregulation of osteoblast differentiation and apoptosis." J Bone Miner Res **16**(10): 1754-1764.
- Bozec, A., L. Bakiri, et al. (2010). "Fra-2/AP-1 controls bone formation by regulating osteoblast differentiation and collagen production." J Cell Biol **190**(6): 1093-1106.
- Capetanaki, Y., I. Kuisk, et al. (1990). "Mouse vimentin: structural relationship to fos, jun, CREB and tpr." Oncogene **5**(5): 645-655.
- Capetanaki, Y., S. Smith, et al. (1989). "Overexpression of the vimentin gene in transgenic mice inhibits normal lens cell differentiation." J Cell Biol **109**(4 Pt 1): 1653-1664.
- Capetanaki, Y. G., J. Ngai, et al. (1984). "Characterization and regulation in the expression of a gene coding for the intermediate filament protein desmin." Proc Natl Acad Sci U S A **81**(22): 6909-6913.
- Caplan, A. I. and S. P. Bruder (2001). "Mesenchymal stem cells: building blocks for molecular medicine in the 21st century." Trends Mol Med **7**(6): 259-264.
- Centrella, M., S. Casinghino, et al. (1995). "Independent changes in type I and type II receptors for transforming growth factor beta induced by bone morphogenetic protein 2 parallel expression of the osteoblast phenotype." Mol Cell Biol **15**(6): 3273-3281.
- Centrella, M., M. C. Horowitz, et al. (1994). "Transforming growth factor-beta gene family members and bone." Endocr Rev **15**(1): 27-39.

- Colucci-Guyon, E., Y. R. M. Gimenez, et al. (1999). "Cerebellar defect and impaired motor coordination in mice lacking vimentin." Glia **25**(1): 33-43.
- Dallas, S. L. and L. F. Bonewald (2010). "Dynamics of the transition from osteoblast to osteocyte." Ann N Y Acad Sci **1192**: 437-443.
- Dignam, J. D., R. M. Lebovitz, et al. (1983). "Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei." Nucleic Acids Res **11**(5): 1475-1489.
- Dobрева, G., M. Chahrour, et al. (2006). "SATB2 is a multifunctional determinant of craniofacial patterning and osteoblast differentiation." Cell **125**(5): 971-986.
- Ducy, P. and G. Karsenty (1995). "Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene." Mol Cell Biol **15**(4): 1858-1869.
- Ducy, P., R. Zhang, et al. (1997). "Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation." Cell **89**(5): 747-754.
- Eckes, B., E. Colucci-Guyon, et al. (2000). "Impaired wound healing in embryonic and adult mice lacking vimentin." J Cell Sci **113 (Pt 13)**: 2455-2462.
- Eckes, B., D. Dogic, et al. (1998). "Impaired mechanical stability, migration and contractile capacity in vimentin-deficient fibroblasts." J Cell Sci **111 (Pt 13)**: 1897-1907.
- Edwards, J. R., J. S. Nyman, et al. (2010). "Inhibition of TGF-beta signaling by 1D11 antibody treatment increases bone mass and quality in vivo." J Bone Miner Res.
- Elefteriou, F., M. D. Benson, et al. (2006). "ATF4 mediation of NF1 functions in osteoblast reveals a nutritional basis for congenital skeletal dysplasias." Cell Metab **4**(6): 441-451.
- Erlebacher, A. and R. Derynck (1996). "Increased expression of TGF-beta 2 in osteoblasts results in an osteoporosis-like phenotype." J Cell Biol **132**(1-2): 195-210.

- Ferrari, S., R. Battini, et al. (1986). "Coding sequence and growth regulation of the human vimentin gene." Mol Cell Biol **6**(11): 3614-3620.
- Filvaroff, E., A. Erlebacher, et al. (1999). "Inhibition of TGF-beta receptor signaling in osteoblasts leads to decreased bone remodeling and increased trabecular bone mass." Development **126**(19): 4267-4279.
- Fitter, S., A. L. Dewar, et al. (2008). "Long-term imatinib therapy promotes bone formation in CML patients." Blood **111**(5): 2538-2547.
- Geiser, A. G., Q. Q. Zeng, et al. (1998). "Decreased bone mass and bone elasticity in mice lacking the transforming growth factor-beta1 gene." Bone **23**(2): 87-93.
- Geoffroy, V., M. Kneissel, et al. (2002). "High bone resorption in adult aging transgenic mice overexpressing cbfa1/runx2 in cells of the osteoblastic lineage." Mol Cell Biol **22**(17): 6222-6233.
- Gingras, A. C., S. P. Gygi, et al. (1999). "Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism." Genes Dev **13**(11): 1422-1437.
- Gingras, A. C., B. Raught, et al. (2001). "Hierarchical phosphorylation of the translation inhibitor 4E-BP1." Genes Dev **15**(21): 2852-2864.
- Hai, T. and T. Curran (1991). "Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity." Proc Natl Acad Sci U S A **88**(9): 3720-3724.
- Hai, T. and M. G. Hartman (2001). "The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis." Gene **273**(1): 1-11.
- Harada, S. and G. A. Rodan (2003). "Control of osteoblast function and regulation of bone mass." Nature **423**(6937): 349-355.

- Harris, S. E., L. F. Bonewald, et al. (1994). "Effects of transforming growth factor beta on bone nodule formation and expression of bone morphogenetic protein 2, osteocalcin, osteopontin, alkaline phosphatase, and type I collagen mRNA in long-term cultures of fetal rat calvarial osteoblasts." J Bone Miner Res **9**(6): 855-863.
- Hartig, R., Y. Huang, et al. (1997). "Binding of fluorescence- and gold-labeled oligodeoxyribonucleotides to cytoplasmic intermediate filaments in epithelial and fibroblast cells." Exp Cell Res **233**(1): 169-186.
- Hartig, R., R. L. Shoeman, et al. (1998). "DNA-mediated transport of the intermediate filament protein vimentin into the nucleus of cultured cells." J Cell Sci **111 (Pt 24)**: 3573-3584.
- Henrion, D., F. Terzi, et al. (1997). "Impaired flow-induced dilation in mesenteric resistance arteries from mice lacking vimentin." J Clin Invest **100**(11): 2909-2914.
- Hering, S., E. Isken, et al. (2001). "TGFbeta1 and TGFbeta2 mRNA and protein expression in human bone samples." Exp Clin Endocrinol Diabetes **109**(4): 217-226.
- Herrmann, H. and U. Aebi (2004). "Intermediate filaments: molecular structure, assembly mechanism, and integration into functionally distinct intracellular Scaffolds." Annu Rev Biochem **73**: 749-789.
- Ivaska, J., H. M. Pallari, et al. (2007). "Novel functions of vimentin in cell adhesion, migration, and signaling." Exp Cell Res **313**(10): 2050-2062.
- James, D., A. J. Levine, et al. (2005). "TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells." Development **132**(6): 1273-1282.
- Janssens, K., P. ten Dijke, et al. (2005). "Transforming growth factor-beta1 to the bone." Endocr Rev **26**(6): 743-774.

- Jian, H., X. Shen, et al. (2006). "Smad3-dependent nuclear translocation of beta-catenin is required for TGF-beta1-induced proliferation of bone marrow-derived adult human mesenchymal stem cells." Genes Dev **20**(6): 666-674.
- Jiang, Y., B. N. Jahagirdar, et al. (2002). "Pluripotency of mesenchymal stem cells derived from adult marrow." Nature **418**(6893): 41-49.
- Jilka, R. L., R. S. Weinstein, et al. (1998). "Osteoblast programmed cell death (apoptosis): modulation by growth factors and cytokines." J Bone Miner Res **13**(5): 793-802.
- Kanatani, N., T. Fujita, et al. (2006). "Cbf beta regulates Runx2 function isoform-dependently in postnatal bone development." Dev Biol **296**(1): 48-61.
- Karsdal, M. A., L. Larsen, et al. (2002). "Matrix metalloproteinase-dependent activation of latent transforming growth factor-beta controls the conversion of osteoblasts into osteocytes by blocking osteoblast apoptosis." J Biol Chem **277**(46): 44061-44067.
- Karsenty, G. (2003). "The complexities of skeletal biology." Nature **423**(6937): 316-318.
- Kern, B., J. Shen, et al. (2001). "Cbfa1 contributes to the osteoblast-specific expression of type I collagen genes." J Biol Chem **276**(10): 7101-7107.
- Kokkinos, M. I., R. Wafai, et al. (2007). "Vimentin and epithelial-mesenchymal transition in human breast cancer--observations in vitro and in vivo." Cells Tissues Organs **185**(1-3): 191-203.
- Komori, T., H. Yagi, et al. (1997). "Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts." Cell **89**(5): 755-764.
- Korpai, M. and Y. Kang (2010). "Targeting the transforming growth factor-beta signalling pathway in metastatic cancer." Eur J Cancer **46**(7): 1232-1240.
- Kratchmarova, I., B. Blagoev, et al. (2005). "Mechanism of divergent growth factor effects in mesenchymal stem cell differentiation." Science **308**(5727): 1472-1477.

- Lamouille, S. and R. Derynck (2007). "Cell size and invasion in TGF-beta-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway." J Cell Biol **178**(3): 437-451.
- Larsson, J., M. J. Goumans, et al. (2001). "Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice." EMBO J **20**(7): 1663-1673.
- Lee, K. W., J. Y. Yook, et al. (2010). "Rapamycin promotes the osteoblastic differentiation of human embryonic stem cells by blocking the mTOR pathway and stimulating the BMP/Smad pathway." Stem Cells Dev **19**(4): 557-568.
- Lian, N., W. Wang, et al. (2009). "Vimentin inhibits ATF4-mediated osteocalcin transcription and osteoblast differentiation." J Biol Chem **284**(44): 30518-30525.
- Liang, G. and T. Hai (1997). "Characterization of human activating transcription factor 4, a transcriptional activator that interacts with multiple domains of cAMP-responsive element-binding protein (CREB)-binding protein." J Biol Chem **272**(38): 24088-24095.
- Liu, D., B. L. Black, et al. (2001). "TGF-beta inhibits muscle differentiation through functional repression of myogenic transcription factors by Smad3." Genes Dev **15**(22): 2950-2966.
- Liu, D., J. S. Kang, et al. (2004). "TGF-beta-activated Smad3 represses MEF2-dependent transcription in myogenic differentiation." EMBO J **23**(7): 1557-1566.
- Liu, W., S. Toyosawa, et al. (2001). "Overexpression of Cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures." J Cell Biol **155**(1): 157-166.
- Lomri, A. and P. J. Marie (1988). "Effect of parathyroid hormone and forskolin on cytoskeletal protein synthesis in cultured mouse osteoblastic cells." Biochim Biophys Acta **970**(3): 333-342.

- Lomri, A. and P. J. Marie (1990). "Changes in cytoskeletal proteins in response to parathyroid hormone and 1,25-dihydroxyvitamin D in human osteoblastic cells." Bone Miner **10**(1): 1-12.
- Lomri, A. and P. J. Marie (1990). "Effects of transforming growth factor type beta on expression of cytoskeletal proteins in endosteal mouse osteoblastic cells." Bone **11**(6): 445-451.
- Lucas, P. A. (1989). "Chemotactic response of osteoblast-like cells to transforming growth factor beta." Bone **10**(6): 459-463.
- Maeda, S., M. Hayashi, et al. (2004). "Endogenous TGF-beta signaling suppresses maturation of osteoblastic mesenchymal cells." EMBO J **23**(3): 552-563.
- Mamane, Y., E. Petroulakis, et al. (2004). "eIF4E--from translation to transformation." Oncogene **23**(18): 3172-3179.
- Manning, B. D. and L. C. Cantley (2003). "Rheb fills a GAP between TSC and TOR." Trends Biochem Sci **28**(11): 573-576.
- Manolagas, S. C. (2000). "Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis." Endocr Rev **21**(2): 115-137.
- Margetts, P. J., P. Bonniaud, et al. (2005). "Transient overexpression of TGF- β 1 induces epithelial mesenchymal transition in the rodent peritoneum." J Am Soc Nephrol **16**(2): 425-436.
- Martin, S. K., S. Fitter, et al. (2010). "NVP-BEZ235, a dual pan class I PI3 kinase and mTOR inhibitor, promotes osteogenic differentiation in human mesenchymal stromal cells." J Bone Miner Res **25**(10): 2126-2137.
- Massague, J. (1998). "TGF-beta signal transduction." Annu Rev Biochem **67**: 753-791.

- Miyazaki, T., N. Kanatani, et al. (2008). "Inhibition of the terminal differentiation of odontoblasts and their transdifferentiation into osteoblasts in Runx2 transgenic mice." Arch Histol Cytol **71**(2): 131-146.
- Mohammad, K. S., C. G. Chen, et al. (2009). "Pharmacologic inhibition of the TGF-beta type I receptor kinase has anabolic and anti-catabolic effects on bone." PLoS One **4**(4): e5275.
- Monteiro, M. J., C. Hicks, et al. (1994). "Determinants for intracellular sorting of cytoplasmic and nuclear intermediate filaments." J Cell Biol **127**(5): 1327-1343.
- Mundlos, S., F. Otto, et al. (1997). "Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia." Cell **89**(5): 773-779.
- Nakashima, K., X. Zhou, et al. (2002). "The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation." Cell **108**(1): 17-29.
- Neve, R. M., K. Chin, et al. (2006). "A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes." Cancer Cell **10**(6): 515-527.
- Nieminen, M., T. Henttinen, et al. (2006). "Vimentin function in lymphocyte adhesion and transcellular migration." Nat Cell Biol **8**(2): 156-162.
- Nomura, M. and E. Li (1998). "Smad2 role in mesoderm formation, left-right patterning and craniofacial development." Nature **393**(6687): 786-790.
- Ogawa, T., M. Tokuda, et al. (1998). "Osteoblastic differentiation is enhanced by rapamycin in rat osteoblast-like osteosarcoma (ROS 17/2.8) cells." Biochem Biophys Res Commun **249**(1): 226-230.
- Olson, E. N. and Y. G. Capetanaki (1989). "Developmental regulation of intermediate filament and actin mRNAs during myogenesis is disrupted by oncogenic ras genes." Oncogene **4**(7): 907-913.

- Otto, F., A. P. Thornell, et al. (1997). "Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development." Cell **89**(5): 765-771.
- Pfeilschifter, J., O. Wolf, et al. (1990). "Chemotactic response of osteoblastlike cells to transforming growth factor beta." J Bone Miner Res **5**(8): 825-830.
- Pinkas, J. and B. A. Teicher (2006). "TGF-beta in cancer and as a therapeutic target." Biochem Pharmacol **72**(5): 523-529.
- Rameh, L. E. and L. C. Cantley (1999). "The role of phosphoinositide 3-kinase lipid products in cell function." J Biol Chem **274**(13): 8347-8350.
- Robey, P. G., M. F. Young, et al. (1987). "Osteoblasts synthesize and respond to transforming growth factor-type beta (TGF-beta) in vitro." J Cell Biol **105**(1): 457-463.
- Rogers, K. R., A. Eckelt, et al. (1995). "Truncation mutagenesis of the non-alpha-helical carboxyterminal tail domain of vimentin reveals contributions to cellular localization but not to filament assembly." Eur J Cell Biol **66**(2): 136-150.
- Schinke, T. and G. Karsenty (1999). "Characterization of Osf1, an osteoblast-specific transcription factor binding to a critical cis-acting element in the mouse Osteocalcin promoters." J Biol Chem **274**(42): 30182-30189.
- Schinke, T., M. D. McKee, et al. (1999). "Extracellular matrix calcification: where is the action?" Nat Genet **21**(2): 150-151.
- Singh, B. and R. B. Arlinghaus (1989). "Vimentin phosphorylation by p37mos protein kinase in vitro and generation of a 50-kDa cleavage product in v-mos-transformed cells." Virology **173**(1): 144-156.
- Sirard, C., J. L. de la Pompa, et al. (1998). "The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo." Genes Dev **12**(1): 107-119.

- Souchelnytskyi, S., K. Tamaki, et al. (1997). "Phosphorylation of Ser465 and Ser467 in the C terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor-beta signaling." J Biol Chem **272**(44): 28107-28115.
- Stein, G. S., J. B. Lian, et al. (2004). "Runx2 control of organization, assembly and activity of the regulatory machinery for skeletal gene expression." Oncogene **23**(24): 4315-4329.
- Steinert, P. M. and R. K. Liem (1990). "Intermediate filament dynamics." Cell **60**(4): 521-523.
- Steinert, P. M. and D. R. Roop (1988). "Molecular and cellular biology of intermediate filaments." Annu Rev Biochem **57**: 593-625.
- Stokoe, D., L. R. Stephens, et al. (1997). "Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B." Science **277**(5325): 567-570.
- Strelkov, S. V., H. Herrmann, et al. (2003). "Molecular architecture of intermediate filaments." Bioessays **25**(3): 243-251.
- Takeuchi, Y., K. Nakayama, et al. (1996). "Differentiation and cell surface expression of transforming growth factor-beta receptors are regulated by interaction with matrix collagen in murine osteoblastic cells." J Biol Chem **271**(7): 3938-3944.
- Tapscott, S. J., G. S. Bennett, et al. (1981). "Intermediate filament proteins in the developing chick spinal cord." Dev Biol **86**(1): 40-54.
- Terzi, F., D. Henrion, et al. (1997). "Reduction of renal mass is lethal in mice lacking vimentin. Role of endothelin-nitric oxide imbalance." J Clin Invest **100**(6): 1520-1528.
- Toivola, D. M., G. Z. Tao, et al. (2005). "Cellular integrity plus: organelle-related and protein-targeting functions of intermediate filaments." Trends Cell Biol **15**(11): 608-617.
- Vallier, L., M. Alexander, et al. (2005). "Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells." J Cell Sci **118**(Pt 19): 4495-4509.

- Walsh, S., C. Jefferiss, et al. (2003). "TGFbeta1 limits the expansion of the osteoprogenitor fraction in cultures of human bone marrow stromal cells." Cell Tissue Res **311**(2): 187-198.
- Wang, W., N. Lian, et al. (2009). "Atf4 regulates chondrocyte proliferation and differentiation during endochondral ossification by activating Ihh transcription." Development **136**(24): 4143-4153.
- Watabe, T. and K. Miyazono (2009). "Roles of TGF-beta family signaling in stem cell renewal and differentiation." Cell Res **19**(1): 103-115.
- Weinstein, M., X. Yang, et al. (1998). "Failure of egg cylinder elongation and mesoderm induction in mouse embryos lacking the tumor suppressor smad2." Proc Natl Acad Sci U S A **95**(16): 9378-9383.
- Wieczorek, E., Z. Lin, et al. (2000). "The zinc finger repressor, ZBP-89, binds to the silencer element of the human vimentin gene and complexes with the transcriptional activator, Sp1." J Biol Chem **275**(17): 12879-12888.
- Wu, Y., X. Zhang, et al. (2007). "TGFbeta1 regulation of vimentin gene expression during differentiation of the C2C12 skeletal myogenic cell line requires Smads, AP-1 and Sp1 family members." Biochim Biophys Acta **1773**(3): 427-439.
- Wullschleger, S., R. Loewith, et al. (2006). "TOR signaling in growth and metabolism." Cell **124**(3): 471-484.
- Yang, X., X. Ji, et al. (2000). "Smad1 domains interacting with Hoxc-8 induce osteoblast differentiation." J Biol Chem **275**(2): 1065-1072.
- Yang, X. and G. Karsenty (2004). "ATF4, the osteoblast accumulation of which is determined post-translationally, can induce osteoblast-specific gene expression in non-osteoblastic cells." J Biol Chem **279**(45): 47109-47114.

- Yang, X., K. Matsuda, et al. (2004). "ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome." Cell **117**(3): 387-398.
- Yu, S., R. T. Franceschi, et al. (2008). "Parathyroid hormone increases activating transcription factor 4 expression and activity in osteoblasts: requirement for osteocalcin gene expression." Endocrinology **149**(4): 1960-1968.
- Yu, V. W., O. Akhouayri, et al. (2009). "FIAT is co-expressed with its dimerization target ATF4 in early osteoblasts, but not in osteocytes." Gene Expr Patterns **9**(5): 335-340.
- Yu, V. W., G. Ambartsoumian, et al. (2005). "FIAT represses ATF4-mediated transcription to regulate bone mass in transgenic mice." J Cell Biol **169**(4): 591-601.
- Zhang, Y. E. (2009). "Non-Smad pathways in TGF-beta signaling." Cell Res **19**(1): 128-139.