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The Role of T-box (Tbx) 2 in Osteoblast Function

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The role of T-box (Tbx) 2 on osteoblast function

Nidhish Francis

B.V.Sc. & A.H., Kerala Agricultural University, 2008

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APPROVAL PAGE

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The role of T-box (Tbx) 2 in osteoblast function

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ABBREVIATIONS

α -MEM	α minimal essential medium
ALP	alkaline phosphatase
ANF	atrial natriuretic factor
AP1	activator protein 1
ATF4	activating transcription factor 4
BMD	bone mineral density
BMPs	bone morphogenetic proteins
BMSC	bone marrow stromal cells
BMU	basic multicellular unit
BSP	bone sialoprotein
Coll-I	collagen type-I
CREB	cAMP response elements binding
CS	calf serum
Cx	connexin
DGS	DiGeorge syndrome
Dlx	distal-less homeobox
ECM	extracellular matrix
FBS	fetal bovine serum
FGF	fibroblast growth factor
GH	growth hormone
GHRH	growth hormone releasing hormone
IGF	insulin-like growth factor

IGFBPs	IGF binding proteins
MITF	microphthalmia associated transcription factor
MMP	matrix metalloproteinases
OCIL	osteoclast inhibitory lectin
OCN	osteocalcin
Osf	osteoblast-specific factor
Osx	osterix
PDGF	platelet derived growth factor
pNPP	p-Nitrophenyl phosphate
POMC	pro-opiomelanocortin
PPAR	proliferation-activated receptor
PPIA	peptidylprolyl isomerase A
PS	penicillin-streptomycin
PTH	parathyroid hormone
RANKL	receptor activator for nuclear factor κ B receptor ligand
Runx2	runt-related transcription factor 2
siRNA	small interfering RNA
Shh	sonic hedgehog
Sox	sry-related HMG box
TBST	Tris-buffered saline tween-20
TH	thyroid hormone
UMS	ulnar mammary syndrome
Vit D3	1, 25-dihydroxyvitamin D3
Wnt	wingless-int

REVIEW OF LITERATURE

Introduction

Bone is composed of dense connective tissue that forms the structural framework of vertebrates. There are approximately 206 bones in an adult human body and 270 in an infant. All these bones vary greatly in their shape and functions. The functions of bone can be organized into three categories: mechanical, synthetic and metabolic. Mechanical functions of bone include protection of internal organs, providing shape to the body and movement. For instance, brain is protected by skull and heart and lungs are enclosed in the ribcage. Bone, along with muscles, tendons, and ligaments function together to aid in movement of a part of or the whole body. A specialized mechanical function of bone is also involved in sound transduction, particularly in the middle ear. The synthetic function of bone tissue primarily comprises of haematopoiesis. This takes place in the medullary cavity of long bones, where blood cells are produced from their progenitors. Bone also functions as a storage organ for minerals, growth factors and fat (energy reservoir) which formulates the bone tissue to play an essential role in body metabolism.

Based on the structure, bone tissue can be categorized as compact bone or spongy bone. Compact bone, also called cortical bone, makes up the shaft of long bones and surfaces of other bones (Galante et al., 1970). Compact bone provides a smooth, white appearance to the bone and accounts for 80% of total bone mass of the skeleton. The fundamental functional unit of compact bone is known as Osteon or

Haversian system. Osteon is characterized as a long narrow cylinder that is approximately 10 mm in length and 0.2 μm in width (Buckwalter et al., 1995).

Each osteon consists of a central canal or Haversian canal, which contains bone's blood and nerve supplies. Surrounding the canal are concentric layers (lamellae) of compact bone tissue. Lamellae consist of osteocytes lodged in spaces (lacunae) of mineralized bony matrix. These osteocytes within an osteon are connected to each other and to the central canal by fine cellular extensions called canaliculi. These canaliculi facilitate the exchange of nutrients and metabolite waste between osteocytes and blood vessels. Osteons are connected to each other and periosteum by oblique channels called as Volkmann's canals.

Cellular and molecular structure

The major cells involved in bone growth and maintenance are osteoblasts, osteoclasts and osteocytes. Osteoblasts are responsible for production of major constituents of bone matrix, thereby commonly referred to as bone forming cells. They originate from multipotent mesenchymal stem cells that later differentiate to preosteoblasts and terminally differentiate into functional mature osteoblasts (Aubin et al., 1995). The transition from osteoprogenitors to mature osteoblasts is induced by several growth factors including insulin-like growth factor (IGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF) and transforming growth factor- β (TGF- β) (Aronow et al., 1990). A mature osteoblast synthesizes a collagen rich matrix (osteoid) which later mineralizes to form the mature bone. During mineralization, some of the osteoblasts get entrapped within the bony matrix and differentiate into osteocytes. On the contrary, some of the osteoblasts remain on the bone surface to form flat lining cells. Osteocytes are essentially osteoblasts that are trapped within the bony matrix. These

osteocytes undergo a change in morphology and develop numerous long cytoplasmic processes that occupy the canaliculi and facilitate the exchange of nutrients and waste. The functional activity of osteocytes is similar to the osteoblasts but has limited capacity of protein synthesis (Nefussi et al., 1991).

Another major cell involved during bone remodeling is the osteoclast which is responsible for bone resorption. Unlike osteoblasts, osteoclasts originate from hematopoietic monocyte-macrophage cell lineage (Gothlin and Ericsson, 1976). Osteoclasts are found in contact with a bone surface and enclosed within a resorption lacuna (Howship's lacuna). They are characterized by a giant size (up to 100 μ m in diameter), multinucleated (usually 10 to 20 nuclei), ruffled border (foldings of the plasma membrane facing the bony matrix), sealing zone (the surrounding zone of attachment of osteoclast to the bony matrix) and a foamy appearance due to a greater concentration of vesicles and vacuoles (Gothlin and Ericsson, 1976).

Significance of bone related research

Over the past years, many research laboratories throughout the world including those at the academic and industrial levels have focused on a better understanding of the molecular mechanisms regulating bone formation and growth. A major cause for this rise in research is explained by a steady increase in bone related diseases in humans. For instance, osteoporosis is one such disease which is on a steady rise in the U.S.A. and all over the world. Statistical estimates by the National Osteoporosis Foundation, U.S.A. revealed that about 44 million people are affected by osteoporosis in the U.S.A. and it is expected to increase to 52 and 62 million by 2010 and 2020, respectively (National Osteoporosis Foundation, 2010). In addition, osteoporosis related fractures

were estimated to cost 19 billion dollars in 2005 and are expected to rise to 25 billion dollars by 2025 (National Osteoporosis Foundation, 2010).

Primary osteoporosis is the most common metabolic disorder of the skeleton and has been extensively studied (Raisz and Prestwood, 2000). This disease has been broadly classified into type 1 (postmenopausal osteoporosis) and type 2 (senile osteoporosis) osteoporosis (Ralston, 1997). Osteoporosis is a disease characterized by reduced bone mineral density (BMD) and fragile bones leading to increased risk of fracture. The reduced BMD occurs due to an imbalance between bone formation and bone resorption. In a normal individual there exists a balance between bone formation and resorption to maintain bone mass. During osteoporosis, there is an increase in bone resorption without a compensatory increase in bone formation (Raisz and Rodan, 2003). The potential methods to improve the BMD in osteoporotic patients are either to increase the bone formation or to reduce the bone resorption (Ralston, 1997). To date, most research has focused on different methods to inhibit the bone resorption. Very recently, interest has been directed in understanding the process of bone formation during bone remodeling. A detailed investigation of the bone formation process would eventually help in developing potential methods to prevent or delay the onset of osteoporosis as well as other bone related diseases.

The process of bone formation depends on the number and activity of osteoblast cells. The number of osteoblasts present at the site of bone formation in turn depends on the lifespan and proliferation potential of osteoblasts (Manolagas, 2000). Bone formation is a complex process and is an integral part of bone remodeling. To identify and understand the regulation of the bone formation process, it is essential to know the different stages and complex processes involved during bone remodeling.

Bone remodeling

Bone remodeling is a very complex process involving a series of highly regulated steps. The overall cascade of bone remodeling depends on the interactions of two cell lineages; osteoblastic and osteoclastic cell lineages (Eriksen, 1986). In a normal individual, there exists a balance between the amount of bone resorbed and the amount of bone formed. Bone remodeling occurs primarily on the inner or endosteal surfaces of bone and much less on the periosteal surfaces (Orwoll, 2003). Bone remodeling occurs in small packets of cells known as basic multicellular unit (BMUs) at focally discrete points on the inner surfaces of bone. Each BMU is geographically and chronologically separated from other packets of cells. At a particular point of time, remodeling at each BMU will be asynchronous; one site may be at the phase of resorption while the other at reversal or formation phases (Parfitt, 2002). This suggests that cellular and molecular events in bone remodeling are controlled by either autocrine or paracrine factors.

Stages of bone remodeling include 1) resorptive phase, 2) reversal phase, 3) formative phase and 4) resting phase.

Resorptive phase

The bone resorption cascade includes a series of steps targeting the removal of bone by osteoclasts. Osteoclasts, also known as bone eating cells, have a haematopoietic origin and are derived from the monocyte macrophage cell lineage (Roodman, 1996). The structure and function of osteoclasts in bone resorption have been well characterized (Roodman, 1996). The first step involves the recruitment of osteoclast progenitors from haematopoietic tissues, such as bone marrow, to the site of

bone resorption. They then proliferate and differentiate to form mature osteoclasts through a cell-cell interaction with osteoblast stromal cells. Differentiation into mature osteoclasts is regulated by receptor activator for nuclear factor κ B receptor ligand (RANK-RANKL) binding. RANKL produced by cells of osteoblast lineage binds to its receptor present on the haematopoietic progenitors to initiate osteoclast differentiation (Martin and Sims, 2005). Once a mature osteoclast is formed, it is followed by removal of osteoid layer on the surface of bone by lining osteoblasts. These osteoblasts secrete several proteolytic enzymes including matrix metalloproteinases (MMPs), collagenase and gelatinase (Meikle et al., 1992). This allows easy access for osteoclasts to the underlying mineralized matrix. Osteoclasts adhere to the bone surface through a receptor–ligand recognition mechanism. Amongst the integrin family of adhesion receptors, a specific type of vitronectin receptor (Lakkakorpi et al., 1991) binds to extracellular matrix (ECM) proteins inducing the polarization of osteoclasts. These processes lead to a ruffled border and clear or sealing zone formation in osteoclasts. The clear zone is an organelle free region of cytoplasm with thick bands of actin rings that serves for tight attachment of osteoclasts, formation of a resorption lacuna commonly known as 'Howship's lacuna' and thus restricting the resorptive surfaces on the bone (Vaananen and Horton, 1995). The next step comprises the activation of adhered osteoclasts and resorbing the bone surfaces within the lacunae. This is mediated by the H^+ - ATPase pump present in the ruffled border of osteoclasts (Blair et al., 1989; Nakamura et al., 1994). The pump transports protons into the Howship's lacunae, providing an acidic environment for the dissolution of bone minerals. Proteolytic enzymes, primarily cathepsin K (Drake et al., 1996) and MMPs including gelatinase B and collagenase (Hill et al., 1994), and lysosomal enzymes are also released from the ruffled border to degrade and dissolve the organic matrix in bone. The

degraded products are endocytosed through the center of the ruffled border, packaged as transcytotic vesicles, transported to the functional secretory domain of the osteoclasts facing the bone marrow and exocytosed (Nesbitt and Horton, 1997). Osteoclasts have a limited life span of 12 to 13 days and therefore, recruitment of osteoclast precursors into the site of bone resorption has to continually take place until desired bone resorption has occurred.

Reversal phase

After a desired amount of bone resorption has occurred, it is followed by a phase of reversal which lasts for approximately 9 days (Hill et al., 1997). During this phase the osteoclastic activity is arrested and thus bone resorption is put to an end. To date, the regulatory mechanisms that occur during this phase have not been well understood. Many researchers have suggested possibilities leading to the end of bone resorption. Firstly, apoptosis or programmed cell death of osteoclasts can be a major cause for the arrest of osteoclastic activity (Wakeyama et al., 2007). Zaidi (1990) provided alternate hypotheses that there is a large increase in the concentration of calcium within Howship's lacunae during bone resorption. The rise in calcium concentration causes rapid cell retraction, inhibition of proteolytic and lysosomal enzyme release into the lacunae, and finally inhibit bone resorption (Zaidi, 1990). A third suggestion put forward is the release of TGF- β or other peptides from the bony matrix during bone resorption which will inactivate osteoclasts and chemotactically attract osteoblasts to the site of bone resorption (Pfeilschifter and Mundy, 1990). Thus, the inactivation of osteoclasts marks the end of reversal phase and the beginning of phase of bone formation.

Formative phase

The process of bone formation includes a series of events beginning from chemotactic attraction of osteoblast precursors to the site of bone formation, proliferation and differentiation of osteoblast progenitors into mature osteoblasts, formation of organic matrix and finally mineralization of bone. The chemotactic attraction of osteoblasts or their precursors onto the site of bone resorption marks the beginning of the process of bone formation. As discussed above, chemotaxis of osteoblast progenitors is mediated by local factors produced during the resorption process. One such factor, TGF- β is chemotactic for bone cells and has been shown to be released by resorbing bone cultures (Pfeilschifter and Mundy, 1987). Similarly, structural proteins like collagen have also been demonstrated to be chemotactic for osteoblast and/or osteoblast progenitors (Mundy et al., 1982).

The second major step in the formative phase is the proliferation and differentiation of osteoblast precursors into mature osteoblasts. The process of bone formation involves a cascade of events starting from recruitment of mesenchymal cells to osteoblastic lineage, differentiation into pre-osteoblasts and then to osteoblasts and maturation of functional osteoblasts that secrete bony matrix (Aubin et al., 1995). Specifically, the mesenchymal stem cells are pluripotent in nature and can give rise to many cell lineages including the osteogenic line. These mesenchymal stem cells have unlimited self renewal capacity implying an extensive proliferative potential. Once these stem cells are recruited to osteogenic line, they are referred to as osteochondroprogenitor cells (Aubin et al., 1995). They are very similar to mesenchymal stem cells but with limited self renewal capacity. The osteochondroprogenitors cells can

either be directed to the osteogenic lineage forming preosteoblasts or directed to the chondrocyte lineage forming prechondroblasts. Although the preosteoblasts are similar to osteoblasts histologically and ultrastructurally, they do not acquire many of the differentiated characteristics of a fully mature osteoblast. The preosteoblasts have limited proliferative capacity and begin to differentiate to functional osteoblasts. A fully mature osteoblast is characterized by the ability to synthesize alkaline phosphatase (ALP), bone matrix proteins including collagen type-I (Coll-I), and several non collagenous proteins including osteocalcin (OCN), bone sialoprotein (BSP), and osteopontin (Aubin et al., 1995). Since the identification of these characteristic proteins, they have been used as markers of osteoblast differentiation. Several studies in mice and rats demonstrate that these differentiation markers are not readily expressed in preosteoblasts (Helder et al., 1993). For instance, BSP is not readily detectable in preosteoblasts but is highly expressed in osteoblasts (Bianco et al., 1993). Furthermore, markers like Coll-I and OCN are weakly expressed in rat preosteoblasts but highly expressed in the mature osteoblasts (Mark et al., 1988; Helder et al., 1993). These functional osteoblasts are the bone forming cells, which form the mineralized bone.

The osteoblastic lineage is also regulated by local growth factors such as TGF- β (Centrella et al., 1991), IGF-I and IGF-II (Schmid et al., 1992), acidic and basic FGF, bone morphogenetic proteins (BMPs) -2, -3, -4, -6, -7 and PDGF (Rydziel et al., 1992), as well as hormones including parathyroid hormone (PTH), growth hormone (GH) and sex hormones, transcription factors including runt-related transcription factor 2 (Runx2), osterix (Osx), activating transcription factor 4 (ATF4), activator protein 1 (AP1) and many others (Nakashima et al., 2002; Xiao et al., 2005; Karsenty, 2008). These transcription factors and their regulation in bone formation are discussed in detail in the following sections.

Finally, mature osteoblasts begin to lay down osteoid from the bottom of the resorption cavity and continue to form until the cavity is filled. Meanwhile the osteoid begins to mineralize at a rate of 1 μ m/day (Aubin et al., 1995). The osteoblasts at the bottom of the cavity are robust in shape and vigorous, forming a thick layer of osteoid. Termination of the osteoblastic activity occurs through a negative feedback inhibition. This has been demonstrated by the actions of osteoclast inhibitory lectin (OCIL) protein that inhibits both osteoblast and osteoclast differentiation (Nakamura et al., 2007). Alternatively, cessation of osteoblastic activity is regulated by the apoptosis of osteoblasts. Hill et al., (1997) reported that tumor necrosis factors released from the neighboring bone marrow cells could induce apoptosis in osteoblasts (Hill et al., 1997). Sclerostin, a protein secreted from the osteocytes, has also been shown to inhibit bone formation (Sutherland et al., 2004). *In vitro* studies have demonstrated that sclerostin inhibits proliferation, impairs mineralization and stimulates apoptosis of osteoblasts by interfering the Wnt and BMP signaling pathways (Sutherland et al., 2004).

Resting phase

The mineralization of newly formed bone marks the beginning of resting phase (Hill et al., 1997). During mineralization, some of the osteoblasts get trapped in the mineralized bony matrix to form osteocytes. These osteocytes later develop processes and form connections with adjacent osteocytes. Osteoblasts on the outer surface differentiate to form lining cells. A complete bone surface is reestablished and maintained until the next wave of remodeling begins.

Regulation of bone remodeling

The process of bone remodeling is tightly regulated by several factors including hormones, growth factors and transcription factors at both systemic and local levels. They exert their effect on recruitment, proliferation and differentiation of both osteoblastic and osteoclastic lineage cells. These factors aid in the maintenance of normal bone mass (Canalis, 1983). The major hormones that regulate bone remodeling include polypeptide hormones (PTH, calcitonin, and GH), steroid hormones [1, 25-dihydroxyvitamin D3 (Vit D3), glucocorticoids and, sex steroids] and thyroid hormones (TH). This review will briefly discuss the role of the major hormones in regulating bone homeostasis and development.

Parathyroid hormone (PTH)

PTH functions as the mediator of bone remodeling and important regulator of calcium homeostasis. It increases the serum calcium concentrations by direct actions on bone and kidney (Swarthout et al., 2002). PTH acts on the kidney to increase the renal tubular calcium reabsorption. Action of PTH on the intestine stimulates the synthesis of Vit D3 increasing the intestinal absorption of calcium (Potts et al., 1997). Besides the role of PTH on calcium homeostasis, recent studies demonstrate the effect of PTH on osteoclast and osteoblast activity. PTH, along with PTH-related peptide (PTHrP) interacts with receptors on osteoblasts to produce osteoclastogenic factor RANKL which initiates osteoclast differentiation. Thus, PTH indirectly activates osteoclasts resulting in increased bone resorption (Teitelbaum, 2000). On the other hand, PTH has been demonstrated to be an anabolic factor *in vitro* and *in vivo*. It is known to stimulate the expression of IGF-I, IGF-II, and IGF binding proteins (IGFBPs) thereby demonstrating anabolic effects of PTH on bone formation (Canalis et al., 1988). Since the intermittent

administration of PTH stimulates the bone formation, PTH has potential medical application for the treatment of osteoporosis (Lindsay et al., 1997; Cosman and Lindsay, 1998).

Sex steroids

Estrogen and testosterone play an important role in bone and mineral metabolism. For instance, reduced estrogen concentrations lead to a marked reduction in BMD, thus describing the major rationale for post-menopausal osteoporosis (Syed and Khosla, 2005). Normal concentrations of estrogen in the body reduce the osteoclast formation by decreasing the receptiveness of osteoclast progenitor cells to RANKL (Srivastava et al., 2001). Estrogen has also been shown to protect bone mass through induction of osteoclast apoptosis (Kameda et al., 1997). Similar to estrogens, androgens also play an essential function in bone growth. They exert their action through androgen receptors present on all types of bone cells (Sato et al., 2002). Normal amount of androgen receptors are required for the suppressive effects of androgens on osteoclastogenesis (Kawano et al., 2003). This explains the reduced risk of osteoporosis, greater mineral density and stronger bones in males.

Growth Hormone (GH) and insulin-like growth factors (IGF)

GH is a polypeptide hormone produced from the anterior pituitary gland. The synthesis and release of GH is regulated by growth hormone releasing hormone (GHRH) and somatostatin (Giustina and Veldhuis, 1998). GHRH promotes while somatostatin inhibit the release of GH from the anterior pituitary gland. One of the most important functions of GH is that it is essential for normal bone development. It has both direct effect and indirect effects on bone. The direct effect of GH on bone is explained by

the presence of GH receptors on osteoblasts and chondrocytes (Barnard et al., 1988). Furthermore, *in vitro* studies have demonstrated that GH stimulates the proliferation rate of cells of osteoblastic lineage (Kassem et al., 1993) and expression of BMPs that are important for osteoblast differentiation (Li et al., 1998; Canalis et al., 2003), thus emphasizing the direct effect of GH on osteoblasts. The indirect effects of GH are mediated by IGF. GH stimulates the release of IGF-I from the liver, which then circulates through blood and exerts its endocrine actions on target tissues such as bone. Additionally, IGF-I is also produced locally from bone and exerts autocrine or paracrine effect on bone (Le Roith et al., 2001). IGF-I and IGF-II are the most abundant growth factors present in the skeletal tissue and the bioavailability of these factors are regulated by IGFBPs. These IGFBPs are often found bound to the circulating IGF-I or complexed with IGF-I produced locally in tissues. To date, six IGFBPs have been identified. Of these, IGFBP-3 and -5 stimulate whereas IGFBP-2, -4 and -6 primarily inhibit the actions of IGF-I on bone (Jones and Clemmons, 1995). *In vivo* studies in mice further confirm the direct and indirect roles of GH and IGF on longitudinal bone growth. Specifically, mutated mice lacking GH and IGF-I had a severe reduction in tibial length and width than either mutant alone (Giustina et al., 2008). These skeletal effects occur as a result of complex interactions between GH, circulating and locally produced IGF and IGFBPs (Giustina et al., 2008).

Growth factors

Besides IGF-I and -II, there are many other growth factors including FGF, PDGF, BMPs and Wnt family of proteins that regulate bone formation and remodeling. Functionally, FGF and PDGF are osteoblast mitogens. One among several types of FGF is FGF-2. *In vitro* studies report that it stimulates DNA synthesis and proliferation of

osteoblast precursors that later mature to form functional osteoblasts (Canalis et al., 1988). However, FGF-2 does not have any action on the differentiation and function of osteoblasts. Infact, it inhibits the expression of markers of osteoblast function such as ALP (marker of early osteoblast diferentiation), OCN (marker of late osteoblast differentiation) and osteopontin (Canalis et al., 1988). This inhibitory effect of FGF-2 on differentiation of osteoblasts is mediated by induction of transcription factor Sox2 and inhibition of Wnt signaling. Similar to FGF-2, PDGF also increases the number of osteoblasts and does not stimulate the differentiation and function of osteoblasts (Canalis et al., 1992).

BMP, a member of the TGF- β super family, is comprised of proteins that are expressed primarily in bone and other extraskkeletal tissues (Daluiski et al., 2001). Of the many different forms, BMP-2,-4 and -6 are the primary BMPs expressed in bone and regulate osteoblast cell differentiation and function via Smad (Sma and Mad related family of proteins) or MAP (mitogen activated protein) kinase signaling pathways (Miyazono, 1999). BMPs induce endochondral ossification and chondrogenesis. They stimulate the maturation and function of chondrocytes with an increase in expression of type II and type X collagens (Canalis et al., 2003). Several BMP antagonists have recently been identified that inhibit BMP mediated signaling pathways by binding BMPs, including noggin, follistatin, chordin and dan family of proteins (Zimmerman et al., 1996; Gazzerro et al., 2007). Activin, a BMP related protein stimulates osteoblast proliferation, collagen synthesis, endochondral formation and enhances fracture healing (Centrella et al., 1991).

Another growth factor that regulates osteoblast differentiation and bone formation is the Wnt (wingless-int) family of proteins. In skeletal tissue, Wnt uses canonical Wnt/ β -

catenin signaling pathway (Westendorf et al., 2004). Wnt receptor mutations lead to severe impairment of bone mass suggesting a critical role in skeletal tissue (Westendorf et al., 2004). Interference of this pathway at different stages leads to many clinical manifestations. Wnt or β -catenin deletion results in decreased osteoblastogenesis and increased osteoclastogenesis, whereas overexpressing the same leads to enhanced osteogenesis and impaired bone resorption (Glass et al., 2005; Holmen et al., 2005), suggesting the importance of Wnt in normal bone growth.

Transcriptional control of osteoblast function

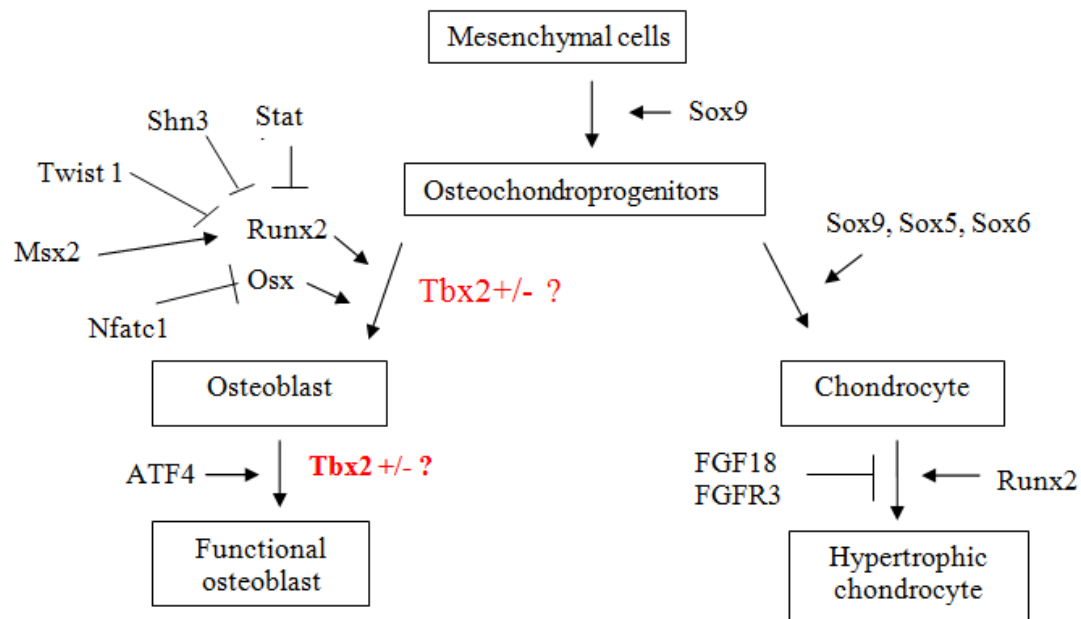
Aside from hormones and growth factors, the process of bone formation is tightly regulated by different transcription factors. Several transcription factors have been identified that play important roles during skeletal development (Komori et al., 1997; Karsenty, 2008; Karsenty et al., 2009). Recent advances in mouse and human genetics have led to identification of these factors. Based on different stages of bone development, these transcription factors primarily function either in chondrocyte or osteoblast differentiation. Within a lineage (osteoblastic or chondrocytic), these transcription factors regulate different stages of differentiation. For a better understanding, transcriptional regulation during skeletal development has broadly been categorized by researchers into two categories: 1) transcription factors that regulate chondrogenesis and 2) transcription factors that regulate osteoblastogenesis. This review will primarily focus on transcriptional regulation of osteoblasts.

As previously discussed, the process of bone formation involves a cascade of events starting from recruitment of mesenchymal cells to osteoblastic lineage, differentiation into pre-osteoblasts and then to osteoblasts and maturation of functional osteoblasts that secrete bony matrix. These different steps are governed by different

transcription factors. Some of the most important transcription factors are discussed below.

Figure 1

Transcriptional control of osteoblast and chondrocyte differentiation



Adapted from Karsenty, 2008

Runt-related transcription factor 2 (Runx2)

Runx2, a member of the runt family of transcription factors is known as the master gene of bone formation (Karsenty, 2008). All members of this family contain a highly conserved domain called the runt domain. It is through this domain whereby Runx2 interacts with nuclear proteins. Runx2 is the earliest and most important determinant of osteoblast differentiation (Ducy et al., 1997; Komori et al., 1997). Molecular and genetic studies revealed that it is involved in the recruitment of mesenchymal cells into osteoblastic lineage. Runx2 also regulates the differentiation of

mesenchymal cells to osteoprogenitor cells and then to mature osteoblasts. The expression of Runx2 occurs at very early stages of mesenchymal cell lineage and is observed in the developing embryo by E9.5 prior to bone formation (Lengner et al., 2002; Romero-Prado et al., 2006). The forced expression of Runx2 causes mesenchymal cells of other lineages to express osteoblast specific genes that are directly regulated by Runx2 (Ducy et al., 1997). Similar to the findings observed *in vitro*, studies in mice lacking Runx2 resulted in skeleton completely deprived of osteoblasts (Komori et al., 1997). These studies demonstrate that Runx2 is one of the most important transcription factors for osteoblast differentiation. Recent studies have shown that an optimal expression of Runx2 is favorable to bone growth (Liu et al., 2001). Transgenic mice over expressing Runx2 in osteoblasts developed osteopenia with multiple fractures. These bones showed impaired bone mineralization with significant reduction in mature osteoblasts (Liu et al., 2001). This suggests that Runx2 negatively controls osteoblast terminal differentiation. The significance of Runx2 in osteoblastogenesis indicates a tight regulation of its activity and expression by many other transcription factors or protein-protein interactions or protein-DNA interactions (Karsenty, 2008).

Osterix (Osx)

Osx, a zinc finger-containing transcription factor, is specifically expressed by osteoblasts and required for osteoblast differentiation. The first evidence of Osx's role in osteoblasts was revealed by Nakashima and coworkers in 2002 (Nakashima et al., 2002). They observed that Osx deficient mice resulted in perinatal lethality and impaired matrix mineralization only in bones that underwent intramembraneous ossification (Nakashima et al., 2002). The endochondral ossified bone showed some matrix

mineralization stating that *Osx*, unlike *Runx2*, is not required for chondrocyte hypertrophy. They also observed that *Osx* is not expressed in *Runx2* deficient mice whereas *Runx2* is expressed in *Osx* deficient mice, suggesting that *Osx* is a downstream target for *Runx2* (Nakashima et al., 2002). *Osx* transcription is positively regulated by *Runx2* and it acts by directing pre-osteoblasts to immature osteoblasts (Celil et al., 2005). Unlike *Runx2*, the mechanism of action and the molecular pathways that regulate *Osx* expression is poorly understood.

Activating transcription factor 4 (ATF4)

ATF4 is an osteoblast enriched transcription factor belonging to cAMP response elements binding (CREB) family of proteins (Xiao et al., 2005). The gene that encodes for ATF4 is expressed in many cell types but accumulation of protein takes place in osteoblasts cells. ATF4 is required primarily for late osteoblast differentiation. The major action of ATF4 on osteoblast differentiation takes place by its interaction with *Runx2* to regulate the transcriptional activity of *OCN*, a late osteoblast differentiation marker (Xiao et al., 2005). ATF4 also controls amino acid import into the osteoblasts. This function of ATF4 is significant for cells like osteoblasts that secrete large amounts of protein (Harding et al., 2003). Besides these functions in osteoblasts, ATF4 also favors osteoclast differentiation and bone resorption (Elefteriou et al., 2005).

Activator protein1 (AP1)

AP1 is a family of transcription factors comprised predominantly of proteins in the Fos and Jun families (Wagner, 2002). Generating mouse models with loss- or gain-of-function of these families of proteins demonstrated AP1 proteins to be important regulators of bone formation (Wagner, 2002). Of these many proteins, c-Fos was the

first transcription factor identified to control osteoblast proliferation and gene expression. Specifically, overexpression of c-Fos in mice resulted in osteosarcoma and deletion of c-Fos in mice led to osteopetrosis signifying the importance of AP1 family of transcription factors (Grigoriadis et al., 1993). Similarly, other members of this family like Fra-1 and JunB also promote bone formation and bone mass with increased number of osteoblasts (Eferl et al., 2004; Kenner et al., 2004) .

Other transcription factors involved in osteoblast function

There are many other transcription factors that regulate osteoblast proliferation, differentiation and apoptosis. They either act directly on osteoblasts or indirectly by interacting with the above described classical transcription factors to control bone formation and bone growth. Some of the key transcription factors involved in bone formation and osteoblast function are discussed in more detail below.

Genetic and molecular studies demonstrated that several transcription factors belonging to the family of homeobox proteins [Msx1, Msx2, distal-less homeobox (Dlx)3, Dlx5 and Dlx6] may play a role in osteoblast differentiation (Shirakabe et al., 2001; Hassan et al., 2004). These proteins act either as a transcriptional activator or repressor to regulate bone growth. Mice deficient in Msx2 demonstrated delayed skull ossification and reduced expression of Runx2 (Shirakabe et al., 2001). Consistent with the above findings, *in vitro* studies demonstrate that Msx2 regulates osteoblast proliferation and apoptosis (Lynch et al., 1998). Similarly, Dlx 3 and Dlx5 are expressed at all stages of osteoblast differentiation, with an increase in expression as osteoblasts mature. They also function indirectly by enhancing the expression of Runx2 and OCN (Holleville et al., 2007). Another group of transcription factors are helix-loop-helix proteins that regulate osteoblast maturation. Of these, Twists (Twist-1 and Twist-2) are the major proteins that

negatively regulate early osteoblast differentiation (Guenou et al., 2005). Twist-2 interacts with Runx2 to reduce the expression of Runx2 in osteoblasts, thereby regulating osteoblast proliferation and differentiation (Guenou et al., 2005). Besides Runx2, there are other transcription factors that play a role in lineage determination. One among them is proliferation-activated receptor γ 2 (PPAR γ 2), which is involved in the differentiation of mesenchymal cells into osteoblasts. In addition, PPAR γ 2 binds to Runx2 and inhibits its transcriptional activity (Akune et al., 2004). This function of PPAR γ 2 has been validated by both *in vitro* and *in vivo* studies in transgenic mice (Akune et al., 2004).

In addition to all these transcription factors, recent advances in mouse and human genetic studies have led to the identification of novel transcription factors in osteoblasts (Gutierrez et al., 2002; Jeon et al., 2003; Govoni et al., 2009). To date, very little information is known about the action of these factors on osteoblast function including proliferation, differentiation and apoptosis. As described earlier, the bone formation cascade involves a complex series of events that are tightly regulated by growth factors, hormones, and, transcription factors during different stages of osteoblast lineage. A novel family of transcription factors involved in early limb development and osteoblast function is described in more detail.

T-box (Tbx) family

One among the novel transcription factors that plays an important role in development of various animal species is Tbx family. The Tbx family of transcriptional factors is very primordial and highly conserved among most species of animals (Papaioannou and Silver, 1998). Tbx family has gained a recent significance in molecular research due to its association with many human diseases including genetic

disorders and various types of cancers. Since then, there has been much interest in understanding the molecular roles of Tbx genes during development.

Evolutionary history and significance of Tbx family

Tbx family was first described in 1927 with the discovery of Brachyury (T locus) that plays an important role in early vertebrate development (Dobrovolskaia-Zavadskaia, 1927). Mutational studies in mice led to the identification of this gene. Mice, heterozygous for Brachyury mutation had a truncated tail and homozygote mutants showed embryonic lethality due to improper notochord and allantois formation (Dobrovolskaia-Zavadskaia, 1927). The short tail in heterozygotes was the hallmark of this study and consequently led to the designation of T (tail) for the Tbx family. Following this discovery of Brachyury, the embryological defects caused by T mutation were studied until the gene was cloned (Herrmann et al., 1990) and found to be a transcription factor (Kispert et al., 1995). This research was a significant breakthrough and since then, many laboratories have reported Tbx genes in species varying from *C.elegans*, *Drosophila*, sea urchins, chick, zebrafish, mouse and humans (Papaioannou, 2001). To date, over 18 T-box transcription factors have been identified in the human genome and have been grouped into five subfamilies: Brachyury, T-brain1, Tbx1, Tbx2 and Tbx6 subfamilies (Papaioannou, 2001; Wilson and Conlon, 2002).

Another attribute of Tbx family to gain much recent medical importance is the linkage of Tbx genes to several human disorders. They include Holt-Oram syndrome (Tbx5), Ulnar-mammary syndrome (UMS; Tbx3), DiGeorge syndrome (DGS; Tbx1), ACTH deficiency (Tbx19) and cleft palate with ankyloglossia (Tbx22). UMS is caused by haploinsufficiency of Tbx3 gene (Bamshad et al., 1997). This syndrome is characterized by posterior limb deficiency or duplication, hypoplasia and dysfunction of mammary

gland, genitals, teeth and hair (Bamshad et al., 1997; Bamshad et al., 1999). Mutations in Tbx5 have been identified as the cause of Holt-Oram Syndrome (Basson et al., 1997). This syndrome is characterized by cardiac and skeletal congenital abnormalities (Basson et al., 1997). Abnormalities in the heart include ventricular and atrial septal defects and impaired conduction system. Skeletal defects primarily affect upper forelimbs (Basson et al., 1997; Li et al., 1997). Another member of this family, Tbx19 (Tpit) was recently identified to activate expression of pro-opiomelanocortin (POMC) leading to reduced ACTH production and ultimately adrenal insufficiency. The DGS is associated with deletion of a small segment, designated as q11, in chromosome 22. The features of this syndrome vary widely from congenital heart defects, palate defects, hypoplastic thymus and parathyroids, facial dysmorphism and learning disabilities (Ryan et al., 1997). Very recent studies show that Tbx1^{-/-} mice can recreate many aspects of DiGeorge syndrome phenotype in mice (Jerome and Papaioannou, 2001; Lindsay et al., 2001). Further studies are needed to confirm the specific role of Tbx1 in DGS.

The five Tbx genes discussed above play significant roles in inherited human disorders. In addition, recent evidence suggests a possible role of Tbx genes, including Tbx2 and Tbx3, in cell cycle regulation in various tissues and thus contributing to the recent medical importance of Tbx family.

T-box structure (general)

All members of this family are transcription factors (DNA binding proteins) that are either activators or repressors of transcription. Tbx proteins generally range in size from 50 to 78 kDa. From many reports on the structure of Tbx proteins, it can be concluded that Tbx proteins are comprised of two functional and structural domains, a

sequence specific DNA-binding domain and a transcriptional regulatory domain (Smith, 1999; Papaioannou, 2001).

The sequence specific DNA binding domain is referred to as T-box (Kispert and Hermann, 1993; Conlon et al., 2001). The T-box is a comparatively large domain making up to one third of the protein. There are varying degrees of homology across this T-box domain within a Tbx subfamily. However, there are specific residues within the T-box that are 100% conserved (Wilson and Conlon, 2002). This observation ultimately promoted the classification of Tbx family into 6 subfamilies. The positions of these domains vary between different members of this family but are regularly placed closer to amino terminus of the protein. The second domain present in these proteins is a transcriptional regulatory domain that functions either as a transcriptional activator or repressor. In most of the Tbx protein studies, these domains are located at the carboxy-terminal portion of the protein.

T-box functions

The different members of Tbx family are expressed during different stages of development and in several tissues including the skeleton, kidney, lungs, liver, mammary gland, muscle and nervous system (Papaioannou and Silver, 1998). The functions of Tbx genes have been best studied through genetics and phenotypes of mice and human mutated for different Tbx genes. Amongst the various tissues studied for the role of Tbx transcription factors, cardiac and skeletal systems attain the prime importance.

Role of Tbx genes in cardiac system

Identification of Tbx5 gene, the responsible candidate for congenital heart defects (Basson et al., 1999), introduced a lot of interest in determining the role of other Tbx genes in cardiac development. The Tbx genes that play an essential role in cardiac development primarily include Tbx1, Tbx2, Tbx3, Tbx5, Tbx18 and Tbx20 (Plageman and Yutzey, 2005). All these factors put together regulate cardiac lineage recruitment, chamber specification, valve formation, epicardial development and specialization of the conduction system. A few of these Tbx factors regulate cardiac gene expression. Specifically, atrial natriuretic factor (ANF; required for water and Na⁺/K⁺ homeostasis) and connexin 40 (Cx40; a gap junction protein), have been identified as Tbx target genes. To date, they are the most extensively studied Tbx target genes in the heart. *In vitro* studies have shown that Tbx5 acts as an activator of ANF and Cx40 while Tbx2 and Tbx3 represses their expression (Habets et al., 2002; Christoffels et al., 2004; Hoogaars et al., 2004). These studies suggest the fact that Tbx genes play an essential role in the embryonic development of heart.

Role of Tbx genes in skeletal system

Another major system tightly regulated by Tbx genes is the skeletal system. Since the identification of Tbx genes, Tbx3 and Tbx5, as the responsible candidates for ulnar-mammary syndrome and Holt-oram syndrome, respectively in 1997, research has focused on the role of Tbx genes on limb bud development (Bamshad et al., 1997). Another Tbx gene, Tbx4 has recently been attributed for small patella syndrome. This is caused by hemizygous mutations in Tbx4 leading to aplasia or hypoplasia of patella and anomalies of pelvis and feet (Bongers et al., 2004). To date, seven Tbx genes, namely T, Tbx2, Tbx3, Tbx4, Tbx5, Tbx15 and Tbx18, have been identified that are highly

expressed in the limb buds. Of these, Tbx4, Tbx5 Tbx2 and Tbx3 have been most extensively studied to understand their specific regulation during embryonic and postnatal skeletal development.

Tbx4 and Tbx5 are two closely related Tbx genes that are essential for normal limb bud development (Chapman et al., 1996a; Gibson-Brown et al., 1996a). The major functions of these Tbx genes include initiation of limb bud outgrowth and specification of forelimb and hindlimb identities. Most of the genes that are involved in limb development are expressed similarly in forelimb and hindlimb, but Tbx5 is only expressed in the forelimb and Tbx4 is only expressed in the hindlimb (Chapman et al., 1996a; Gibson-Brown et al., 1996a). Due to the differential expression of Tbx4 and Tbx5 during limb bud growth, they are supposedly involved in specifying the structural differences between forelimb and hindlimb.

Tbx15 and Tbx18 are two closely related Tbx genes with 93% sequence homology (Singh et al., 2005). Tbx15 is expressed in the murine mesenchyme, pharyngeal apparatus and the craniofacial region (Candille et al., 2004). Genetic models of transgenic mice were created to determine the function of Tbx15 on skeletal development. Homozygous null mutation of Tbx15 led to many skeletal defects in mouse embryos (Singh et al., 2005). These embryos showed a reduced bone size and altered bone shape including limbs. On the other hand, mice carrying only one functional copy of Tbx15 were normal and survived to adulthood (Singh et al., 2005). Similar to Tbx15, Tbx18 is also greatly expressed during early embryonic development in mouse. It is expressed in the somites, resulting sclerotome (vertebrae) and also in the limb bud mesenchyme. Inactivation of one allele of Tbx18 did not result in any malformations, while inactivation of both alleles of Tbx18 resulted in early postnatal death and were

characterized by severe skeletal malformations of vertebrae and ribs (Bussen et al., 2004; Singh et al., 2005).

Tbx3 is another member of T-box family playing essential role during bone development both embryonically and postnatally. As explained earlier, UMS demonstrated the significance of Tbx3 during embryonic limb bud development (Bamshad et al., 1997). Further studies on Tbx3 have shown that it may play an important role during postnatal bone development (Govoni et al., 2006). It has been demonstrated that Tbx3 is expressed in adult mouse bone and is regulated by many growth factors (GH, BMP-7 and Wnt3a) and regulates osteoblast function (Govoni et al., 2006; 2009). Specifically, knocking down the expression of Tbx3 using siRNA inhibited osteoblast proliferation (Govoni et al., 2006). Further studies showed that over expression of Tbx3 suppresses the differentiation of osteoblasts and is involved in regulating the expression of key transcription factors involved in osteoblast differentiation (Govoni et al., 2009).

Tbx2

Structure

Tbx2 belongs to the Tbx2 subfamily of Tbx transcription factors that includes Tbx3, Tbx4 and Tbx5. Tbx2 is located on chromosome 11 in humans and has been mapped to chromosome 17q23 in mice (Bollag et al., 1994; Campbell et al., 1995). Tbx2 is comprised of 3,562 bp and 3,378 bp in mouse and human respectively with seven exons. Similar to other Tbx genes, Tbx2 has a T-box DNA binding domain and a transcriptional repressor domain. The human T-box domain shows 90% DNA and 96% peptide sequence homology to the mouse (Law et al., 1995). The T-box domain is

located at amino acid position 106-289 within the T-box protein both in humans (712 amino acids) and mouse (711 amino acids).

Similar to Tbx3, Tbx2 also function as a transcriptional repressor (Carreira et al., 1998; Sinha et al., 2000). Recent advances in proteomics have identified two separate repression domains for Tbx2 (Paxton et al., 2002). Along with a carboxy terminal repression domain located between amino acids 529 and 573, a novel amino-terminal repression domain between amino acids 1 and 53 have been identified (Paxton et al., 2002). Further studies by Paxton and coworkers found that the carboxy terminal repression domain by itself was not sufficient to produce the repressor activity of Tbx2 (Paxton et al., 2002). Deletion of both the repression domains completely abolished the repressor activity of Tbx2, suggesting that amino terminal repression domain play an important role in mediating the repressor activity of Tbx2 on target genes (Paxton et al., 2002). Besides these, a weak activation domain has also been identified in Tbx2. Because of the presence of both activation and repressor domains, Tbx2 protein is suggested to play multiple specific roles based on the species and cell lines. For instance, over expression of Tbx2 increased the expression of type 1 collagen in NIH 3T3 fibroblasts but decreased col-1 expression in rat ROS 17/2.8 osteoblast cell line (Chen et al., 2001). In most research carried out to date, Tbx2 primarily acts as a transcriptional repressor. Further studies will help to elucidate the specific role of the activation and repression domains of Tbx2 protein.

Functions of Tbx2

Most studies demonstrate that Tbx2 is expressed primarily during embryonic development both in mouse and humans. It is expressed in mouse and human heart, lung, kidney, ovary, and bone (Campbell et al., 1995; Law et al., 1995). Transgenic

mouse models were created to investigate the specific role of Tbx2 during embryonic development. A homozygous mutant for Tbx2 is embryonically lethal in mice due to cardiac insufficiency whereas heterozygous mutants are normal (Plageman and Yutzey, 2005).

Role of Tbx2 in cardiac development

To date, the function of Tbx2 has only been identified in a few tissues, although it is expressed in a wide variety of tissues. In particular, the role of Tbx2 has been extensively studied during heart development. It is involved in the proliferation and compartmentalization of the developing heart (Harrelson et al., 2004). Tbx2 is expressed as early as 8 days post coitum in the myocardium of the developing mouse heart. Many studies demonstrate that Tbx2 may be a downstream target of BMP signaling pathways that are important during the development of non chamber myocardium (Zhang and Bradley, 1996). Recent studies demonstrate that Tbx2 along with Tbx3 represses the differentiation and formation of cardiac chambers (Christoffels et al., 2004) whereas Tbx5 and Tbx20 positively regulate the formation of chamber myocardium (Cai et al., 2005). This in fact suggests that Tbx2 and Tbx3 may have similar function in cardiac tissue and other tissues.

Role of Tbx2 in mammary development

Furthermore, recent studies suggest a role of Tbx2 along with Tbx3 during mammary gland development both in humans and mice (Rowley et al., 2004). Tbx3 is specifically expressed in the epithelial cells, while Tbx2 is expressed in the underlying mesodermal cells of a mammary placode (Rowley et al., 2004). The importance of Tbx3 on mammary gland was demonstrated by developing a Tbx3 knockout mouse. Heterozygous mice showed mammary hypoplasia and abnormalities of nipples, whereas

homozygous mice did not develop mammary glands (Davenport et al., 2003a). However, the role of Tbx2 during the development of mammary glands is poorly understood with comparison to Tbx3. The role of Tbx2 along with Tbx3 was supposedly brought into attention when double heterozygous mice for Tbx2 and Tbx3 showed severe deficiencies in the formation of mammary placodes than Tbx3 single heterozygote mice (Jerome-Majewska et al., 2005).

Role of Tbx2 in brain, eye and melanocyte development

In vitro and *in vivo* studies suggest a role of Tbx2 during brain development, specifically the hypothalamus. The evidence for this suggestion arises from the interaction of Tbx2 with sonic hedgehog (Shh) in ventral tubero-mammillary cells (a subset of hypothalamic cells) (Manning et al., 2006). Besides hypothalamus, Tbx2 is also involved in embryonic development of eye and skin. Tbx2 along with other Tbx members (Tbx3 and Tbx5) are differentially expressed in the neural retina (Behesti et al., 2006). Many studies using mouse embryo cultures reveal that these Tbx genes (Tbx2, Tbx3 and Tbx5) regulate the embryonic development of eye via BMP4 signaling (Behesti et al., 2006). They also reported that homozygous mutant mouse embryos show a reduced retinal volume, delayed optic vesicle invagination ultimately leading to small and abnormal optic cups. Tbx2 is also expressed in several melanocyte and melanoma cell lines but not in pre-melanoblasts (Carreira et al., 1998; Carreira et al., 2000). The role of Tbx2 in melanocyte was demonstrated by the interaction of MITF (microphthalmia associated transcription factor) with Tbx2. MITF is a key regulator of melanocyte proliferation, differentiation and apoptosis (Levy et al., 2006). MITF binds to the Tbx2 promoter and activates the expression of Tbx2 suggesting a specific role of Tbx2 in melanocytes (Carreira et al., 2000).

Role of Tbx2 in cell cycle regulation

Besides the role of Tbx2 during embryonic development in different tissues described above, recent advances in molecular biology and genetics reveal a potential role for Tbx2 in cell cycle regulation and cancer. The expression of Tbx2 along with a closely related factor, Tbx3, was upregulated in several cancers including breast (Fan et al., 2004), pancreatic (Mahlamaki et al., 2002), melanoma (Vance et al., 2005), liver (Renard et al., 2007) and bladder (Ito et al., 2005). The upregulation of Tbx2 in these cancers is explained by the fact that it functions as an immortalizing gene and enables the cells to bypass senescence (Dobrzycka et al., 2006). Tbx2 along with Tbx3 suppresses the expression of p19^{ARF}, a tumor suppressor, and promotes the bypass of senescence through inactivation of p53 pathway (Dobrzycka et al., 2006). Another explanation for the tumorigenic potential of Tbx2 was put forward by Davis and coworkers in 2008 (Davis et al., 2008). They proposed that Tbx2 may contribute to oncogenesis through bypassing cell cycle check points (Davis et al., 2008). Similarity in the functions of Tbx2 and Tbx3 in cell cycle regulation suggests that Tbx2 and Tbx3 may have overlapping functions in different tissues.

Role of Tbx2 in skeletal development

Tbx2 along with other Tbx genes (Tbx3, Tbx4 and Tbx5) is expressed in mouse embryonic limb buds. Besides mice, the expression of Tbx2 has been extensively studied in chicks (Isaac et al., 1998). The spatial and temporal expression of Tbx2 along with Tbx3 is very similar in the forelimb and hindlimb buds of mouse and chicks (Gibson-Brown et al., 1996b; Gibson-Brown et al., 1998). Tbx2 and Tbx3 are required for the identity of digit III and IV. Recent advances in the identification of different signaling pathways further strengthen the potential role of Tbx2 during limb development. For

instance, Shh pathway, involved in the determination of cell fate determination, embryonic patterning and organogenesis during early vertebrate development, is regulated by the expression of Tbx2 and Tbx3 within the posterior limb bud (Suzuki et al., 2004; Nissim et al., 2007).

Besides the role of Tbx2 during early embryonic limb bud development, recent studies *in vitro* suggest a potential role of Tbx2 during bone development. It was demonstrated that Tbx2 is expressed in mouse hypertrophic chondrocytes, periosteal osteocytes and osteoblasts (Chen et al., 2001). Furthermore, microarray analysis of Tbx2 directed gene expression in NIH3T3 cells, a fibroblast cell line, showed that among 8734 genes, 107 genes were upregulated and 66 genes were down-regulated by two fold or greater. The over expression of Tbx2 in NIH3T3 cells increased the expression of several genes [pleiotrophin (osf-1), osteoblast-specific factor-2(osf-2) and collagen type 1 α] that are involved in osteoblastic or chondrocytic lineage (Chen et al., 2001). They also carried out transfection experiments with sense and anti-sense Tbx2 in a rat osteosarcoma cell line (ROS 17/2.8) further suggesting that Tbx2 could potentially be involved during bone development (Chen et al., 2001). Specifically, osteoblast specific factor 2 (osf-2) was downregulated in ROS 17/2.8 cells transfected with antisense Tbx2 whereas osf-2 was upregulated in cells transfected with sense Tbx2. In addition, recent studies demonstrate that Tbx2 regulates the expression of Connexin 43 (Cx43) in osteoblast-like cells (Chen et al., 2004). Cx43 is a gap junction protein found in neural crest cells, myocytes, osteoblasts and many other tissues. Cx43 plays an essential role in cell-cell communication and is the predominant gap junction protein found in bone and plays critical role in normal ossification and osteoblast function (Lecanda et al., 2000).

These studies described above prove that Tbx2 is closely related to Tbx3 with 90% homology in sequence and is functionally similar to Tbx3 in cardiac development (Christoffels et al., 2004) and cell cycle regulation (Dobrzycka et al., 2006). Both Tbx2 and Tbx3 are transcriptional repressors and regulate various stages during development. Recent studies have proved the important roles of Tbx3 during postnatal bone development (Govoni et al., 2006; Govoni et al., 2009). Identification of such novel transcription factors and their role in osteoblast function will provide a better understanding of the bone formation process which would further aid in the development of potential medical therapeutics for the prevention or treatment of osteoporosis or other bone related diseases. To date, there has been neither *in vitro* nor *in vivo* data on the role of Tbx2 during post natal bone development. Based on the close homology of Tbx2 and Tbx3, functional similarity of Tbx3 and Tbx2 in different tissues, regulation of osteoblast function by Tbx3, and the role of Tbx3 and Tbx2 in embryonic limb bud development, we have developed the following hypothesis.

HYPOTHESIS

We hypothesize that Tbx2 is an important transcriptional regulator of osteoblast function (proliferation and differentiation).

AIMS

1. To transfect and knockdown the expression of Tbx2 in MC3T3-E1 (a mouse preosteoblast cell line) using small interference RNA (siRNA).
2. To evaluate the role of Tbx2 in osteoblast proliferation determined by changes in cell number and DNA synthesis.

3. To evaluate the role of Tbx2 in osteoblast differentiation determined by changes in alkaline phosphatase (ALP) activity.

MATERIALS AND METHODS

Cell culture

The murine preosteoblast-like cell line (MC3T3-E1) was purchased from American Type Culture Association (ATCC), Manassas, VA. The media used for the cell culture was α -minimal essential medium (α -MEM; invitrogen, cat. no.12571-049) containing 10% calf serum (CS; Thermo Scientific, cat. no. SH30072.03) and 1% penicillin–streptomycin (PS). The murine bone marrow stromal cells (BMSC) were maintained in α -minimal essential medium (α -MEM) containing 10% fetal bovine serum (FBS; Thermo Scientific, cat. no. SH30910.03) and 1% PS. The cell culture media containing α -MEM + 10% CS without antibiotics were used to plate the cells the day before transfection. The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air with media being changed in every 2 to 3 days. Cultures at approximately at 70% to 80% confluence were harvested with diluted trypsin-EDTA (0.5 % trypsin-EDTA). The cells were passaged at least once after thawing and then used for each experiment mentioned below (Appendix Section I).

Isolation of mouse bone marrow stromal cells

The mouse BMSC were isolated from 4 to 6 week old mice as previously described (Govoni et al., 2006) and plated at a density of 20×10^6 cells per 120 mm petri dish. The media used for the maintenance of the mouse BMSC contained α -MEM with 10% FBS and 1% PS. The cells were allowed to adhere to the plate for 5 to 7 days during which 3 mL of the media was added to each plate. Once most of the BMSC adhered to the bottom of the plate (approximately 6 days), media was changed every three days until the cells reached 70% confluent. The confluent plates were then used

for protein extraction. The details of protein extraction are described in the following sections.

siRNA transfection

The MC3T3-E1 cells were transfected with siRNA for mouse Tbx2 and scrambled negative control (NC siRNA) purchased from Dharmacon (Tbx2 siRNA: siGENOME SMART pool, cat. no. M-048790-01; NC siRNA: siGENOME Non-Targeting siRNA Pool #2, cat. no. D-001206-14-05). The cells were transfected using Lipofectamine 2000 (Invitrogen, cat. no. 11668-019) according to the manufacturer's instructions. The transfection conditions were optimized by using a siGLO Red transfection indicator (Dharmacon, cat. no. D-001630-02-05). Different concentrations of siGLO red/well (16.5, 33, and 66 nM) and Lipofectamine 2000/well (0.5, 1, and 1.5 μ L) were selected to optimize the transfection efficiency. 24 hr after the transfection, the number of fluorescent cells versus non-fluorescent cells were examined. After the transfection conditions were optimized, the transfections were performed using Tbx2 siRNA and NC siRNA. Appropriate controls were used for these experiments. The MC3T3-E1 cells with Lipofectamine 2000 was used as a control to subtract any cytotoxic effect of Lipofectamine 2000. MC3T3-E1 cells transfected with Lipofectamine 2000 and NC siRNA was another control to subtract any effect of siRNA on cell proliferation. All data presented are compared with the NC siRNA control samples. Briefly, the cells were plated at a density of 2,000 cells/well for osteoblast proliferation, 5,000 cells/well for osteoblast differentiation in 96-well plates and 70,000 cells/well for RNA extraction in 6-well plates. After 24 hrs, the cells were transfected with 66 nM siRNA and Lipofectamine 2000 (0.25 μ L/well for 96-well plate, 1 μ L/well for 24-well plate, and 5 μ L/well for 6-well plate). The cells were either transfected with Tbx2 siRNA or NC

siRNA that served as the negative control. The amount of siRNA and the lipofectamine were diluted in Opti-MEM I Reduced Serum Medium (Invitrogen, cat. no. 31985-062). After the 5 min of incubation, the diluted siRNA was combined with the diluted Lipofectamine 2000, mixed gently and incubated for 25 min at room temperature. Then, the siRNA-Lipofectamine 2000 complexes were added to each well containing the cells and incubated at 37°C for 24 hr (Appendix Section II).

RNA extraction

The cells were plated at a density of 70,000 cells/well in a 96 well plate. The following day, the cells were transfected with siRNA (Tbx2 siRNA or NCsiRNA). 24 hr after transfection, the media was removed from the wells, the cells were rinsed with PBS twice, and 1 mL of TRI reagent (Sigma Aldrich, cat. no. T9424-200) was added. The cells were scraped and transferred to a microcentrifuge tube, vortexed and incubated for 5 min at room temperature. This was followed by the addition of one-fifth volume of >99% chloroform (Sigma Aldrich, cat. no. C2432-500) mixed, and incubated for 2 to 3 min at room temperature. After the incubation, the tubes were centrifuged at 12,000 X g for 15 min at room temperature. The aqueous clear supernatant phase was carefully transferred to a new microcentrifuge tube. Thereafter, RNA was extracted using Rneasy Mini Kit (Qiagen, cat.no. 74104) according to the manufacturer's instructions. The total RNA was quantified by using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Following extraction, the residual DNA was removed from RNA samples by a 30 min digestion at 37°C with DNase using a DNA-free kit (TURBO DNA-free, Ambion, cat. no. AM1907). The RNA quality was determined using Experion Automated Electrophoresis Station (Bio-Rad, Hercules, CA). (Appendix Section III)

Reverse transcription (RT)-PCR

Each RNA sample was diluted to 300 ng/10 μ L for the RT-PCR. The reverse transcription was performed under standard conditions with SuperScript II reverse transcriptase (Invitrogen, cat. no. 18064-014) and Oligo (dT) primers (Ambion, cat.no. 5730G) in a 20 μ L final volume. Briefly, 1 μ L of Oligo (dT) primers was incubated with 10 μ L of the 300 ng RNA sample at 70°C for 10 min in the S1000 Thermo Cycler (Bio-Rad, Hercules, CA) followed by the addition of the master mix containing the dNTP, DTT and SuperScript II (Invitrogen, cat. no. 18064-014). Thereafter the reaction conditions were 37°C for 3 min, 42°C for 60 min, 4°C for 3 min, and 90°C for 2 and ½ min. Once the reactions were complete, 80 μ L of nuclease free water was added to the samples. (Appendix Section IV, V and VI)

Real-time RT-PCR

Quantitative real-time RT-PCR analysis was used to determine the expression levels of Tbx2, Tbx3, and PPIA (peptidylprolyl isomerase A; endogeneous control). Real-time RT-PCR was performed using Power SYBR Green Master Mix (Applied Biosystems, cat. no. 4367659) and 7500 Fast Real-Time PCR system (Applied Biosystems, Foster city, CA). Specifically, 20 μ L of the reaction was loaded onto a Fast Optical 96 well reaction plate (Applied Biosystems, cat. no. 4346906). Each reaction contained 10 μ L of 2X Power SYBR Green Master Mix, 1 μ L each of 10mM of forward and reverse primer, 3 μ L of nuclease free water, and 5 μ L of cDNA. The PCR conditions were 50°C for 20 s, 95°C for 10 min, 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, 60°C for 1 min, 95°C for 30 s, and 60°C for 15 s. The ΔC_T values were determined by subtracting the C_T value for the control gene from the C_T value for the gene of interest. Once the ΔC_T was determined for the control and the treatment group, $\Delta\Delta C_T$ was

calculated to express the relative quantification of gene expression (Govoni et al., 2006).

The primers used to amplify mouse Tbx2 were forward: 5' -TGA AGC TCC CAT ACA GCA CCT T -3', reverse: 5' -TTG TCG ATC TTC AGC TGT GTA ATC T -3', PPIA; forward: 5' -TCC TGG ACC CAA AAC GCT CC -3', reverse: 5' -CCA TGG CAA ATG CTG GAC CA -3', Tbx3; forward 5' -TTC CTA CCT CAC CGG GCG -3', reverse: 5' -CCG TTG GGA GGC AGC GT -3'. (Appendix Section VIII)

Western Blot

Preparation of whole cell lysate

Cells were seeded at a density of 70,000 cells/well in a 6-well plate. After 24 hr, the cells were transfected with siRNA. Forty eight hr after transfection, they were scraped into ice-cold phosphate-buffered saline (PBS, pH7.4) and centrifuged at 12,000 X RPM for 5 min. The pellet was lysed with 25 μ L of modified RIPA lysis buffer (TEKnova, cat. no. R3792). The modified RIPA buffer was prepared by the adding 10 μ L of the 100 X Halt protease inhibitor cocktail (PIC; Sigma Aldrich, cat. no. 1862209). Then the lysate was incubated on ice for 30 min followed by centrifugation at 12,000 X RPM for 5 min at 4°C. The supernatant was transferred to a pre-cooled microcentrifuge tube and stored at -80°C until used.

Preparation of whole tissue lysate

The bone, lung and heart were isolated from a 3 to 4 week old mouse and snap frozen in liquid nitrogen. A mortar and pestle was used to grind the tissues in the liquid nitrogen. This was followed by the addition of 3 mL RIPA lysis buffer, mixed properly and transferred to a microcentrifuge tube. The lysate was then incubated on ice for 1 hr

followed by centrifugation at 12,000 X RPM for 5 min at 4°C. The supernatant was transferred to a pre-cooled microcentrifuge tube and stored at -80°C until used.

Protein estimation

The protein concentration was estimated using D_C protein assay (Bio-Rad, cat.no. 500-0114). Bovine serum albumin (BSA; Sigma Aldrich, cat no. A7888-100G) was used as the standard. Specifically, different dilutions of BSA standards ranging from 0.125 mg/mL to 2 mg/mL were prepared. RIPA buffer with PIC was used as the diluent to prepare these BSA standards. Additionally, the protein samples for the estimation were also diluted in a ratio of 1:5 using the same diluent. Then 5 µl of the standards and the samples were pipetted into a clean 96-well plate followed by the addition of reagent A and reagent B. After 15 min of incubation at room temperature, the absorbances were read at 750 nm in the plate reader (Synergy 2, BioTek, VT, USA).

SDS-PAGE and immunoblotting

Equal amounts of protein (40 µg) were placed in sample buffer (63 mM Tris HCl, pH 6.8, 2 mM EDTA, 10 mM β-mercaptoethanol, 10% glycerol, 2% SDS, and 0.0025% Bromophenol blue) and heated to 65°C for 10 min. The denatured proteins were vortexed, briefly centrifuged and loaded onto a 10% sodium dodecyl sulphate-polyarylamide gel (SDS-PAGE) along with a SDS-PAGE protein standard (Precision plus protein standards kaleidoscope, Bio-Rad, cat.no.161-0375). For a better separation of proteins, the gel was run at 100 volts for 170 min at room temperature. After this, the proteins were transferred to a nitrocellulose membrane at 100 volts for 150 min. To confirm the complete transfer of proteins from the gel onto the membrane, the gel was stained with Coomassie blue solution (0.5%; Fisher scientific, cat. no. D203010) for 30

minutes at room temperature and the blot was stained with Ponceau S solution (0.1%; Fischer Scientific, cat. no. BP103-10). The blot was destained by a thorough rinsing with Tris-buffered saline tween-20 (TBST). The membrane was blocked in 5% non fat milk in TBST for 2 hr at room temperature. After washing with TBST, the membrane was probed with rabbit anti-Tbx2 antibody (1:500; abcam, cat. no. ab33298) overnight at 4°C. The following day, the membrane was washed thrice with TBST and incubated with a horseradish peroxidase conjugated anti-rabbit antibody (Sigma Aldrich, ca. no. A0545) for 60 min at room temperature. The excess antibody was rinsed off by washing with TBST three times. The Tbx2 protein was then visualized using Super Signal West Pico Chemiluminescent Substrate (Thermo scientific, cat. no. 34077). For reblotting, the membrane was stripped at 55°C for 30 min in stripping buffer [62.5 mM tris HCl (pH 6.8), 2% SDS, and 0.7% β -mercaptoethanol]. The blot was then washed thrice in TBST with 10 min interval followed by blocking with 5% non fat milk in TBST for 60 min at room temperature. After blocking, the membrane was reprobed with rabbit anti- β -actin antibody (1:500, Sigma Aldrich cat. no. A2066) overnight at 4°C and served as the loading control. On the next day, the membrane was washed with TBST, followed by incubation with horseradish peroxidase conjugated anti-rabbit antibody (Sigma Aldrich, ca. no. A0545) for 60 min at room temperature, and visualized as described above. (Appendix Section IX)

Cell proliferation assay

alarBlue assay

The alamarBlue assay is used to determine the cell number. MC3T3-E1 cells were plated at a density of 2,000 cells/well in a 96-well plate in culture media (α -MEM containing 10 % CS). A day after, the cells were transfected with siRNA (negative control

siRNA or Tbx2siRNA). 24 hr after transfection, the cells were deprived of serum for another 24 hr, and then treated with either 0.1% BSA or 1% CS. The alamarBlue assay was used to determine the cell number 48 hr after treatment. In particular, the cells were rinsed twice with 100 μ L of PBS followed by the addition of diluted alamarBlue indicator (Invitrogen, cat. no. DAL1025). The alamarBlue indicator was diluted in a ratio of 1:10 using α -MEM. After 4 hr of incubation at 37°C, the fluorescence was determined using a fluorescent plate reader (Synergy 2, BioTek) at 540/35 X 600/40 nm with sensitivity set at 54. (Appendix Section X)

5-bromo-2'deoxyuridine (BrdU) assay

BrdU assay is a chemiluminescent assay for the quantification of cell proliferation, based on the measurement of BrdU incorporation during DNA synthesis (Roche Applied Science, cat. no. 11669915001). Similar to the alamarBlue assay, the cells were plated at a density of 2,000 cells/well in a 96-well plate. After 24 hr of incubation, the cells were transfected with siRNA. 24 hr after transfection, the cells were deprived of serum for another 24 hr, and then treated with either BSA or 1% CS. The BrdU assay was performed to determine the DNA synthesis 48 hr after treatment. Specifically, the cells were incubated with BrdU labelling solution at a concentration of 10 μ L/well for 2 hrs at 37°C. After the incubation, the labelling solution was removed. Then the cells were fixed and DNA was denatured. This was followed by the addition of anti-BrdU-POD antibody (100 μ L/well) which binds to the BrdU incorporated in the newly synthesized DNA. The excess antibody was removed. 100 μ L/well of the substrate solution was added to each well and incubated for 5 min at room temperature. The chemiluminescence was detected using the plate reader (Synergy 2, BioTek, VT, USA). (Appendix Section XI)

Cell differentiation assay

Alkaline Phosphatase (ALP) activity

The cells were plated at a density of 5,000 cells/well in a 96-well plate. On the next day, the cells were transfected with siRNA (NCsiRNA or Tbx2siRNA). After 24 hr of incubation at 37°C, the cells were deprived of serum. The media used for serum deprivation was α -MEM containing 0.1% BSA, 1% PS, 50 μ g/mL ascorbic acid (Sigma Aldrich, cat. no. A8960), and 10mM of β -glycerophosphate (Sigma Aldrich, cat. no. G9891). Twenty four hr later, the cells were treated with either 0.1% BSA or human bone morphogenetic protein 2 (hBMP-2) at a concentration of 30 ng/mL and incubated for 72 hr at 37°C. The ALP activity was measured as previously described (Govoni et al., 2008). Briefly, the cells were washed with PBS two times, permeabilized with 100 μ L of 0.1% triton followed by a freeze thaw procedure. Of the 100 μ L, 40 μ L and 20 μ L of the lysate was transferred to a new 96-well plate for determining the ALP activity and protein concentration, respectively. 200 μ L of the ALP substrate, p-Nitrophenyl phosphate (pNPP; Sigma Aldrich, cat. no. P7998-100), was added to each well. The absorbance was read immediately (0 hr) and after 5 hr of substrate addition at 405 nm using the plate reader (Synergy 2, BioTek, VT, USA). The protein concentration was measured by using the Quick Start Bradford Protein assay (Bio-Rad, cat. no. 500-0205) and bovine serum albumin (BSA, Sigma Aldrich, cat no. A7888-100G) that served as the protein standard. The ALP activity was standardized to the cellular protein content. (Appendix Section XII).

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) and/or student's t-test where appropriate. Data were analyzed by using SAS 9.2 software (SAS Institute Inc., Cary, NC, U.S.A.). Data are presented as mean \pm SE and a significant difference was determined at $P \leq 0.05$.

RESULTS

Efficiency of siRNA transfection

To determine the efficiency of transfection, MC3T3-E1 cells were transfected with siGLO red transfection indicator, a negative control (NC) siRNA at 16.5, 33, and 66 nM and Lipofectamine 2000 transfection reagent at 0.5, 1.0, and 1.5 μ L, followed by the quantification of fluorescent cells (Figure 2). Overall, the cells transfected with 16.5 nM and 66 nM of siRNA had the lowest and greatest transfection efficiency, respectively ($P \leq 0.001$; Figure 3). Specifically, the cells transfected with 16.5 nM of siRNA and 0.5, 1.0, and 1.5 μ L of Lipofectamine 2000 had 30, 43 and 55 % of cells transfected with siRNA, respectively ($P \leq 0.001$). Similarly, the cells transfected with 33 nM of siRNA and 0.5, 1.0, and 1.5 μ L of Lipofectamine 2000 had 38, 75 and 74 % of cells transfected with siRNA, respectively ($P \leq 0.001$). The cells transfected with 66 nM of siRNA and 0.5 μ L, 1.0 μ L, and 1.5 μ L of Lipofectamine 2000 had 54, 83 and 86 % of cells transfected with siRNA, respectively (Figure 3). We determined the greatest transfection efficiency with 66 nM of siGLO red and 1.5 μ L of Lipofectamine 2000. We did not detect any significant difference ($P \leq 0.596$) in the efficiency of transfection when treated with 1.0 μ L or 1.5 μ L of Lipofectamine 2000/well and 66 nM of siGLO red. Since the Lipofectamine 2000 is slightly cytotoxic, we decided to use 1.0 μ L of Lipofectamine 2000/well for transfection. Thus, it was deduced through optimization to use 66 nM of siRNA with 1.0 μ L of Lipofectamine 2000 per well for our experiments.

Knockdown of Tbx2 expression using siRNA

The mRNA expression of Tbx2 was reduced by 81 ± 2 and 78 ± 2 % after 24 and 48 hr of transfection, respectively when compared to the control ($P \leq 0.015$; Figure 4). To determine if the knockdown of Tbx2 altered Tbx3 expression, the mRNA

expression of Tbx3 was also determined 24 and 48 hr after transfection with Tbx2 siRNA. Surprisingly, the expression of Tbx3 was reduced by $40 \pm 2\%$ at 24 and 48 hr after transfection when compared to the control ($P \leq 0.015$; Figure 5).

Western blot analysis

To confirm the knockdown of Tbx2 protein, western blot analysis was performed using Tbx2 specific antibody. The whole cell lysate proteins were extracted from the MC3T3-E1 cells after transfection with siRNA (Tbx2 siRNA and NC siRNA). Surprisingly, we did not detect significant amounts of Tbx2 protein in the NC siRNA and Tbx2 siRNA transfected samples (data not shown). In addition, we also did not detect Tbx2 protein in the normal nontransfected MC3T3-E1 cells (Figure 6 A). To confirm that the antibody used for the western blot was effective, we extracted protein from mouse lung, which has been shown to have high expression of Tbx2 (Chapman et al., 1996a). We were able to detect Tbx2 protein in mouse lung at 75 kDa, the expected molecular size for this protein (Figure 6 A). To determine if the lack of Tbx2 protein expression in MC3T3-E1 cells was specific to the cell line, we looked at the Tbx2 protein expression in mouse bone tissue lysate, mouse BMSC, and human osteosarcoma cell line (Saos-2; Figure 6 A and B). We did not detect Tbx2 protein in mouse bone tissue lysate and BMSC (Figure 6 A). However, a band at approximately 100 kDa was observed in Saos-2 cells (Figure 6 B). To confirm that 75 kDa band in MC3T3-E1 cells and 100 kDa band in Saos-2 cells were indeed Tbx2, we performed western blot with Tbx2 blocking peptide. The blocking peptide successfully removed the 75 and 100 kDa bands (Figure 7), thus confirming that the bands were indeed Tbx2.

Role of Tbx2 in osteoblast function

We did not observe a significant effect ($P \geq 0.29$) of knockdown of Tbx2 mRNA expression on osteoblast cell number (Figure 8) and DNA synthesis (Figure 9) in the presence or absence of serum. Similarly we did not observe a significant change ($P \geq 0.255$) in ALP activity both in the presence or absence of BMP-2 in cells transfected with Tbx2 siRNA (Figure 10).

Figure Legends

Figure 2. Optimization of siRNA transfection. Cells were transfected with various concentrations of siGLO red transfection indicator (16.5, 33, and 66 nM) and Lipofectamine 2000 (lipo; 0.5, 1.0, and 1.5 μ L). Data are presented as mean \pm SE. Bars with different letters are significantly different at $P \leq 0.001$. $n = 4/\text{treatment}$.

Figure 3. Transfection efficiency of MC3T3-E1 cells using 66 nM of siGLO red siRNA and 1.0 μ L of Lipofectamine 2000. The efficiency was determined by the number of fluorescent cells vs. non-fluorescent cells. Each image is a representative of an experiment which included 4 plates/treatment and had 86 % of cells transfected with siRNA.

Figure 4. Knockdown of Tbx2 expression. MC3T3-E1 cells were transfected with 66 nM Tbx2 siRNA or negative control siRNA (NC siRNA; control) with $n = 4/\text{treatment}$ and the mRNA expression were determined by real-time RT-PCR analysis 24 and 48 hr after transfection. Data are presented as mean \pm SE and expressed as % of control. * indicates a significant difference at $P \leq 0.015$.

Figure 5. Expression of Tbx3 in cells transfected with Tbx2 siRNA. MC3T3-E1 cells were transfected with 66 nM Tbx2 siRNA or negative control siRNA (NC siRNA; control) with $n = 4/\text{treatment}$ and the mRNA expression were determined by real-time RT-PCR analysis 24 and 48 hr after transfection. Data are presented as mean \pm SE and expressed as % of control. * indicates a significant difference at $P \leq 0.015$.

Figure 6. Tbx2 protein expression in various tissues and cell lines. A) Western blot analysis was used to detect Tbx2 protein in mouse lung, mouse bone marrow stromal cells (BMSC), MC3T3-E1 cells and Saos-2 cells. B) Western blot analysis was used to detect Tbx2 protein in mouse lung, MC3T3-E1 cells, and Saos-2 cells. β -actin served as loading control for all the western blot analysis.

Figure 7. Confirmation of Tbx2 protein in mouse and human samples. The 100 kDa band in Saos-2 cells and 75 kDa band in mouse lung tissue lysate were confirmed as Tbx2 protein by peptide neutralisation.

Figure 8. Role of Tbx2 in osteoblast cell number. Osteoblast cell number was determined by alamarBlue assay. The MC3T3-E1 cells were transfected with 66 nM Tbx2 siRNA and negative control siRNA (control) in the absence (0.1 % BSA) or presence (0.1 % BSA + 1 % CS) of calf serum. Data are presented as mean \pm SE and expressed as % of control. n = 4/treatment.

Figure 9. Role of Tbx2 in osteoblast DNA synthesis. DNA synthesis was determined by BrdU assay. The MC3T3-E1 cells were transfected with 66 nM Tbx2 siRNA and negative control siRNA (control) in the absence (0.1 % BSA) or presence (0.1 % BSA + 1 % CS) of calf serum. Data are presented as mean \pm SE and expressed as % of control. n = 4/treatment.

Figure 10. Role of Tbx2 in osteoblast differentiation. Osteoblast differentiation was determined by ALP activity and corrected for protein. The MC3T3-E1 cells were transfected with 66 nM Tbx2 siRNA and negative control siRNA (control) in the presence or absence of BMP-2 at a concentration of 30 ng/mL. Data are presented as mean \pm SE and expressed as % of control. n = 4/treatment.

Figure 2.

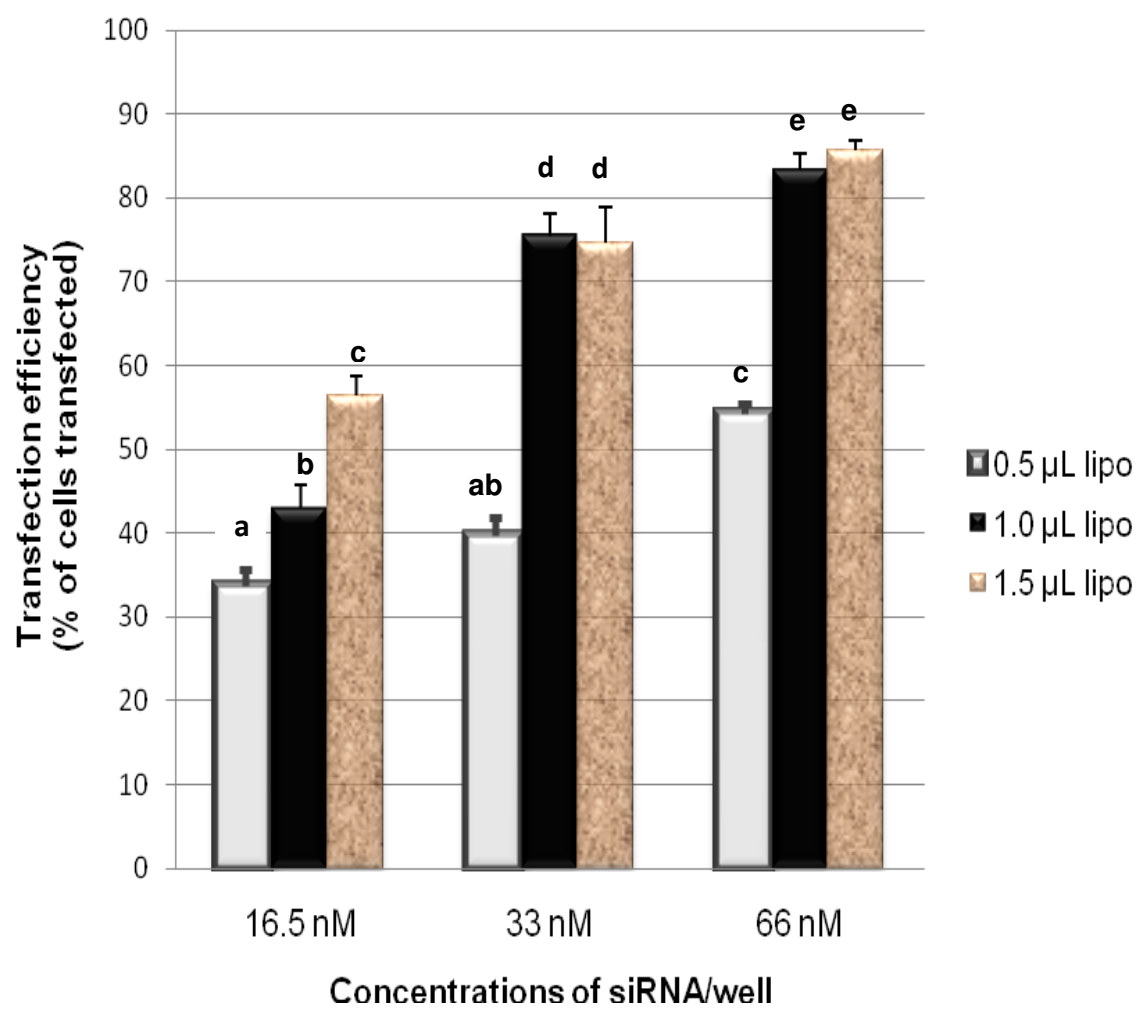
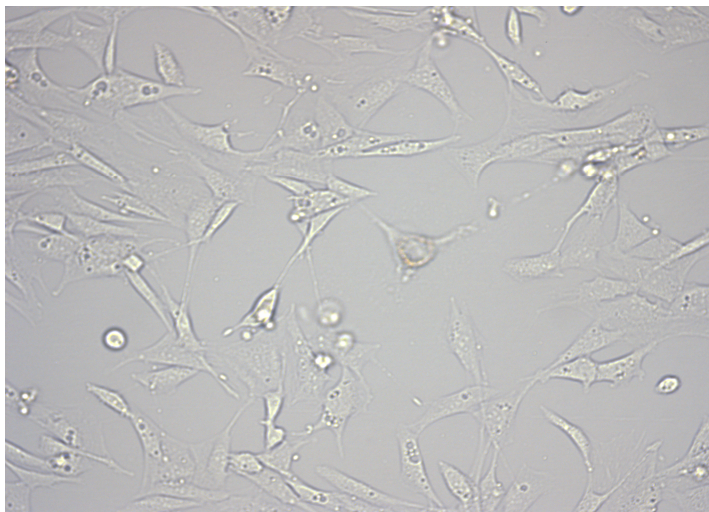
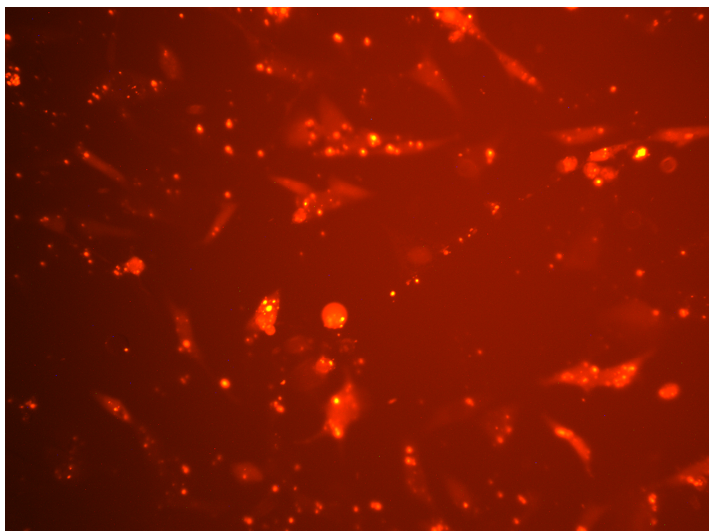


Figure 3.



Non-fluorescent



Fluorescent

Figure 4.

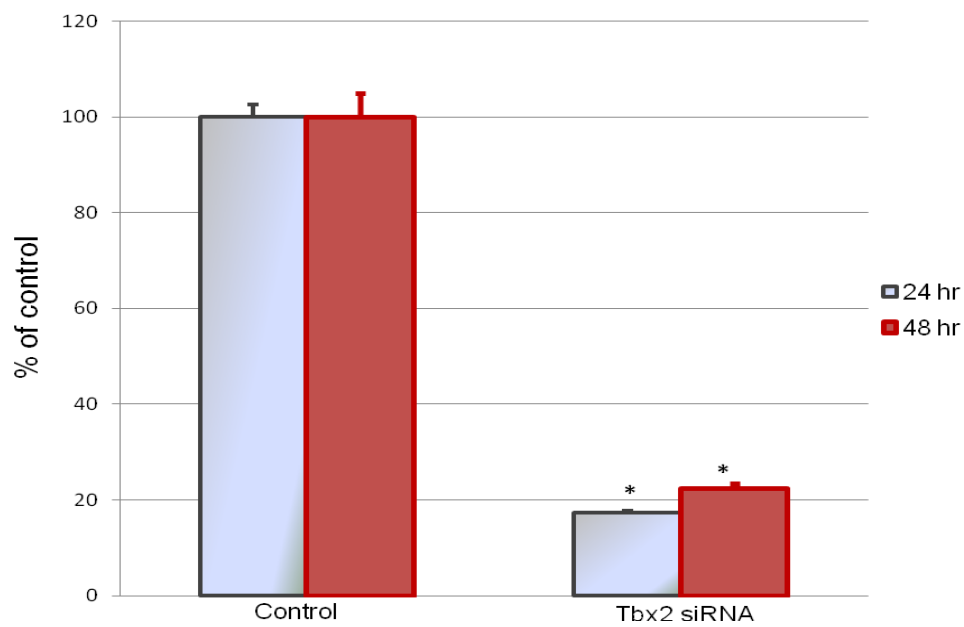


Figure 5.

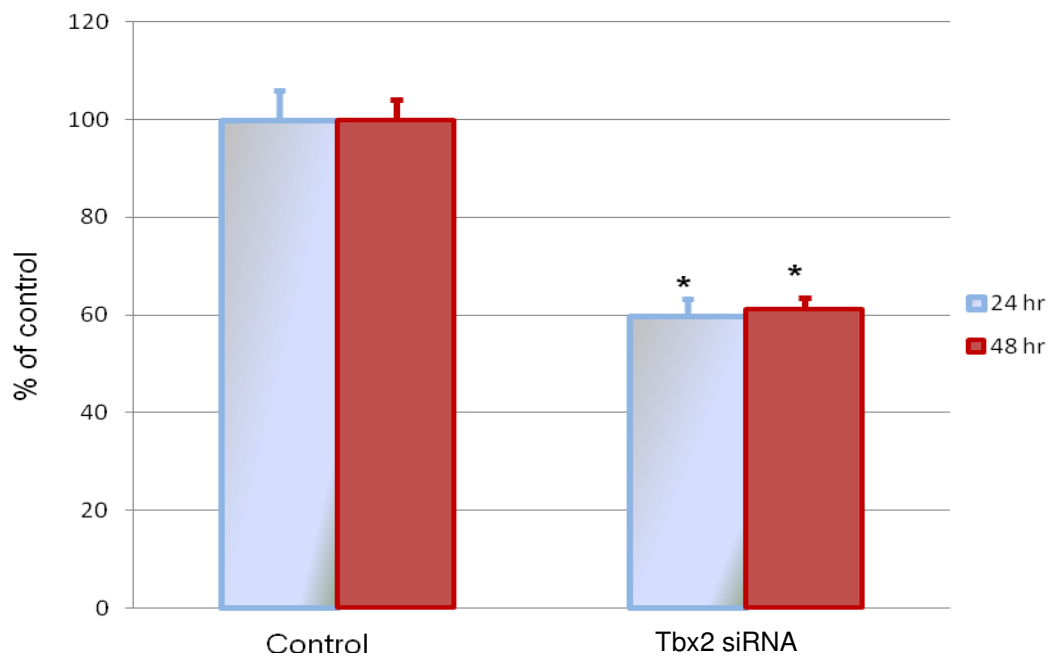
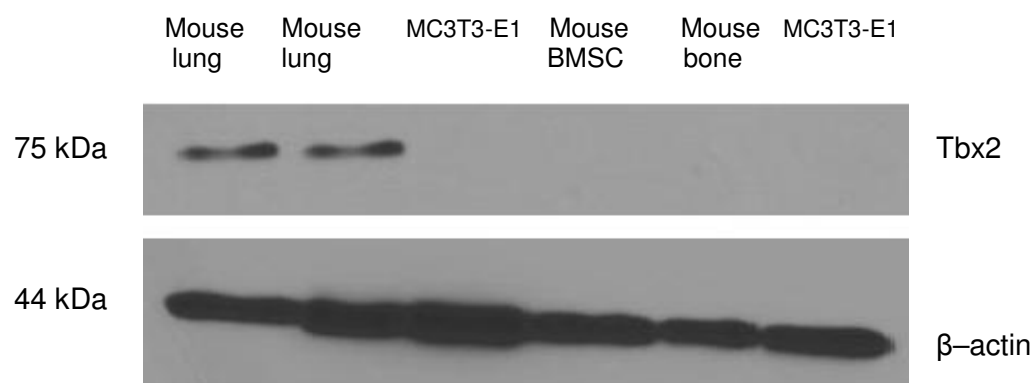


Figure 6.

A.



B.

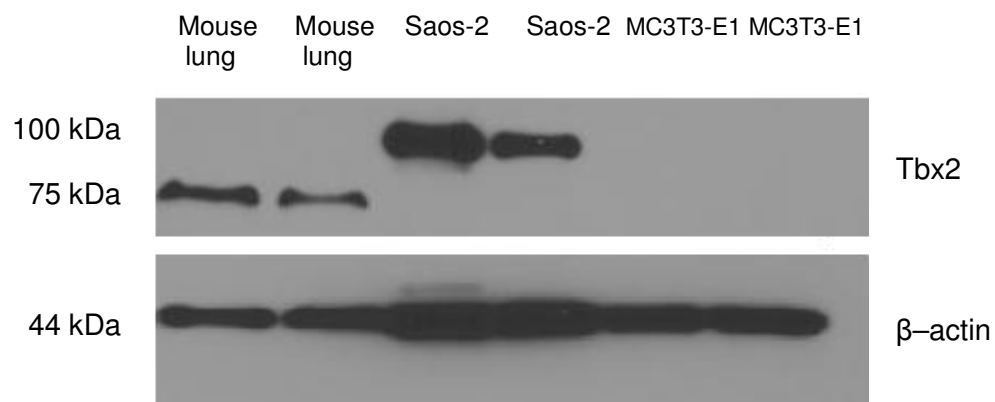


Figure 7

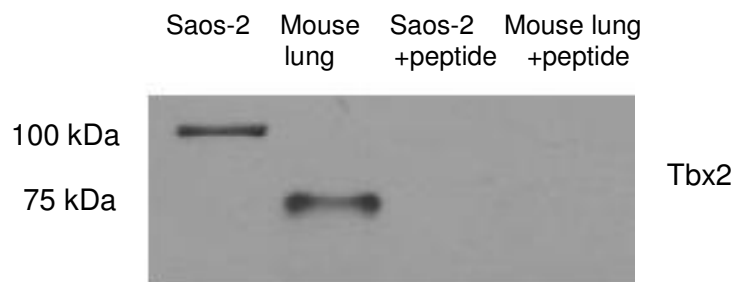


Figure 8.

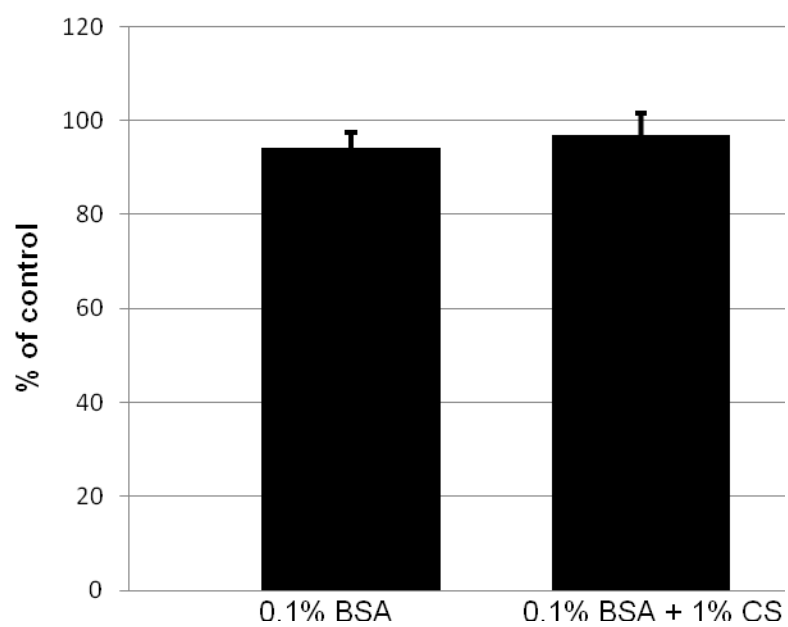


Figure 9.

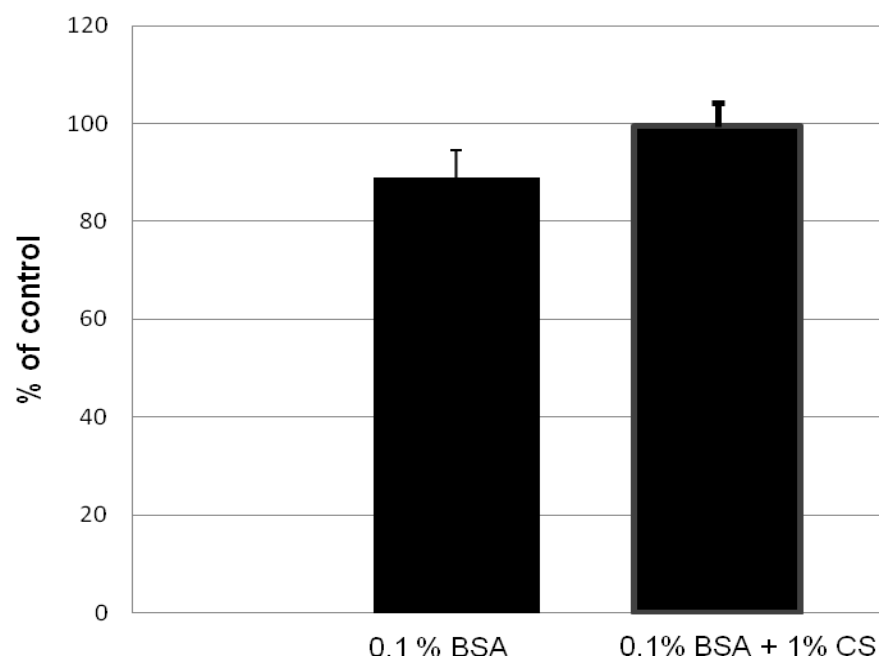
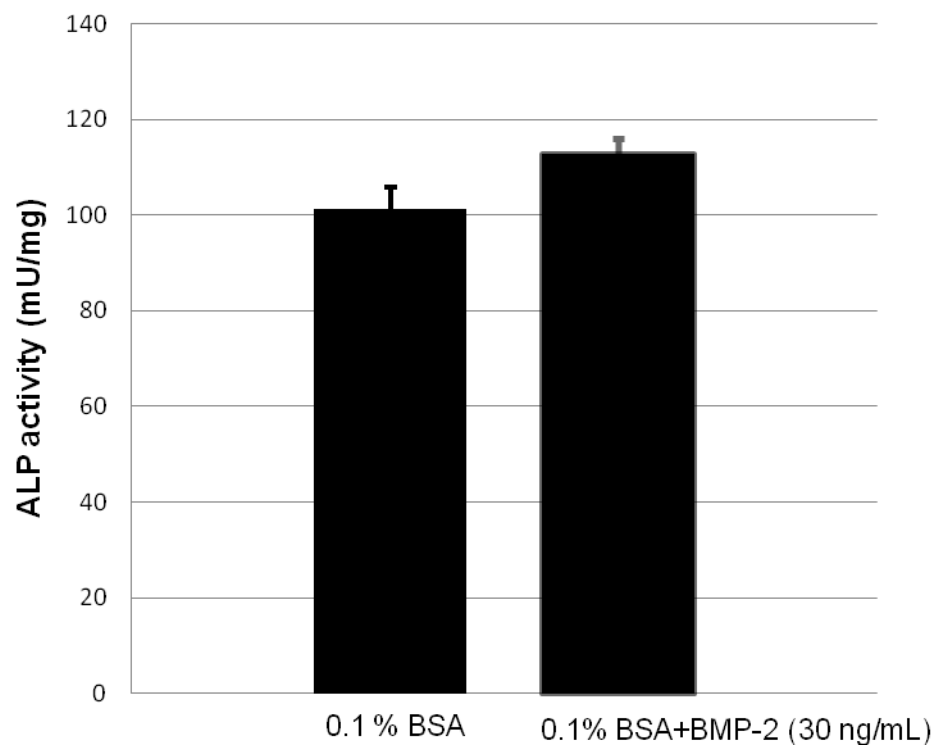


Figure 10.



DISCUSSION

The process of bone formation is very complex and tightly regulated by hormones, growth factors and transcription factors. With the recent advances in mouse and human genetic information, studies have led to the identification of novel transcription factors that regulate bone formation. Identification of these novel transcription factors will provide a better understanding of the bone formation process and will ultimately aid in the development of potential therapies for the prevention and/or treatment of osteoporosis or other bone related diseases.

The Tbx family is one among the novel transcription factors that has very recently been shown to regulate embryonic limb development and osteoblast function. Specifically Tbx2, Tbx3, Tbx4, and Tbx5 are highly expressed in the limb buds and extensively studied to understand their role during embryonic skeletal development (Chapman et al., 1996; Candille et al., 2004). Very recent studies demonstrate that Tbx transcription factors are also expressed postnatally. In particular, Tbx3 expressed in adult mouse bone, is regulated by several growth factors and regulates osteoblast function (Govoni et al., 2006). A very closely related member to Tbx3 is Tbx2 with 90% homology in the mRNA sequence. In addition, both Tbx2 and Tbx3 are transcriptional repressors and regulate various stages during embryonic development (Plageman and Yutzey, 2005). They are functionally similar in different tissues including embryonic heart development (Christoffels et al., 2004), embryonic eye development (Behesti et al., 2006) and cell cycle regulation (Dobrzycka et al., 2006). As described above, the role of Tbx3 in osteoblast function has been well characterized. However, to date there are no data available on the role of Tbx2 in osteoblast function during postnatal bone development.

Through the use of RNA interference we have attempted to elucidate the function of Tbx2 in osteoblast proliferation and differentiation. Based on the knowledge that Tbx2 is very closely related to Tbx3 in sequence homology, has overlapping functions in embryonic heart, eye development and cell cycle regulation (Christoffels et al., 2004; Dobrzycka et al., 2006), we hypothesized that similar to Tbx3, Tbx2 also regulates osteoblast function. For this study, we successfully transfected a murine preosteoblast cell line (MC3T3-E1) and knocked down the mRNA expression of Tbx2 using Tbx2 siRNA and Lipofectamine 2000. Previous studies have successfully used the transfection reagent Lipofectamine 2000 to transfect the MC3T3-E1 cells with siRNA (Alford et al., 2010). In particular, the concentration of Lipofectamine 2000 used by the Alford and coworkers was similar to the concentration used for this study (Alford et al., 2010). Similarly, Tbx2 siRNA has also been previously used to knockdown the expression of Tbx2 in breast cancer cell lines (Redmond et al., 2010). Specifically, they knocked down the expression of Tbx2 by more than 90 % at 24 hr after transfection (Redmond et al., 2010). This is consistent with our findings that we were able to successfully transfect the MC3T3-E1 cell lines and knock down the expression of Tbx2 by more than 80 % at 24 hr after transfection.

As described above, Tbx2 is very closely related to Tbx3, sharing 90 % homology in sequence and overlapping functions in many tissues. Based on these findings, we anticipated that Tbx2 and Tbx3 may have similar expression patterns and functions in osteoblasts. A previous study in which Tbx3 was knocked out in mouse suggested a compensatory upregulation of Tbx2 with the loss of Tbx3 in embryonic heart development (Plageman and Yutzey, 2005). To determine if the knockdown of Tbx2 altered Tbx3 expression, the mRNA expression of Tbx3 was determined in MC3T3-E1 cells treated with Tbx2 siRNA. Surprisingly, we observed a significant

decrease in the expression of Tbx3 by 40 % in cells transfected with Tbx2 siRNA.

Although the non-specificity of Tbx2 siRNA used for this study may be a possible explanation for reduced Tbx3 expression in MC3T3-E1 cells treated with Tbx2 siRNA, it is unlikely. To determine the specificity of Tbx2 siRNA, the four Tbx2 siRNA sequences present in the mouse Tbx2 siGENOME smart pool were blasted against the mouse genome and the results matched only to the mouse Tbx2 mRNA and were not homologous to Tbx3. Hence, the non specificity of Tbx2 siRNA is less likely an explanation for the reduced expression of Tbx3 with knock down of Tbx2.

A biological effect within the cells is more likely an explanation for the reduced expression of Tbx3 mRNA in MC3T3-E1 cells transfected with siRNA. Contrary to our findings, it has been demonstrated that transfection of a melanoma cell line with Tbx3 siRNA led to upregulation of Tbx2 suggesting that Tbx3 represses the expression of Tbx2 in melanoma cells (Rodriguez et al., 2008). This study suggests that the change in Tbx3 mRNA expression may be a biological effect of reduced Tbx2 and substantiates for the variation in the expression of Tbx3 and Tbx2 when transfected with siRNA as a biological effect. Studies involving Tbx3 deficient mice also demonstrate the variation in the expression of Tbx2 (Davenport et al., 2003b; Bakker et al., 2008). Specifically, in situ hybridization of the Tbx3 homozygous mutant embryos demonstrated a greatly reduced and absence of Tbx2 expression in the posterior margin of the forelimb and hindlimb, respectively whereas the expression of Tbx4 and Tbx5 was not altered (Davenport et al., 2003b). This suggests that knocking down Tbx3 expression reduces the expression of Tbx2 in embryonic limb bud. This is similar to our findings where knocking down the expression of Tbx2 in a mouse preosteoblast cell line reduced the mRNA expression of Tbx3. It is still not clear if the expression of Tbx2 and Tbx3 appear to be dependent on each other, however further studies are necessary to elucidate the relative expression of

Tbx2 or Tbx3 within the osteoblast and determine if they are indeed dependent on each other.

Tbx2 is expressed in mouse and human heart, lung, kidney, bone, and mammary tissue during embryonic development (Fan et al., 2004; Ito et al., 2005). Although Tbx2 is expressed in various tissues, the functions of Tbx2 are not yet well understood in these tissues. Based on the fact that Tbx2 is widely expressed in several tissues including embryonic bone, it is expected that Tbx2 is also expressed in MC3T3-E1 cells, a preosteoblast cell line. As predicted, we observed Tbx2 mRNA expression in the MC3T3-E1 cells. To further verify the fact the Tbx2 is indeed expressed in the MC3T3-E1 cells, the two Tbx2 primers used for this study were validated by sequencing the PCR product and blasted against the mouse genome. Results of the blast analysis matched only to the mouse Tbx2 mRNA. Therefore, it was confirmed that Tbx2 is expressed in MC3T3-E1 cells at the mRNA level.

To confirm the knockdown of Tbx2 at the protein level, whole cell lysate proteins were extracted from the siRNA transfected cells and western blot was performed. Surprisingly, we could not detect significant amounts of Tbx2 protein in either of the siRNA transfected MC3T3-E1 cells (NC siRNA or Tbx2 siRNA) or in non transfected MC3T3-E1 cells. Previous studies demonstrate that Tbx2 is highly expressed in mouse lung (Chapman et al., 1996a). To confirm that the antibody used for the western blot was effective, we extracted mouse lung protein and used it as a positive control. The Tbx2 protein could be detected in the mouse lung at 75 kDa but not in the MC3T3-E1 cell lysate. Furthermore, to validate the lack of Tbx2 protein expression was not specific to the cell line, we looked at the expression of Tbx2 protein in mouse BMSC, bone tissue lysate, and human osteosarcoma cell line (Saos-2). A previous study demonstrated the

presence of Tbx2 protein in many osteosarcoma cell lines including Saos-2, ROS 17/2.8, and MG63 (Chen et al., 2001). Similar to their findings, we detected the Tbx2 protein in Saos-2 cells but at approximately 100 kDa. The predicted molecular size of Tbx2 protein is 75 kDa but our western blot showed an intense band at 100 kDa that was later confirmed as Tbx2 protein by the peptide neutralization. It is unclear as to why the Tbx2 protein was detected in two molecular sizes by western blot analysis. The possible explanations for the disparity in molecular size may be due to alternative splicing of Tbx2 mRNA. Previous studies have demonstrated that alternate splicing can occur in human Tbx2 (generally denoted as TBX2) and lead to proteins of varying molecular sizes (Campbell et al., 1998). Moreover, in communication with a research lab extensively working on Tbx2 in South Africa further substantiated for the band observed at 100kDa (Dr. Sharon Prince Lab, Department of Human Biology, University of Cape Town, South Africa). Similar to our findings, they also detect the Tbx2 protein at approximately 100kDa (Abrahams et al., 2008). Although it is unclear as to why two different band sizes were obtained for Tbx2, it was confirmed by peptide neutralization that the bands observed at 75 kDa in mouse lung and approximately at 100 kDa in Saos-2 cells were indeed Tbx2. Further studies on the structure of Tbx2 protein are necessary to address the disparity in molecular sizes of Tbx2 protein in western blot analysis.

We failed to detect Tbx2 protein is not present in detectable amounts in MC3T3-E1 cells, mouse BMSC, and bone tissue lysate but is detected in Saos-2 cells. This finding is consistent with the detection of varying amounts of Tbx2 protein in melanocytes (Vance et al., 2005). In particular, it has been demonstrated that the Tbx2 protein is poorly expressed or absent in both primary human melanocytes and mouse melanocyte cell line whereas greatly expressed in melanoma cell lines (Vance et al., 2005). Very similar to their findings, we could not observe significant amounts of Tbx2

protein in mouse BMSC and mouse osteoblast cell line but greatly expressed in Saos-2, a human osteosarcoma cell line. Similarly, the expression of Tbx2 is upregulated in many cancers, including breast, pancreatic, melanoma, liver, and bladder (Fan et al., 2004; Ito et al., 2005). Tbx2 along with Tbx3 prevents senescence in these cancer cells by a mechanism involving their ability to transcriptionally repress p14^{ARF}, a tumor suppressor gene (Lingbeek et al., 2002). This demonstrates that Tbx2 is involved in cell cycle regulation suggesting that Tbx2 may be an important regulator of cancer cell progression. A better understanding of the upstream and downstream targets for Tbx2 will provide a better understanding of the upregulation of Tbx2 in several cancerous tissues.

The expression of Tbx2 also varies greatly within a cell type. For instance, the expression of Tbx2 at the mRNA level and at the protein level were examined in a variety of breast cancer cell lines and it was observed that the Tbx2 protein was detected in only three out of eight breast cancer cell lines used for the study (Redmond et al., 2010). Similarly, Tbx2 protein was detected in five of the twelve human melanoma cell lines examined by Rodriguez and coworkers (Rodriguez et al., 2008). These studies suggest that the expression of Tbx2 varies greatly within a cell type. Therefore, the selection of a cell line that expresses Tbx2 both at the mRNA and protein level forms the basic criterion to study the functional role of Tbx2 within a cell type.

Thus, our study revealed that there are detectable amounts of Tbx2 expression at the mRNA level but Tbx2 protein was not detected in MC3T3-E1 cells. This suggests that the Tbx2 mRNA expression profile does not correlate with expression profile of Tbx2 protein. Similar findings have been observed in many eukaryotic proteomic studies (Gygi et al., 1999; Chen et al., 2002). A previous study determined the relationship between

mRNA and protein expression levels for 150 genes in the yeast *Saccharomyces cerevisiae* (Gygi et al., 1999). They demonstrated that the quantification of mRNA is insufficient to predict the protein levels within the cell. In particular, they observed that the correlation between mRNA and protein expression is greatly reduced in lesser abundant proteins when compared to greater abundant proteins (Gygi et al., 1999). This suggests the importance of posttranslational mechanisms regulating gene expression especially in less abundant proteins. In this context, Tbx2 being a less abundant protein can explain for the disparity in the mRNA and protein expression profile. Further studies are needed to address this variation in the mRNA and protein expression profile of Tbx2 in MC3T3-E1 cells.

Surprisingly, we did not observe any effect of knockdown of Tbx2 on osteoblast proliferation and differentiation. Specifically, knocking down the expression of Tbx2 using siRNA did not affect the proliferation or differentiation of osteoblasts both in the presence or absence of serum. Previous studies have demonstrated the role of Tbx2 in cell cycle regulation (Dobrzycka et al., 2006). As described earlier, Tbx2 along with Tbx3 act as anti senescence factors in several cancers (Fan et al., 2004; Ito et al., 2005). Bypassing the senescence leads to uncontrolled cell proliferation thereby ultimately leading to cancer. Additionally Tbx3, closely related to Tbx2 with 90% homology in sequence and functional similarity in tissues, has been demonstrated to be an important mediator of osteoblast proliferation by Govoni and coworkers in 2006 (Govoni et al., 2006). They observed that knocking down the expression of Tbx3 in MC3T3-E1 cells reduced osteoblast proliferation both in the presence or absence of serum (Govoni et al., 2006). Several other studies have also demonstrated the regulatory effect of Tbx3 on cell proliferation. For instance, the proliferation rates of rat bladder hyperplastic epithelial and carcinoma cells are regulated by Tbx3 (Ito et al., 2005). In addition to the role of Tbx3 on

osteoblast proliferation, studies have demonstrated that Tbx3 regulates osteoblast differentiation (Govoni et al., 2006; Govoni et al., 2009). Specifically, they reported that the expression of Tbx3 increased 2 fold during *in vitro* differentiation of MC3T3-E1 cells. In addition, Tbx3 increased the expression of BMP-7 and Wnt3a (Govoni et al., 2006), known stimulators of osteoblast differentiation further confirming the fact that Tbx3 regulates osteoblast differentiation. Based on the close homology in sequence and functional similarity of Tbx3 to Tbx2, we hypothesized that Tbx2, like Tbx3 regulates osteoblast proliferation and differentiation. However, we could not determine any effect of Tbx2 on osteoblast proliferation and differentiation. But this could be due to lack of Tbx2 protein in these cells and did not knock it down.

Our preliminary results are comparable to previous studies which demonstrate that although Tbx2 and Tbx3 are very closely related, they may differ in their expression and have distinct roles within a cell type (Rodriguez et al., 2008; Peres et al., 2010). In several melanoma cell lines used for the study, no cell lines demonstrated significant amounts of both Tbx2 and Tbx3 protein detected by western blot analysis (Rodriguez et al., 2008). The cell lines that greatly expressed Tbx3 protein poorly expressed Tbx2 protein and vice versa suggesting a significant biological function of only one of the two (Tbx2 or Tbx3) in melanoma cells. These factors could account for the poor expression of Tbx2 protein in MC3T3-E1 cells, where Tbx3 protein is expressed (Govoni et al., 2006). Very recently, a study demonstrates that Tbx3 and Tbx2 have distinct roles in the melanoma and breast cancer cell line (Peres et al., 2010). They report that Tbx2 promotes cell proliferation and does not affect the cell migration whereas Tbx3 is required for tumor formation and cell migration and in fact inhibits cell proliferation (Peres et al., 2010). The differential expression and distinct role of Tbx3 and Tbx2 within

a cell type suggests that Tbx2 is not an important regulator of osteoblast function like Tbx3.

To conclude, we transfected and knocked down the expression of Tbx2 in MC3T3-E1 cells to evaluate the role of Tbx2 in osteoblast function. We did not determine any effect of Tbx2 in osteoblast function including proliferation and differentiation. There are two possible explanations for why we did not observe any effect of Tbx2 in osteoblast function. Firstly, it is possible that Tbx2 is not required for the osteoblast function which is suggested by the absence or poor Tbx2 protein expression in MC3T3-E1 cells. Secondly, based on little to no Tbx2 protein expression in MC3T3-E1 cells, knock down of Tbx2 was not an optimal method to evaluate its role on osteoblast proliferation and differentiation. Further studies involving the over expression of Tbx2 in MC3T3-E1 cells are needed to better understand the role of Tbx2 on osteoblast function. Use of other preosteoblast cell lines that express Tbx2 both at the mRNA and at the protein level is an alternate method to elucidate the role of Tbx2 on osteoblast function.

APPENDIX

Section I. Cell culture - maintenance of MC3T3-E1 cell lines

1. Warm the bottles of α -MEM + 10% CS + 1% PS media, PBS, and 50% Trypsin-EDTA + 50% PBS in water bath at 37°C. Note: Do not place the bottle with Trypsin-EDTA directly in water.
2. Wipe down the working area in the hood with a 70% ethanol soaked gauze sponge. Spray or wipe the pipette gun and a 50 mL Falcon tube with 70% ethanol and place them in the hood.
3. Wipe the bottles from the water bath with 70% ethanol gauze and place them into the hood.
4. Once all above steps are done remove the cell culture dishes from the incubator and place them into the hood.
5. Tilt the plate of cells. Using a glass Pasteur pipette and the vacuum pump, remove the media from the plate without touching the bottom of plate with the pipette tip.
6. Tilt the plate and use a transfer pipette to gently add PBS to the plate, from the side wall. Then swirl the PBS over the plate to rinse the cells.
7. Tilt the plate and using a glass Pasteur pipette and the vacuum pump remove the PBS from the plate.
8. Add 50% trypsin-EDTA + 50% PBS directly onto the cells.
9. Incubate the plate for 3 min at 37°C.
10. Remove the plate from incubator and place it in the hood. Tilt the plate and with a transfer pipette draw up some of the 50% trypsin-EDTA + 50% PBS. Using the liquid in the pipette rinse the bottom of plate. When the cells are detached, use a transfer pipette to add α -MEM + 10% CS + 1% PS media to inactivate the trypsin-EDTA.
11. Keep rinsing the plate with 50% trypsin-EDTA + 50% PBS and α -MEM + 10% CS + 1% PS media until bottom of the plate has no visible residue.
12. Transfer the 50% trypsin-EDTA + 50% PBS and α -MEM + 10% CS + 1% PS media solution from the plate to a 50 mL Falcon tube.
13. Centrifuge the 50mL Falcon tube at 1000 rpm for 3 min at room temperature (22°C to 26°C). Note: remember to balance the centrifuge with a water blank.
14. Once the centrifuge is complete move the Falcon tube back into the hood. Use a glass Pasture pipette and the vacuum pump to remove the supernatant from cell pellet that remains at the bottom of the tube.
15. Add at least 10mL of α -MEM + 10% CS + 1% PS media to the cell pellet in the Falcon tube (Note: amount of media to use to re-suspend the pellet varies according to the number of plates available and type of experiment). Add the media slowly, and then pipette up and down until the pellet is broken up.
16. Once cells are re-constituted in the α -MEM + 10% CS + 1% PS media add more media as needed for the number of new plates that the cells are being expanded to.

17. Add about 7 to 8 mL of α -MEM + 10% CS + 1% PS media to new plates, and then add your pre-determined amount of the α -MEM + 10% CS + 1% PS containing the cells to each plate. Note: add the cells in small drops all around the plate so that the cells do not clump.
18. Swirl the plates to ensure that the cells are evenly distributed.
19. Label each plate with cell type and origin, passage number, initials, date, and plate number.
20. Check plates under the microscope to ensure cells are evenly distributed in an appropriate confluency.
21. Incubate the plates, clean up, and wipe down the hood with 70% ethanol.

Section II. siRNA transfection with Lipofectamine 2000

1. The MC3T3-E1 cells (2,000 cells/well in a 96-well plate, 14,000 cells/well in a 24-well plate, and 70,000 cells/well in a 6-well plate) are plated in α -MEM +10% CS (100 μ L of media/well for 96-well plate, 500 μ L of media/well for 24-well plate and 2 mL of media/well for 6-well plate).
2. Dilute the siRNA (10 pmols/well for 96-well plate, 40 pmols/well for 24-well plate, and 200 pmols/well for 6-well plate) in Opti-MEM I Reduced Serum Media (25 μ L/well for 96-well plate, 50 μ L/well for 24-well plate, and 250 μ L/well for 6-well plate) and mixed gently.
3. Mix the lipofectamine 2000 gently before use.
4. Dilute the Lipofectamine 2000 (0.25 μ L/well for 96-well plate, 1 μ L/well for 24-well plate, and 5 μ L/well for 6-well plate) in Opti-MEM I Reduced Serum Media (25 μ L/well for 96-well plate, 50 μ L/well for 24-well plate, and 250 μ L/well for 6-well plate) and incubated for 5 min at room temperature.
5. Combine the diluted siRNA and diluted Lipofectamine 2000 (to form the siRNA-Liporectamine 2000 complex) as soon as possible and incubate for 25 min at room temperature.
6. Add siRNA-Lipofectamine 2000 complex to each well (50 μ L/well for 96-well plate, 100 μ L/well for 24-well plate, and 500 μ L/well for 6-well plate).
7. Incubate the plates for 24 hours at 37°C.

Section III. RNA extraction—Qiagen Kit

1. The transfected cells are grown in 6-well plates (70,000 cells/well).
2. Tilt the plate of cells. Using a glass Pasteur pipette and the vacuum pump, remove the media from the plate without touching the bottom of plate with the pipette tip.
3. Tilt the plate and use a transfer pipette to gently add PBS to the plate, from the side wall. Then swirl the PBS over the plate to rinse the cells.
4. Tilt the plate and using a glass Pasteur pipette and the vacuum pump remove the PBS from the plate.
5. Add 1 mL TRI Reagent (trizol) to each well and scrape with pipet tip.
6. Transfer it to a 1.5 mL microcentrifuge tube, vortex, and homogenize.
7. Incubate for 5 min at room temperature.
8. Add 200 μ L chloroform to each tube and mix by inverting 5 to 7 times.
9. Incubate for 2 to 3 min at room temperature.
10. Centrifuge at 12,000 x g for 15 min.
11. Transfer the aqueous phase (transparent, clear top layer) into a new micro centrifuge tube (approximately 500 μ L).
12. Add one volume (500 μ L) of 70% EtOH to each tube and vortex the tubes.
13. Transfer 700 μ L of the sample into the RNA easy spin column.
14. Centrifuge at 8000 x g for 30 seconds and discard the flow through.
15. Add 700 μ L Buffer RWI and centrifuge at 8000 x g for 30 seconds.
16. Discard the flow through and the collection tube, and place the filter into a new collection tube.
17. Add 500 μ L of Buffer RPE and centrifuge at 8000 x g for 30 seconds.
18. Discard the flow through and add 500 μ L of RPE buffer.
19. Centrifuge at 8000 x g for 2 min and discard the flow through.
20. Centrifuge at 8000 x g for 1 minute.
21. Discard the flow through and the collection tube, and put the filter into a new capped collection tube.
22. Add 50 μ L of Rnase free water and centrifuge at 8000 x g for 1 minute.
23. Take 35 to 40 μ L from the collection tube and put back on the filter. Centrifuge at 8000 x g for 1 minute.
24. Collect the eluted RNA and spec it in the Nanodrop spectrophotometer.
25. Aliquot and store in the -80°C freezer

Section IV. Normalizing the concentration of RNA to 300 ng/10µL

Sample

1. We need 300 ng/10 µL or 30 ng/µL of RNA to be used for reverse transcription. The final volume should be 26 µL (20 µL to be divided into two sets of reverse transcription and 6 µL for spectrophotometer analysis).

$$C1V1 = C2V2$$

$$C2 = 30 \text{ ng/}\mu\text{L}$$

$$V2 = 26 \mu\text{L}$$

C1 = concentration of RNA obtained after DNA-free treatment

$$V1 = ?$$

This calculation should give V1, the amount of stock solution of RNA to be taken. The volume is corrected to 26 µL using nuclease free water.

2. The RNA concentration should come to 30 ng/µL, variations are adjusted to 30 ng/µL by adding Nuclease Free water (0.5-1.0 µL) or adding RNA (0.2 µL).
3. Transfer 10µL of normalized RNA (300 ng/10 µL) into one tube of an 8 tube strip (x 2) for reverse transcription.

Section V. Reverse transcription

1. Thaw the reagents on ice (Ambion Oligo (dT), Invitrogen Superscript kit II containing DTT, 5X buffer and reverse transcriptase)
2. Add 1 μL of Oligo (dT) to each 10 μL of normalized RNA sample (300 ng/10 μL) in 8 tube strips. This makes it a total of 11 μL . NOTE: Include a Positive control too.
3. Identify, or set the program on the PCR machine to run:
 - 70°C for 10 min
 - 4°C for 20 min
 - 37°C for 3 min
 - 42°C for 1 hr
 - 4 °C for 3 min
 - 90°C for 2.30 min
 - 10°C forever
4. Run the tubes in the PCR machine and start step1, 70°C for 10min.
5. During step1, make the master mix (amounts are per sample, multiply by the number of samples plus two for pipetting errors).
 - Master Mix
 - 5X Buffer- 5.5 μL
 - dNTP- 1.0 μL
 - DTT- 2.0 μL
 - Superscript II 0.5 μL
 - NOTE: Add SS II last.
6. Hit pause on the PCR machine when the temperature is dropping to 4°C.
7. Take the samples with RNA and Oligo (dT) out of the machine and keep on ice.
8. Add SSII to the master mix, and mix gently.
9. Add 9 μL of master mix to each sample, making it a total of 20 μL (keep samples on ice while adding the master mix).
10. Place the samples back into the machine, hit "proceed" and hit "proceed" again to move to step # 3. The step #2 is skipped.
11. When reverse transcription is complete add 80 μL of Nuclease free water to samples and store in -20°C freezer.

Section VI. Polymerase Chain Reaction (PCR)

1. Thaw reagents on ice (Promega Green Master mix, Tbx2 114 forward and reverse primer working aliquots, cDNA sample).
2. Make Master mix (amounts are per sample, multiply by the number of samples plus two for pipetting errors)

Nuclease free water 5.5 μ L

Green master mix 12.5 μ L

Forward Primer 1.0 μ L

Reverse Primer 1.0 μ L

3. Into 8-tube strips transfer 20 μ L of the master mix per tube.
4. Into the same 8-tube strips add 5 μ L of the cDNA per tube, making it a total of 25 μ L/tube. This should be done just before it is ready to go into the PCR machine.
5. Place the tubes into PCR machine and run the program with the steps below:

94°C for 3min

94°C for 30s

60°C for 1min

72°C for 1min

72°C for 5 min

10°C forever

X 40cycles

6. Once the PCR is complete (about 2 and 1/2 hr), the sample is stored at 4°C in the 8-tube strips.

Section VII. Agarose gel electrophoresis

Preparation of a 2% agarose gel (mini gel)

1. Weigh out 1 g of agarose.
2. Add it to a conical flask containing 50 mL of Tris-acetate-EDTA (TAE) buffer.
3. To dissolve, heat the mixture in a microwave for about 1.5 min (solution starts boiling by this time). NOTE: take the flask out and swirl it every 30 seconds to avoid spillage and to aid proper dissolution.
4. Allow the flask it to cool to about 50°C to 60°C (i.e. cool to touch).
5. Add 3 µL of ethidium bromide stock solution (10 mg/mL) to the liquid gel. Gently swirl to mix. NOTE: WEAR GLOVES! Ethidium bromide is TOXIC MUTAGEN. Proper care is to be taken in disposal.
6. Pour the liquid gel into the gel forming cassette.
7. Place the combs at one end to form the wells, allow it to solidify (about 20 min).

Loading the wells

1. Load ladder to the first well: 1 µL of loading dye + 5 µL of DNA ladder (100bp ladder, Promega). The mixing can be done over a small piece of parafilm.
2. 10 µL of sample (PCR products) are loaded into the rest of the wells. There is no need to add the loading dye to it, since the PCR product by itself contains the dye (green master mix).
3. Fill the mangle apparatus with TAE Buffer.
4. Close the lid, making sure that the electric field is generated as desired (Run to Red).
5. Connect the electrodes to the battery and turn it on. Look for bubbles rising up on either side of the electrodes.
6. Run it at 115 volts for about 45 min (i.e. until the dye front reaches almost bottom of the gel).
7. Visualize the gel.

Section VIII. Real time RT-PCR

1. Thaw the reagents (Syber green wrapped in foil, forward and reverse primers of Tbx2, PPIA, and any other targets, and cDNA) on ice.
2. Turn the lights off before you begin the experiment.
3. Make Master Mix (depending on the number of target genes). If Tbx2 and PPIA, then prepare two Master mixes (Eg. If 6 samples of cDNA, then two master mixes of each Tbx2 and PPIA for 8 reactions, 2 extra reactions for pipetting error)
4. Amount per reaction:

2X Syber Green	10 μ L
Forward Primer	1 μ L
Reverse primer	1 μ L
NFree water	3 μ L
5. Add 15 μ L of master mix to each well that you will be using in a 96 PCR optical plate.
6. Add 5 μ L of cDNA (diluted 1:5, 20 μ L of cDNA + 80 μ L of nuclease free water) to the previously identified tubes. Make sure to pipette to the bottom of the tube.
7. Seal the plate with a PCR adhesive film tightly to prevent evaporation.
8. Centrifuge the plate at 1,000 rpm for 1 to 2 min.
9. Run it in Q-PCR Machine.

Section IX. Western Blot

SDS-PAGE

1. Take the casting stand and place the rubber bases (gray color) on them.
2. Place the 1.5 mm glass spacer plates with the green holder on to a casting stand. (large plate facing the casting stand and small plate facing you). (*Use black clips to secure both sides of the plates carefully*).
3. Prepare resolving gel (12%) and use a 10 mL pipette to pour the solution between the 1.5 mm spacer plates up to the mark (make sure you leave a 1 TO 2 cm gap). Pour 300 μ L of isobutanol to remove bubbles and to level the gel by dragging the pipette tip across (the isobutanol is more dense so it will float on top of the resolving gel solution).
4. Allow the gel to solidify for 1 hour. Carefully remove the glass plates with the resolving gel from the green holder. Wash the plates with deionized water without separating plates. Take syringe and remove the isobutanol and wash thoroughly with water. Return the plates to the casting stand.
5. Now place the glass plates with gel in to the electrode stand (Large plate facing you and the small plate facing the stand). Now use the blotting paper (3 mm chromatography paper) to remove the remaining water off the top of the resolving gel.
6. Prepare stacking gel (4%) solution and pour on top of the resolving gel. Fill with this solution up to the top. Carefully insert a 10 well comb (1.5mm) (smooth side facing the large plate) and allow it to solidify for 30 min. Make sure there are no air bubbles. (comb-BioRad should be facing you after you turn apparatus)
7. Turn on the hot water bath to 65°C.
8. Meanwhile, calculate the amount of sample required for loading the gel. The sample will be loaded at 20 μ L per well and the ladder will be loaded at 5 μ L of sample per well. Each sample contains 3.3 μ L of 6X loading dye, 20 μ g of protein sample. The total volume will be adjusted with water.
8. Vortex the sample tubes with the dye for 15 seconds. Place the samples in the hot water bath for 10 min.
9. Remove comb carefully from the gel. Add ladder (5 μ L) and samples (20 μ L each) using sequencing pipette tips.
10. Pour the running buffer into the apparatus. Close the unit and connect it to the power supply. Electrophoreses the samples at 100 volts for 150 min at 300 amps.

Transfer

1. Carefully, take the gel out of the electrode assembly.
2. Cut the top of the (stacking) gel using a blade.
3. For each gel, cut two 3 mm chromatography paper (filter paper) and 1 PVDF or nitrocellulose membrane (cut according to the size of the gel). Make a small cut for the membrane on the right hand top corner.
4. Wet the filter paper and the PVDF membrane in Milli-Q water for 5 seconds followed by soaking in transfer buffer (Tris/Glycine/Methanol) for 15 to 20 min.
5. Wet the scotch pads (white pad that comes with the apparatus) in water and then in transfer buffer for 5 min.
6. Make a sandwich:
 - Open transfer cartridge (plastic case with white and black sides)
 - Place the scotch pad, then filter paper, then gel on the black side of the cartridge
 - Carefully place membrane on the gel such that the right hand cut matches with right side of the gel and roll out air bubbles with a glass pipette.
 - Place the other filter paper and scotch pad on top.
 - Roll air bubbles again with glass pipette.
 - Close and seal cartridge.

NOTE: The gel and the membrane were stacked into a sandwich with scotch pads, presoaked filter paper membrane in such a way that the transfer of proteins occurs from the negative to the positive electrode.
7. Place cartridge in transfer apparatus or Mini Transblot Electrophoretic Cell (Bio-Rad Laboratories) (make sure that the black side of cartridge faces black side of the apparatus).
8. Fill the apparatus with transfer buffer until the gel or membrane is covered.
9. Add stir bar to the transfer apparatus and place the apparatus in a cold room on a stir plate. Turn on the stir plate and connect the apparatus to power.
10. Transfer overnight at 25 volts in 4°C or at 100 volts for 2 ½ hrs at room temperature. The membrane thus obtained was either used immediately or stored at 4°C.

Western Immunoblotting

1. After transfer, take out the membrane from the cassette.
2. Place the membrane with proteins in a container (usually lid of a pipette box) and blocking buffer (~ 10 mL) such that the membrane is fully immersed in the buffer. Incubate the membrane in blocking buffer for 1 hour at 4°C on a rocking platform.
3. Wash the membranes three times for 5 min each wash with TBS/T.

4. After washing, add primary antibody (Tbx2 1: 500, abcam) to the blocking buffer (~10 mL) with respective dilutions in a container. Incubate membrane in the primary antibody dilution overnight.
5. Wash membrane three times for 5 min each wash with TBS/T.
6. After washing, add secondary antibody (Anti Tbx2 1: 2000, abcam) to the blocking buffer (~10 mL) with respective dilutions in a container and incubate for 1 hour.
7. Again wash the membrane three times for 5 min each wash in TBS/T prior to developing.

Stripping the membrane:

Stripping the membrane enables the removal of the antibodies from the membrane without losing proteins and reprobing the same membrane with a different antibody. Each membrane can be stripped and reused 3 to 4 times. For example: stripping allows checking of the expression of the protein of interest as well as housing keeping gene on the same membrane.

1. Wash the membrane for 5 times for 5 min each wash in TBS/T.
2. Incubate the membrane in a plastic container with 10 mL of stripping buffer at 50°C to 55°C.
3. Wash again in TBS/T twice with each wash lasting 5 min.
4. Block the membrane with 5% non fat milk.
5. Follow the same protocol for the regular western blotting from this step onwards.

Buffers used for western blot

10X TRIS/GLYCINE STOCK SOLUTION

For 1000 mL of 10X Tris/Glycine:

30 g Tris base

144 g Glycine

1000 mL Milli-Q H₂O

Need not autoclave/sterilize

RUNNING BUFFER

For 1000 mL 1X solution:

100 mL 10X Tris/Glycine stock solution

900 mL Milli-Q H₂O

Add 1g SDS

Need not autoclave/sterilize

TRANSFER BUFFER

For 1000 mL 1X solution:

80 mL of 10X Tris/Glycine stock solution

720 mL of Milli-Q H₂O

200 mL MeOH

Need not autoclave/sterilize

TRIS BUFFERED SALINE (TBS)

For 1000 mL 10X stock solution:

24 g Tris base

80 g NaCl

1000 mL Milli-Q H₂O

Need not autoclave/sterilize

TBS/T

For 1000 mL 1X solution:

100 mL 10X TBS

1 mL Tween-20

900 mL Milli-Q H₂O

Need not autoclave/sterilize

100mM Ammonium Per Sulfate (APS)

54.9 mg APS

549 µL of Milli-Q H₂O

Dissolve and aliquot 100 µL into each MCF tube

Store at -20°C

Do not use more than once after thawing.

1.5 M TrisHCl pH 8.8

For 500 mL 1.5M solution:

Tris base 90.76 gm

Add 450 mL Milli-Q water

Adjust to pH 8.8 with HCl.

Adjust volume to 500 mL with Milli-Q water

Store at 4 °C

1 M TrisHCl pH 6.8

For 500 mL 1 M solution:

Tris base 60.57 gm

Add 450 mL Milli-Q water

Adjust to pH 6.8 with HCl.

Adjust volume to 500 mL with Milli-Q water

Store at 4 °C

0.5 M TrisHCl pH 6.8

For 500 mL 0.5M solution:

Tris base 30.5 gm

Add 450 mL Milli-Q water

Adjust to pH 6.8 with HCl.

Adjust volume to 500 mL with Milli-Q water

Store at 4 °C.

Sodium Dodecyl Sulfate (SDS)

For 100 mL 10% SDS stock solution:

10 g electrophoresis grade SDS

Add the powder carefully to 90 mL Milli-Q H₂O

Place the container in a 65 °C water bath (with frequent stirring) or a hot plate stirrer to dissolve the SDS.

When SDS has dissolved, bring volume to 100 mL Milli-Q H₂O.

Do not autoclave/sterilize. Store at room temperature (At room temperature SDS solidifies. Before use, reheat to dissolve).

30% Acrylamide/ 0.8 % Bis-acrylamide For 100 mL solution:

Acrylamide 30 gm

N'N'-bis-methylene-acrylamide 0.8 gm

Adjust volume to 100 mL with Milli-Q water

Sterilize the solution by filtration through a filter (0.45 micron pore size)

Store in amber bottle at 4 °C in the dark (30 days max)

Blocking Buffer:

To a 50 mL tube add

5 mL 10XTBS

45 mL Milli-Q water

2.5 gm skim milk

50 µL Tween

Stripping Solution:

For 100 mL of stripping solution:

12.5 mL of 0.5M Tris-HCl

20 mL of 10% SDS

700 µL of b-mercaptoethanol

Adjust the volume to 100 mL with dH₂O

Reagents	Resolving Gel		Stacking Gel	
	20 mL	10 mL	20 mL	10 mL
1.5 M TrisHCl pH 8.8	5 mL	2.5 mL	-----	-----
0.5 M TrisHCl pH 6.8	-----	-----	5 mL	2.5 mL
Distilled water	6.7 mL	3.35 mL	10 mL	5 mL
10% SDS	200 µL	100 µL	100 µL	50 µL
30% Acrylamide/Bis-acrylamide	8 mL	4 mL	2.4 mL	1.7 mL
100mM APS (Ammonium Persulfate)	100 µL	50 µL	100 µL	50 µL
(freshly prepared)				
TEMED	10 µL	5 µL	10 µL	5 µL

Coomassie Brilliant Blue Staining on PVDF/Nitrocellulose Membrane:

For 1L staining solution add the following:

0.5% Coomassie brilliant blue (0.5 g)

40% methanol (400 mL)

10% acetic acid (100 mL)

Q/S with milli-Q H₂O to 1000 mL

Destaining Solution

For 1L destaining solution add the following

30% methanol (300 mL)

10% acetic acid (100 mL)

Q/S with milli-Q H₂O to 1000 mL

Gel Staining/Destaining Protocol

Stain gel/membrane in CBB solution for *30 min* on shaking platform

Destain until background is transparent and bands are visible

Wash gel 3 to 5 times with H₂O

Section XI. alamarBlue assay

Day 0 (Plating cells in 10% calf serum)

1. The cells are passed once (as per the Cell passage protocol) before plating.
2. Cells are collected from the plates, centrifuged at 1,000 rpm for 2 to 3 min, resuspended in 10 mL of media and finally counted using a haemocytometer.
3. A 96 well plate is plated at a concentration of 2,000 cells/well in 50 μ L/well of media.

Day 1 (Cells in serum free environment)

1. Stock Media (α -Minimal Essential Medium, MEM + 0.1% Bovine serum albumin, BSA + 1% Pencillinstreptomycin) is thawed in water bath at 37°C.
2. Dump off the media in 96-well plate on to a paper towel placed in fume hood.
3. Load 50 μ L of media to all the wells, mix gently, and dump it off.
4. Load 50 μ L of media to all the wells and mixe.
5. Place the plate in a 5% CO₂ incubator at 37°C.

Day 2 (Treatment of cells)

1. Layout a design for the control and different treatment wells (stock media with 0.1% BSA-control, 0.1% CS, 0.5% CS, 1.0% CS, 5.0% CS, 10% CS etc. – treatment wells).
2. Prepare treatment groups with different concentrations of calf serum and stock media in 15 mL tubes. (Be sure to make double the volume required for each treatment group).
3. Dump off the media in 96 well plate on to a paper towel placed in fume hood.
4. Load 50 μ L of control and treatment media to the designated wells using a multipette.
5. Mix gently and kept in CO₂ incubator.

Day 4 (alamarBlue assay)

1. Set Synergy 2 to read plate to allow for warm up.
2. Dump off the media (control and treatment) in 96 well plates.
3. Rinse all the wells with 100 μ L of PBS.
4. Dump off the PBS, and re-wash with another 100 μ L of PBS.
5. Add alamarBlue indicator to plain α -MEM medium in 1:10 dilution (9 mL of α -MEM + 1mL of alamarBlue for 96 wells) NOTE : Take care not to expose alamarBlue to sunlight. Add alamarBlue indicator just before wells are loaded, keeping it in fumehood for a long time greatly reduces activity.
6. Dump off the PBS from the 96 well plates.
7. Load 100 μ L of solution (α - MEM + alamarBlue) to all wells and mix gently.
8. Wrap the plate with aluminum foil and placed in CO₂ incubator.
9. After 4 hours, fluorescence is detected by Biotek plate reader 540/35 x 600/40 nm, Sensitivity 54.

Section XII. BrdU assay

Day 0 (Plating cells in 10% calf serum)

1. The cells are passed once (as per the Cell passage protocol) before plating
2. Cells are collected from the plates, centrifuged at 1,000 rpm for 2 to 3 min, resuspended in 10 mL of media and finally counted using a haemocytometer.
3. A 96 well plate is plated at a concentration of 2,000 cells/well, 50 μ L media/well.

Day 1 (Cells in serum free environment)

1. Stock Media (α -Minimal Essential Medium, MEM + 0.1% Bovine serum albumin, BSA + 1% Pencillin-streptomycin) is thawed in water bath at 37°C.
2. Dump off the media from the 96-well plate on to a paper towel placed in fume hood.
3. Load 50 μ L of media to all the wells, mix gently and dump it off.
4. Load 50 μ L of media to all the wells and mixed.
5. Place the plate in 5% CO₂ incubator at 37°C.

Day 2 (Treatment of cells)

1. Layout a design for the control and different treatment wells (stock media with 0.1% BSA-control, 0.1% CS, 0.5% CS, 1.0% CS, 5.0% CS, 10% CS etc. – treatment wells).
2. Prepare treatment groups with different concentrations of calf serum and stock media in 15 mL tubes. (Be sure to make double the volume required for each treatment group).
3. Dump off the media in 96well plate on to a paper towel placed in fume hood.
4. Load 50 μ L of control and treatment media to the designated wells using a multipette.
5. Mix gently and return to the CO₂ incubator.

Day 4 (alamarBlue assay)

1. Add 10 μ L/well BrdU labeling solutions to the plate and incubate for 2 hrs at 37°C.
2. The labeling solution is removed.
3. Add 200 μ L/well FixDenat to the cells and incubate for 30 min at room temperature.
4. Remove the FixDenat solution.
5. Add 100 μ L/well Anti-BrdU-POD working solution and incubate for 90 min at room temperature.
6. Excess antibodies are removed by dumping off the solution and rinse the wells three times with 200 μ L/well of washing solution.
7. The washing solution is dumped off.
8. The bottom of the plate is sealed with a black adhesive film.
9. Add 100 μ L/well of the substrate solution to each well.
10. The plate is incubated for 5 min at room temperature.
11. The luminescence is read at BioTek plate reader with auto sensitivity

Section XIII. Alkaline phosphatase (ALP) assay

Day 1: Plate cells

1. Plate cells at 5,000 cells/well in 96 well plate in α -MEM + 10% CS + 1% PS at 100 μ L/well.
2. Incubate for 24 hours at 37°C.

Day 2: Change to serum free media

1. Rinse with 100 μ L of α -MEM + 0.1% BSA + 1% PS two times.
2. Make a stock media with 10 mL of α -MEM + 0.1% BSA + 1% PS, 100 μ L Vit C (AA2P), and 100 μ L β -glycerophosphate.
3. Add 100 μ L/well of the above solution and incubate for 24 hours.

Day 3: Add Appropriate Growth Factors

1. Make a stock media of 5 mL of α -MEM + 0.1% BSA + 1% PS, 50 μ L Vit C (AA2P), and 50 μ L β -gal (or make 10 mL as needed).
2. Used the above media and to make your treatments.
3. Remove old media from wells without rinsing.
4. Add 100 μ L/well for control and treatments and incubate for 72 hours.

Day 6: Permealize the cells and perform ALP assay

1. The procedures for this day can be performed under non-sterile conditions.
2. Dump off the media from the cells.
3. Rinse two times with warm PBS using 100 μ L/well.
4. Add 100 μ L of 0.1% triton to each well.
5. Shake at medium speed at room temperature for 15 to 20 min.
6. Wrap in a parafilm.
7. Freeze at -80°C for at least 15 min (optional – after shaking the cells can be stored at -80°C until future use).
8. Obtain two 96 well plates.
9. Thaw the ALP substrate by placing the tube in the water bath and also thaw out the 96-well plate at room temperature.
10. Scrape the cells using the tips on a multipipettor and pipette mix the cell lysates. Make sure that no bubbles are contained in the pipette tip when the lysate is transferred to the new 96-well plate: Set multipipettor to 40 μ L, hold the button on the pipettor down then begin scrapping the wells; once well is sufficiently scrapped release the button and remove the lysate from the well.
11. Transfer 40 μ L of the cell lysate (for ALP) into one 96-well plate.
12. For protein analysis add 80 μ L Distilled H₂O then add 20 μ L of the cell lysate (for protein concentration) into one 96-well plate.

ALP activity

1. Prepare two Blank wells with 40 μL of 0.1% Triton/well.
2. Add 200 μL of the ALP Substrate to all of the wells (including blank).
3. Immediately read (0 hour) absorbance at 405 nm, and then incubate at 37°C and read after 5 hours of incubation.

Protein estimation

BLANK- in the next available column, make two blank wells. First well: 20 μL of 0.1% Triton and 80 μL of Distilled H_2O . Second well: 100 μL of Distilled H_2O

SAMPLE- 20 μL lysate and 80 μL Distilled H_2O . (H_2O added first)

DYE- 2 mL Bio-Rad Protein Assay Reagent and 8 mL H_2O (1:5 dilution)

STANDARDS-

Stock: 100 μg BSA/ 1 mL dH_2O = for example: 30 mg BSA/300mL dH_2O

1. Of the 300mL stock take 150 μL and add 150 μL dH_2O
{final concentration is 50 $\mu\text{g}/\text{mL}$ }
 2. Of the resulting solution take 150 μL and add 150 μL dH_2O
{final concentration is 25 $\mu\text{g}/\text{mL}$ }
 3. Of the resulting solution take 150 μL and add 150 μL dH_2O
{final concentration is 12.5 $\mu\text{g}/\text{mL}$ }
 4. Of the resulting solution take 150 μL and add 150 μL dH_2O
{final concentration is 6.25 $\mu\text{g}/\text{mL}$ }
 5. Of the resulting solution take 150 μL and add 150 μL dH_2O
{final concentration is 3.125 $\mu\text{g}/\text{mL}$ }
-
1. Into the next available column add 100 μL of each standard/well (7 wells total; include 100 $\mu\text{g}/\text{mL}$ (from stock) and 0 $\mu\text{g}/\text{mL}$ (distilled H_2O))
 2. Add 200 μL of the dye to all wells on the plate.
 3. Incubate the plate for 5 min and read the plate absorbance at 595 nm.

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