A LYMPHOID ENHANCER BINDING FACTOR (LEF) 1 ISOFORM REGULATES OSTEOBLAST MATURATION

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ABSTRACT

The canonical Wnt signaling pathway has emerged as an important regulator of bone formation, regeneration and repair. Studies over the past decade have demonstrated that activation of canonical Wnt signaling generally promotes osteoblast proliferation and enhances bone mass, while suppression of Wnt signaling results in bone loss. Gaining a better understanding of Wnt signaling in the context of skeletal metabolism is important because current skeletal regeneration and repair treatments have limitations, anti-resorptive therapies have unknown long-term health consequences and the demand for therapies is rising as our population ages. Osteoporosis is a growing healthcare challenge that affects about 44 million Americans. Cancer survival rates drop drastically in patients suffering from bone metastases. A complete, molecular understanding of the canonical Wnt signaling cascade in the context of skeletal biology will promote the development of new therapies for the treatment of osteoporosis, skeletal cancer metastasis, and other skeletal diseases that result in uncoupling of bone formation and resorption.

Lymphoid Enhancer Binding Factor (Lef) 1 is a transcription factor in the canonical Wnt/Lrp5/6/β-catenin signaling cascade, which regulates osteoblast differentiation, bone density and skeletal strength. In this thesis, I describe the expression and function of an alternative Lef1 isoform in osseous cells. Lef1ΔN is a naturally occurring isoform driven by a promoter (p2) within the intron between exons 3 and 4 of *Lef1*. Lef1ΔN is induced during late osteoblast

differentiation. This is opposite to the expression pattern of the full-length Lef1 protein, which as we previously showed, decreases during differentiation. We showed that the Lef1ΔN p2 promoter is active in osteoblasts and Runx2 positively regulates its activity. BMP2 also promotes Lef1ΔN expression, whereas Wnt3a represses it. Lef1ΔN overexpression in differentiating osteoblasts induced osteocalcin and type 1 collagen expression, which suggests Lef1ΔN is a crucial regulator of terminal differentiation in osseous cells. We found Lef1ΔN interacts with β-catenin to activate a Lef1-responsive promoter and stimulate the transcription of genes involved in late osteoblast differentiation. We mapped the region of Lef1ΔN that associates with β-catenin to an element within the first 61 amino acids of Lef1ΔN and showed this region was required to induce type 1 collagen and osteocalcin expression during osteoblast maturation. Taken together, Lef1ΔN interacts with β-catenin to regulate terminal differentiation in osseous cells.

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Introduction and Literature Review

Section 1: Bone

Function. The skeleton is an essential organ that performs a variety of important functions. The adult human skeleton consists of 206 bones. The two most abundant types of bone are flat bones, such as the skull, mandible, and scapula, and long bones, including the femur, tibia, and radius. Long bones assist in movement when muscle contractions cause them to move. Flat bones protect vital organs such as the brain, heart, and lungs. The skeleton plays an integral role in regulating the body's serum calcium homeostasis and maintaining an appropriate acid-base equilibrium. Bone produces growth factors and cytokines, which are utilized within the bone microenvironment and in other tissues. For example, factors secreted by bone forming osteoblasts are essential for survival, renewal, and maturation of hematopoietic stem cells (Taichman, 2005).

Development. Bone development occurs through two distinct mechanisms, intramembranous and endochondral bone formation. At sites of bone formation, mesenchymal cells condense and produce areas of high osteoprogenitor cell density which provides the foundation for future skeletal formation. During intramembranous bone formation, mesenchymal cells differentiate directly into osteoblasts. The osteoblasts produce a disorganized collagen matrix called woven bone, which eventually becomes remodeled and replaced by more organized lamellar bone. Flat bones such as the skull, scapula, and mandible are formed through intramembranous bone formation. During endochondral bone formation, mesenchymal cells differentiate into chondrocytes and form a cartilage model for future bone development, termed a cartilage anlage. Chondrocytes at the center of the anlage stop proliferating as endochrondral bone formation progresses and eventually reach a stage of terminal differentiation (hypertrophy). Mesenchymal cells surrounding the hypertrophic region of the cartilage anlage differentiate into osteoblasts to form a bone collar. Unlike proliferating chondrocytes, hypertrophic chondrocytes express high levels of connective tissue growth factor (Ctgf) and vascular endothelial growth factor (VEGF). These factors promote the invasion of blood vessels, osteoblast progenitor cells, and cartilage/bone-resorbing cells forming the primary ossification center where hypertrophic cartilage is eroded and replaced with bone marrow and trabecular bone, thus completing the process of endochondral bone formation. Endochrondral bone formation produces long bones including the femur, tibia and radius.

Structure. Long bones are comprised of a hollow shaft, called the diaphysis, which widens at the ends to form the metaphyses (regions below the growth plate) and the epiphyses (regions above the growth plate). The diaphysis consists mainly of cortical bone, which is compact and makes up the outer cortex of most bones. The metaphyses and epiphyses are composed primarily of trabecular (cancellous) bone surrounded by an outer sheath of cortical bone. About 80% of the human adult skeleton is comprised of cortical bone, while the remaining 20% is cancellous bone. The fibrous outer coating of bones is referred to as the periosteum and contains blood vessels, osteoblasts, and fibroblasts.

The lining of the endocortical surface is called the endosteum and houses blood vessels, nerve endings, osteoblasts, and osteoclasts (Figure 1).

Bone Maintenance. Four types of cells play significant roles in bone maintenance: osteoblasts, osteoclasts, osteocytes, and bone lining cells. The cooperative action of osteoblasts and osteoclasts is described as the basic multicellular unit (BMU). Under normal physiological conditions, BMU cellular activity is coupled, meaning the relative amount of bone resorbed by osteoclasts is equal to the amount of osteoblast-mediated bone formation (Khosla et al., 2008). However, this balance can be disrupted by changes in mechanical stress, hormone levels, and aging. Osteoblasts and osteoclasts constantly direct bone remodeling in response to paracrine and endocrine factors, variations in mechanical loading, and changes in serum calcium levels. Osteoblasts are derived from mesenchymal cells, which are also capable of becoming chondroblasts, smooth muscle cells, or adipoctyes. Osteoclasts are of hematopoietic origin, or more specifically, originate from the myelomonocytic lineage and share a common precursor with macrophages. Osteocytes, which are terminally differentiated osteoblasts, reside in bone encapsulated structures referred to as lacunae, and function primarily to regulate bone homeostasis in response to mechanical loading (Bonewald, 2006). Osteocytes remain in direct contact with osteoblasts and other osteocytes and communicate their responses to physiological stimuli to help mediate the bone remodeling process.

Bone Remodeling. Bone remodeling is a stepwise process that involves initiation of osteoclast formation, bone resorption by osteoclasts, a “reversal period", osteoblast-mediated bone matrix formation to fill in the resorbed pit formed by osteoclasts, and finally bone matrix mineralization (Sims and Gooi, 2008). Osteoclast formation is triggered by a variety of cytokines released by cells within the BMU and neighboring immune cells residing in the bone marrow. Signaling initiated by these factors results in the expression of receptor activator for nuclear κ B ligand (RANKL) on the surface of premature and developing osteoblasts, and the interaction between RANKL and its receptor (RANK) on mononuclear hemopoietic osteoclast precursor cells is essential for the formation of a functional, multinucleated osteoclast . Mature osteoblasts express a RANKL decoy receptor called osteoprotegerin (OPG), which inhibits osteoclast formation. RANKL and OPG are expressed during different stages of osteoclast and osteoblast differentiation, and modulation of these receptors allows osteoblasts to tightly regulate osteoclast formation. For example, RANKL expression is stimulated by microdamage that occurs within the bone matrix as a result of pathological conditions and normal skeletal loading (Parfitt et al., 2000). Osteoblasts direct osteoclast differentiation by releasing chemoattractants, such as osteocalcin, α_2 HS-glycoprotein, and collagen-I fragments, that cause osteoclast precursors to move together and toward the bone surface (Malone et al., 1982). Bone lining cells, which are defined as terminally differentiated osteoblasts, mediate the fusion and attachment of osteoclast precursors to the bone surface. A thin layer of nonmineralized matrix called the osteoid surrounds the mineralized matrix. Cytokines generated during osteoclast formation cause bone lining cells to produce collagenase and digest the osteoid, which allows the osteoclasts to resorb the mineralized matrix (Chambers and Fuller, 1985). Integrins on osteoclasts associate with matrix proteins such as osteopontin and bone sialoprotein to mediate osteoclast attachment to the bone matrix (Ross and Teitelbaum, 2005). As the bone matrix is resorbed through the action of acid and lysosomal enzymes produced by osteoclasts, factors embedded in the matrix, including IGFs, BMPs, TGFβ, PDGF, and FGF, are released and stimulate osteoblast differentiation. Once osteoclasts complete resorption and leave the bone surface, the "reversal phase" takes place. Mononuclear cells of unknown origin secrete MMPs and serine proteases to clean the resorbed pit thus creating a "reversal line" (Tran Van et al., 1982).

EphrinB4, which is produced by both osteoclasts and osteoblasts, interacts with ephrinB2 to stimulate bone formation. EphrinB2 is expressed by osteoblast lineage cells and its expression is enhanced by PTHrP, which is produced by preosteoblasts and cells within the bone marrow (Suda et al., 1996). PTHrP and circulating PTH are bound by PTH1R on preosteoblasts and mature osteoblasts to promote osteoblast differentiation (Dobnig and Turner, 1995) and prevent osteoblast apoptosis (Jilka et al., 1999). Osteoblasts produce osteoid to fill in the resorbed pit by working together and communicating with each other through gap junctions and the expression of numerous membrane-bound and secreted factors. As the secretion of osteoid by osteoblasts slows and bone remodeling concludes, osteoblasts differentiate into lining cells or osteocytes. After bone formation is finished and the bone surface is covered by lining cells, osteocyte signaling stimulates continued matrix mineralization.

Osteoporosis. Bone undergoes continuous remodeling during a human's lifespan. From childhood throughout early adulthood, bone formation surpasses bone resorption, resulting in a net increase in bone which peaks around the age of 30 years. In women, from the time of peak bone mass to the onset of menopause, bone formation and bone resorption are approximately equal and overall changes in bone mass are negligible (Bonura, 2009). Postmenopausal women experience a decline in the production of estrogen, which has been associated with increased osteoblast apoptosis, accelerated bone resorption and susceptibility to osteoporotic fractures (Bradford et al., 2010; Manolagas et al., 2002). By the age of 80, most women have lost about 30% of their peak bone mass (Looker et al., 1998). This age-associated decrease in bone mass puts postmenopausal women at a high risk for osteoporosis. While not as common or sudden as menopause, an age-related loss of androgens in men or decreased androgen levels from chemical or surgical castration has the same negative effect on the skeleton (Manolagas et al., 2002). Osteoporosis is characterized by low bone mass and microarchitectural deterioration of bone tissue accompanied by decreased bone strength and increased risk for fracture (2003).

Approximately 44 million Americans, representing 55% of men and women over the age of 50, have osteoporosis or low bone density that can lead to fractures (Lewiecki, 2009). It is estimated that 1 in 2 women and 1 in 4 men over the age of 50 years old will have an osteoporosis-related fracture in their lifetime (Bonura, 2009). In 2005, over 2 million fractures associated with osteoporosis were reported in the US and cost over $17 billion in health care expenses (Burge et al., 2007). Despite the frequency of osteoporosis cases, the rates of screening and treatment remain low. A 2005 report indicates that nearly 9 out of 10 women are neither screened nor treated following a fragility-related fracture (Foley et al., 2007). Bone mineral density testing enables physicians to screen patients and diagnose osteoporosis before a fracture occurs. Osteoporosis can also be clinically diagnosed in a patient who has suffered a fragility fracture, independently of bone mineral density.

Postmenopausal osteoporosis is primarily treated by drugs which inhibit bone resporption. A variety of antiresorptive drugs with different mechanisms of action are approved by the US Food and Drug Administration (FDA) for the treatment/prevention of osteoporosis including bisphosphonates, estrogen therapy, raloxifene (a selective estrogen response modulator or SERM), and calcitonin. Bisphosphonates are attracted to bone mineral and inhibit osteoclast function by blocking the action of farnesyl diphosphate synthase, an enzyme important for osteoclast activity (Russell et al., 2008). Estrogen and raloxifene prevent osteoclast formation by inhibiting RANKL and promoting OPG expression in osteoblasts (Eghbali-Fatourechi et al., 2003; Hofbauer et al., 1999), while calcitonin directly blocks osteoclast activity (Bonura, 2009). The only anabolic agent currently approved by the FDA for treatment of osteoporosis is a recombinant form of parathyroid hormone called Teriparatide, which stimulates osteoblastic bone formation leading to increased trabecular bone density and connectivity (Hodsman et al., 2005).

Like osteoporosis, skeletal tumor metastasis disrupts the balance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption. The skeleton is the preferred site of metastasis for breast and prostate cancer cells (Kingsley et al., 2007). While the 5 year survival rates for breast and prostate cancers detected at the earliest stages are near 100%, the survival rate drops to 26% for those with breast cancer metastases and 33% for individuals with prostate cancer metastases (Jemal et al., 2007). Within the skeleton, metastatic bone lesions are either osteoblastic or osteolytic, the majority of breast cancer bone lesions being osteolytic in nature (Kozlow and Guise, 2005). Conversely, an excess of osteoblast activity relative to resorption by osteoclasts leads to osteoblastic lesions in most skeletal prostate cancer metastases (Saad et al., 2006).

Skeletal Tumor Metastases. Tumor metastasis to bone occurs during the late stages of cancer progression. First, tumor cells detach from the primary tumor and enter the bloodstream. Cancer cells preferentially hone to the bone microenvironment because it is rich in factors that promote tumor cells growth, such as TGF-β and insulin-like growth factors. Once tumor cells infiltrate the bone microenvironment, they adhere to the endosteal surface and begin colonizing bone. A “vicious cycle” occurs in which tumor cells release factors that stimulate osteolysis and cause the release of growth factors embedded within the bone matrix that promote cancer cell proliferation, a more aggressive tumor phenotype, the migration of tumor cells and further bone destruction (Kingsley et al., 2007). Tumor cells thrive in the bone microenviroment because they use many of the same signaling cascades and transcription factors as bone cells, a scenario termed “osteomimicry” (Koeneman et al., 1999). For instance, during prostate cancer metastasis tumor cells secrete Wnt ligands that stimulate osteoblasts, while simultaneously promoting cancer cell proliferation in an autocrine manner (Hall et al., 2006).

Current Treatments. The clinical need to develop new bone anabolic agents is high because current bone regeneration options have limitations, antiresorptive therapies have unknown long-term health consequences, and the demand for therapies is rising as the population ages. The current standard of care is an anti-resorptive treatment such as bisphosphates, hormone replacement, and selective estrogen receptor modulators. Although these treatments are effective, controversy exists about their long-term effects on general skeletal health, particularly with regards to repair of microfractures, and on breast cancer risk (Drake et al., 2008; Rossouw et al., 2002). Teriparatide (Forteo™, parathyroid hormone (PTH) residues 1-34) is the only anabolic drug available at this time. The regulations on Teriparatide use vary by country (e.g. two year maximum in the USA) and it is usually only prescribed to patients with established osteoporosis, those who already suffered a fracture, or those in which bisphosphanates are ineffective or contraindicated. It is administered daily via subcutaneous injection and stimulates new bone formation, but has several side effects, including hypercalcemia and hypercalciuria, and has been shown to cause osteosarcomas in rats when given at high doses (Vahle et al., 2002). Understanding basic skeletal biology is important because it will promote the development of new therapies for the treatment of osteoporosis, skeletal cancer metastasis, and other skeletal diseases that result in uncoupling of bone formation and resorption.

Section 2: Runx2 in Bone Formation

Runx2 is part of the Runt domain family of transcription factors. Like the two other members of this family (Runx1 and Runx3), Runx2 contains a DNA binding and protein-protein interaction motif, termed the Runt homology domain due to its high homology to the *Drosophila* transcription factor Runt. Runx proteins bind DNA with the amino acid sequence, 5'-PuACCPuCA-'3 (or its complement, 5'-TGPyGGTPy-3'), to regulate the transcription of target genes (Meyers et al., 1993).

Runx2 is essential for osteoblast development from mesenchymal stem cells and maturation into osteocytes. The first evidence indicating that Runx2 is the master regulator of bone formation came in 1995 when a cell-specific, promoter-based search for osteoblast-specific transcription factors showed that Runx2 binds to a *cis*-acting element in the promoter of osteocalcin, a gene expressed exclusively in fully differentiated osteoblasts that regulates energy metabolism (Ducy and Karsenty, 1995; Ducy et al., 1997; Geoffroy et al., 1995; Lee et al., 2007). Subsequently, it was shown that inactivation of both *Runx2* alleles in mice results in arrested osteoblast differentiation and complete lack of mineralized bone throughout the entire skeleton (Komori et al., 1997; Otto et al., 1997). Heterozygous *Runx2* mice have hypoplastic clavicles and delayed closure of the fontanelles, similar to the skeletal abnormalities observed in the autosomal-dominant human skeletal disease cleidocranial dysplasia (CCD) (Mundlos et al., 1997; Otto et al., 1997). Not surprisingly, *Runx2* mapped within the CCD locus and most forms of human CCD were found to be caused by inactivating Runx2 mutations (Lee et al., 1997; Mundlos et al., 1997; Zhou et al., 1999). Conditional overexpression of *Runx2* in murine osteobasts using a collagen type 1 promoter blocked terminal differentiation and increased bone resorption (Geoffroy et al., 2002; Liu et al., 2001) indicating that Runx2 levels must be tightly regulated throughout osteoblast differentiation and maturation. This strong molecular and genetic evidence provides the foundation for the widely accepted view that Runx2 controls osteoblast differentiation.

Runx2 interacts with a variety of transcription factors and associates with the nuclear matrix to modulate numerous signals and regulate the expression of several genes crucial for osteoblast development and maturation including osteocalcin, bone sialoprotein, osteopontin, and collagen type 1 (Ducy, 2000). Although Runx2 and Osterix, a transcription factor that acts downstream of Runx2, are the only factors known to be essential for osteoblast differentiation in mice, evidence exists that crosstalk between Runx2 and several other signaling pathways play a role in the process.

The canonical Wnt/LRP5/β-catenin pathway promotes osteoblast proliferation and is important in the maintenance of bone mass (Day et al., 2005; Hill et al., 2005; Kato et al., 2002; Mbalaviele et al., 2005). This pathway will be discussed in detail later. Briefly, during canonical Wnt signaling, Wnt ligand binding to the Frizzled (Fzd) receptor and lipoprotein receptor-related protein (LRP) 5 or 6 coreceptors prevents glycogen synthase kinase (GSK) 3 from targeting β-catenin for degradation and enables β-catenin to translocate to the nucleus where it binds to Lef1/Tcf transcription factors to regulate genes involved in osteoblast proliferation (Hoeppner et al., 2009b). Lymphoid enhancer-binding factor (Lef) 1 is a transcriptional nuclear effector of the Wnt/LRP5/β-catenin signaling pathway, which interacts with Runx2 protein through an association between their DNA binding domains (Kahler and Westendorf, 2003). Lef1 represses Runx2-mediated activation of the osteocalcin promoter and this repression is enhanced in the presence of constitutively active β-catenin (Kahler and Westendorf, 2003), indicating that Lef1 and the canonical Wnt signaling pathway modulate Runx2 activity. Furthermore, Runx2 activity has been linked to secreted frizzled-related protein (Sfrp) 1, a natural antagonist of Wnt signaling. *Sfrp1*-deficient mice display overactive Wnt signaling and a high bone mass phenotype with 2-5 fold excess Runx2 promoter activity, 7-8 fold induction of Runx2 mRNA and a substantial increase in osteocalcin expression (Gaur et al., 2005). A functional Tcf regulatory element responsive to canonical Wnt signaling was identified in the promoter of the Runx2 gene providing additional support that Runx2 is a target of Wnt signaling during osteoblast maturation (Gaur et al., 2005).

Bone morphogenetic protein (BMP) 2 is an osteogenic agonist that contributes to regulation of Runx2 levels while promoting osteoblast proliferation and gene expression. BMP2 binds to type I and II BMP receptors to phosphorylate Smad 1, 5, and 8 proteins, which associate with Smad 4 and translocate to the nucleus. The activated Smad complex binds to target genes, including Runx2, to regulate their expression during osteoblast differentiation (Li and Xiao, 2007). In the presence of BMP2, the pluripotent mesenchymal precursor cell line C2C12 is induced to differentiate into osteoblast lineage cells and terminal differentiation into myoblasts is inhibited (Katagiri et al., 1994). This osteogenic effect of BMP2 can be completely abolished when mutated Smad 1or Smad 5 are overexpressed in C2C12 cells (Nishimura et al., 1998; Yamamoto et al., 1997). Runx2 has been shown to interact with Smad 1 and 4 (Alliston et al., 2001; Zhang et al., 2000), which provides further evidence that the BMP2-Smad 1/5/8-Smad 4-Runx2 pathway plays an important role in osteoblast differentiation. Correspondingly, the pathogenesis of CCD may be related to impaired BMP/Smad signaling that targets Runx2 during bone formation. As discussed, CCD is caused by primarily by heterozygous mutations in Runx2. Zhang and colleagues studied a nonsense mutant of Runx2 identified in a CCD patient that results in a truncated Runx2 protein (Zhang et al., 2000). The authors demonstrate that the truncated Runx2 protein failed to interact with Smads and was not able to induce osteogenic differentiation in C2C12 cells upon stimulation with BMP2 (Zhang et al., 2000). Recently, the Smad interacting domain (SMID) was mapped to the C-terminal region of Runx2 (391-432) within the nuclear matrix targeting signal (NMTS), and it was shown that the SMID/NMTS domain is essential for BMP2-mediated osteoblast differentiation (Afzal et al., 2005).

In vitro and *in vivo* studies have demonstrated that Runx2 is an essential transcription factor for osteoblast differentiation and bone formation. Interestingly, two signaling pathways essential to skeletal homeostasis, the Wnt/LRP5/6/β-catenin and BMP/Smad cascades, converge to regulate Runx2 gene expression and activity. Such signaling cross-talk clearly illustrates the importance of Runx2 as the key regulator of bone development.

Section 3: Wnt/LRP5/6/β-catenin Signaling in Bone Formation

Wnts are cysteine-rich, secreted glycoprotein ligands that activate cell surface receptor-mediated signaling pathways involved in gene expression, cell fate determination, proliferation, and migration. Wnts are required for embryogenesis, organogenesis, postnatal development and regeneration of adult tissues including lymphocytes, skin, colon, hair follicles and bone (Westendorf et al., 2004). In humans and mice, there are 19 Wnts that bind to receptor complexes containing one of 10 Frizzled (Fzd) receptors and sometimes one of two low-density lipoprotein receptor-related protein (LRP) 5 or 6 co-receptors, theoretically creating at least 380 potential receptor combinations. Complicating the issue further, Wnts also bind to Ryk and Ror2 receptors. Wnt ligands can thus stimulate multiple signaling cascades, including the "canonical" β-catenin pathway, the planar cell polarity (PCP) pathway, and the calcium (Ca^{2+}), protein kinase A, Src, and JNK pathways (van Amerongen et al., 2008) (Figure 2). Wnts have historically been classified as "canonical" (e.g. Wnt 1, 3a, 8, 10b) on the basis of their ability to inhibit GSK3 phosphorylation of β-catenin and its subsequent degradation or as “non-canonical” (e.g. Wnt 4, 5a, 11) if they do not affect β-catenin levels. These simplistic classifications are now being challenged by studies demonstrating that some Wnts (including Wnt1, 5a and 11) stimulate several of the above-mentioned signaling pathways in context-dependent manners that can depend on which receptor is available and active (Mikels and Nusse, 2006).

In the absence of canonical Wnt signaling, β-catenin is phosphorylated by GSK3β, which leads to ubiquitination of β-catenin by E2-ligase and subsequent degradation by the proteosome. During active canonical Wnt signaling, Wnt ligand binding to the Fzd receptor and LRP5 or LRP6 coreceptor leads to activation of Disheveled (Dsh), which inhibits the cytoplasmic destruction complex composed of GSK3β, Axin, and APC, thus preventing GSK3β from targeting β-catenin for protesomic degradation. Cytoplasmic β-catenin levels increase and β-catenin translocates to the nucleus where it displaces nuclear co-repressors (CoR) from Lef1/Tcf transcription factors to regulate expression of genes involved in cell cycle progression (e.g. cyclin D1), survival (e.g. c-myc), and osteoblast maturation (e.g. osteocalcin) (Hoeppner et al., 2009b; Westendorf et al., 2004) (Figure 2).

The canonical Wnt pathway dictates osteoblast specification from osteo/chondro-progenitors, stimulates osteoblast proliferation, regulates synthesis of matrix proteins, enhances osteoblast and osteocyte survival and aids in the transmission of mechanical loading signals to bone lining cells (reviewed in (Bonewald and Johnson, 2008; Khosla et al., 2008)). The role of

Wnt signaling in bone formation gained significant recognition in 2001 when Gong and colleagues reported a direct link between loss-of-function mutations in the LRP5 co-receptor and the autosomal recessive disorder osteoporosis-pseudoglioma syndrome (OPPG), a disease associated with severe osteoporosis (Gong et al., 2001). These findings were recapitulated in *Lrp5*$^{-/-}$ mice, which developed a low bone mass phenotype similar to patients with OPPG because of decreased osteoblast proliferation (Kato et al., 2002). *Lrp6* hypomorphic mice also have low bone mass, while mice lacking *Lrp5* and one copy of *Lrp6* display an additive reduction in bone mass (Holmen et al., 2004; MacDonald et al., 2007). Other groups identified a gain of function mutation in LRP5 at amino acid G171 in individuals with high bone mass and remarkable skeletal strength (Boyden et al., 2002; Little et al., 2002). Transgenic mice overexpressing the G171V mutation in preosteoblasts using the Col1a1(3.6) promoter recapitulated the high bone mass phenotype found in humans (Akhter et al., 2004; Babij et al., 2003). It was recently demonstrated that expression of the gain-of-function G171V mutation in more mature osteoblasts using the Col1a1(2.3) promoter did not affect bone density (Yadav et al., 2008). The latter study also demonstrated that *Lrp5*-deficiency in the duodenum, rather than in osteoblasts, decreased osteoblast proliferation indirectly via gut-derived serotonin binding its osteoblast receptor, 5-hydroxytryptamine receptor 1b (Htr1b) and the cAMP response binding element (CREB) transcription factor (Yadav et al., 2008). These data suggest that *Lrp5* deficiency causes bone loss in a Wnt-independent manner, but does not rule out a crucial role for Wnt signaling in osteoblasts. Lrp6 or another co-receptor might be more crucial for Wnt signaling in osteoblast lineage cells, particularly in immature osteoblasts and in progenitor cells.

Since the LRP5 discoveries earlier this decade, many studies have documented a role for Wnt pathway components in bone formation, regeneration and repair. **Table 1** summarizes the bone phenotypes associated with genetic altered expression of Wnt signaling pathway components. The overarching conclusion derived from these studies is that activation of the Wnt pathways facilitates osteoblast specification from mesenchymal progenitors and enhances bone mass and strength, while suppression causes bone loss. The interesting and perplexing caveat is that several mechanisms are responsible for altered bone mass. For example, LRP5 appears to regulate osteoblast numbers and proliferation (Kato et al., 2002), perhaps in a Wnt-independent fashion (Yadav et al., 2008), while β-catenin regulates osteoprotegerin (OPG) production in mature osteoblasts and affects bone resorption without affecting osteoblast numbers (Glass et al., 2005). In progenitor cells, β-catenin activation facilitates osteoblast differentiation at the expense of chondrocyte development (Day et al., 2005; Hill et al., 2005; Hu et al., 2005; Rodda and McMahon, 2006), while Wnt5a and Wnt10b increase bone volume by suppressing peroxisome proliferator activator receptor (PPAR) γ activity to block adipogenesis and promote osteoblast lineage maturation (Bennett et al., 2005; Bennett et al., 2007; Takada et al., 2007). These genetic studies, as well as ones showing that Wnt pathway activation enhances osteoblast and osteocyte survival in vitro (Babij et al., 2003; Day et al., 2005; Hens et al., 2005) and that Wnt pathways are active in bone regeneration sites (Silkstone et al., 2008), strongly support crucial roles for Wnts pathways in bone mass accrual. However, recent data suggest that more needs to be done to understand how cells at different stages of maturity respond to specific Wnts.

Section 4: Lymphocyte Enhancer-binding Factor 1

Lymphocyte Enhancer-binding Factor (Lef1) 1 is a transcriptional effector of the Wnt/β-catenin signaling cascade. Activation of canonical Wnt signaling is initiated upon binding of Wnt ligands to receptor complexes comprised of Frizzled and Lrp5/6 proteins, resulting in β-catenin translocation to the nucleus and subsequent displacement of transcriptional co-repressors (i.e. CtBP, Groucho/TLE, and histone deacetylases) from Tcf7 and Lef1 transcription factors (Billin et al., 2000; Brannon et al., 1999; Brantjes et al., 2001; Levanon et al., 1998). Tcf7 and Lef1 recruit co-activators (i.e. p300, Bcl9, and Pygopus) (Behrens et al., 1996; Billin et al., 2000; Daniels and Weis, 2005; Hecht et al., 2000; Hsu et al., 1998; Huber et al., 1996; Kramps et al., 2002; Miyagishi et al., 2000; Sun et al., 2000; Tutter et al., 2001) leading to chromatin relaxation and gene transcription (Westendorf et al., 2004). However, in some contexts, Wnt signaling and β-catenin facilitate Lef1/Tcf7-mediated repression through poorly understood mechanisms (Baker et al., 1999; Cadigan et al., 1998; Cadigan et al., 2002; Jamora et al., 2003; Kahler and Westendorf, 2003; Payre et al., 1999; Piepenburg et al., 2000; Willert et al., 2002; Yang et al., 2000). In the absence of nuclear β-catenin, Lef1/Tcf7 remains associated with transcriptional co-repressors and weakly interacts with chromatin (Tutter et al., 2001).

In adults, the expression of the Lef1/Tcf7 transcription factors is generally restricted to mitotically active cells in renewable tissues. Examples of such tissues include lymphoid follicles, skin, hair follicles, colon, intestine, testis and human tumors (Mayer et al., 1997; Porfiri et al., 1997). Bone is also a renewable tissue, and accordingly, Lef1/Tcf7 activity is increased in areas of bone remodeling and regeneration, and in proliferating osteogenic cells (de Jong et al., 2002; Hadjiargyrou et al., 2002; Kahler and Westendorf, 2003; Kato et al., 2002; Qi et al., 2003). Lef1/Tcf7 expression is usually downregulated as cells stop proliferating and is lost in terminally differentiated cells (Hadjiargyrou et al., 2002; Kato et al., 2002; Kratochwil et al., 1996; Mariadason et al., 2001; Oosterwegel et al., 1993; Shibamoto et al., 2004; Travis et al., 1991; Zhou et al., 1995) including osteoblasts (Kahler et al., 2006). Genetic studies indicate that Lef1 and Tcf7 play different roles in bone cells. *Tcf7*-deficient animals are viable, but have low bone mass caused by decreased OPG expression and increased osteoclastogenesis (Glass et al., 2005). This bone loss is enhanced when *Tcf7*-null mice are crossed with *β-catenin*-deficient mice (Glass et al., 2005). *Lef1* deficiency is postnatally lethal (van Genderen et al., 1994); however, *Lef1* haploinsufficiency decreased trabecular bone mass in female mice by reducing osteoblast activity (Noh et al., 2009). Animals lacking both *Lef1* and *Tcf7* most resemble *Wnt3a*$^{-/-}$ mice (Galceran et al., 1999). Thus, there is strong evidence that Lef1 and Tcf7 act in concert during development to mediate canonical Wnt3a signaling.

First discovered in lymphocytes, Lef1 is one of four transcription factors (the others are Tcf7 (a.k.a. Tcf1), Tcf7L1 (a.k.a. Tcf3) and Tcf7L2 (a.k.a. Tcf4)) that bind β-catenin and regulate gene expression (Arce et al., 2006; Hoppler and Kavanagh, 2007). Lef1 contains a well-characterized N-terminal β-catenin binding domain and a C-terminal high mobility group (HMG) domain that associates with DNA. When Lef1 interacts with DNA, it induces a sharp 130° bend in the double helix and alters the DNA binding of other transcription factors to regulate gene transcription (Carlsson et al., 1993; Giese et al., 1991; Giese and Grosschedl, 1993; Giese et al., 1995; Giese et al., 1997; Love et al., 1995; van de Wetering and Clevers, 1992) The DNA binding domain interacts one of the most common motifs in human promoters, CTTTGT (Xie et al., 2005), and regulates of gene expression. A context-dependent activation (CAD) domain mediates interaction with co-factors, such as Groucho (Arce et al., 2009) and pituitary homeobox (PITX) 2 (Amen et al., 2007), and is located between the β-catenin binding domain and the DNA binding domain (Giese and Grosschedl, 1993). Lef1ΔN is a naturally occurring Lef1 isoform that is transcribed from a promoter (p2) within the intron between exons 3 and 4 in mice (exons 2 and 3 in humans) (Arce et al., 2006; Hovanes et al., 2001). The 2.3 kb transcript of Lef1ΔN lacks the first 113 amino acids of Lef1, the well characterized β-catenin binding domain, and a portion the of CAD domain, but retains the DNA binding motif and the nuclear localization signal.

Each of the four Lef1/Tcf7 genes generate functional diversity by encoding multiple isoforms through differential promoter usage and alternative splicing (Hovanes et al., 2001; Hovanes et al., 2000; Van de Wetering et al., 1996). Isoforms lacking the N-terminal high affinity β-catenin binding domain sometimes act as competitive inhibitors of full-length Lef1/Tcf7 proteins (Arce et al., 2006; Hoppler and Kavanagh, 2007). Differential expression of the full-length and shorter ΔN transcripts is now extensively documented in lymphocytes, myeloid cells, tumors, skin and the intestine (Merrill et al., 2001; Niemann et al., 2002; Skokowa et al., 2006; Takeda et al., 2006; Wang et al., 2005).

Section 5: Rationale and Specific Aims

The clinical need to develop new bone anabolic agents is high because current bone regeneration treatments have limitations, anti-resorptive therapies have unknown long-term health consequences, and the demand for therapies is rising as our population ages. In order to manipulate osteoblast function to treat diseases such as osteoporosis and skeletal cancer metastases, we must first understand the process of osteoblast differentiation at the molecular level. Since the Wnt signaling pathway was implicated as an important regulator of bone formation early this decade, much work has been done to describe the role of various components of this pathway during osteoblast differentiation. Transgenic and knockout mouse models have demonstrated that activation of the Wnt signaling pathway results in increased bone mass, whereas inhibition causes decreased bone mass. However, nuclear effectors of this pathway warrant further study. Our laboratory has focused on identifying the role of Lef1 during osteoblast maturation. We previously demonstrated that overexpression of Lef1 blocks terminal differentiation of osteoblasts, whereas suppression of Lef1 by shRNA accelerates osteoblast differentiation (Kahler et al., 2006). Lef1 was also shown to inhibit Runx2-mediated transcriptional activation of the osteocalcin promoter (Kahler and Westendorf, 2003). While studying the role of Lef1 in osteoblast differentiation, we observed expression of Lef1ΔN, an isoform that is transcribed from a naturally occurring, intronic promoter. Given that the full-length isoform of Lef1 inhibits terminal differentiation of osteoblasts, the focus of my thesis work was to examine the role of Lef1ΔN in osteoblast maturation. The goals of my thesis are three-fold: first, to determine the temporal expression pattern of Lef1ΔN and how its expression is regulated; second, to understand how Lef1ΔN regulates osteoblast maturation; and third, to define a region of Lef1ΔN that is capable of interacting with β-catenin. The following chapters describe my research addressing the role and regulation of Lef1ΔN during osteoblast maturation.

The first section of this thesis defines the temporal expression of Lef1ΔN during osteoblast maturation and describes what factors regulate Lef1ΔN expression. Lef1ΔN is transcribed from an intronic promoter between exons 3 and 4 of mouse Lef1 and lacks the N-terminal high-affinity β-catenin binding domain that is retained in the full-length isoform. We analyzed Lef1 mRNA levels during MC3T3 osteoblast maturation by northern blotting and observed a 2.3 kb transcript corresponding to Lef1ΔN appearing after 6 days of differentiation and prior to detection of osteocalcin, a gene expressed during late osteoblast differentiation. To confirm the temporal expression of Lef1ΔN, we developed a differential quantitative PCR approach to measure Lef1ΔN transcript expression during osteoblast differentiation. Using this technique, we observed Lef1ΔN mRNA is expressed around four to six days of osteoblast differentiation, similar to the results obtained by northern blotting. Next, we sought to identify factors that regulate the expression of Lef1ΔN in osteoblasts. We scanned the Lef1 p2 promoter, from which Lef1ΔN is transcribed, for transcription factor binding sites and identified a potential Runx2 binding element. Our lab has previously shown that Lef1 blocks Runx2-dependent transcriptional activation of the osteocalcin promoter and demonstrated that Lef1 and Runx2 are capable of interacting through their DNA binding domains (Kahler and Westendorf, 2003). Given this data, the presence of a Runx2 binding element in Lef1 p2, and the essential role of Runx2 in osteoblast development, we hypothesized that Runx2 regulates the activity of Lef1ΔN in osseous cells. As discussed previously, the BMP2-Smad 1/5/8-Smad 4-Runx2 pathway promotes osteoblast differentiation. Lef1ΔN is expressed during the later stages of osteoblast differentiation when cells are switching from a proliferative to terminal differentiation state. Since BMP2 is known to induce osteoblast differentiation, we hypothesized that BMP2 may regulate Lef1ΔN expression in osteoblasts.

The second aim of this thesis is to understand the role of Lef1ΔN in osteoblast maturation. Previous work from our lab indicates that Lef1 full-length protein is highly expressed during the early stages of osteoblast differentiation and declines as differentiation proceeds (Kahler et al., 2006). Conversely, Lef1ΔN protein expression begins around day six and is present throughout late osteoblast differentiation. During early osteoblast differentiation, preosteoblasts undergo proliferation. As differentiation proceeds and osteoblasts mature, they switch from a proliferative phase to terminal differentiation. Given that Lef1ΔN is expressed during the later stages of osteoblast differentiation, we hypothesize that Lef1ΔN helps mediate a switch from proliferation to terminal differentiation in maturing osteoblasts. Lef1ΔN does not block Runx2-mediated activation of the osteocalcin, a gene involved in late osteoblast differentiation, whereas the full-length isoform of Lef1 does block activation of osteocalcin. We addressed our hypothesis by stably overexpressing Lef1ΔN in C2C12 myo-osteoblast progenitor cells and evaluating the effect this had on the expression of late osteoblast differentiation genes such as osteocalcin and type 1 collagen.

Lef1ΔN lacks the high-affinity β-catenin domain present in the full-length isoform of Lef1. Consequently, Lef1ΔN inhibits activation of a Lef1-responsive promoter called Top-Flash in the presence of β-catenin in human T lymphocyte Jurkat cells (Hovanes et al., 2001). We sought to determine whether Lef1ΔN inhibits β-catenin-mediated activation of Top-Flash in osseous cells. Surprisingly, Lef1ΔN actually enhanced the activation of Top-Flash in the presence of β-catenin in all the osteoblast cell lines evaluated. In 2005, Daniels and Weis reported a novel second, low-affinity β-catenin binding domain within Lef1 in a region retained by the Lef1ΔN isoform, N-terminal to the DNA binding domain (Daniels and Weis, 2005). We hypothesized that in osteoblasts Lef1ΔN enhances activation of a Lef1-responsive promoter by interacting with β-catenin through this second, low-affinity β-catenin binding domain. To test this hypothesis, we evaluated whether the Lef1 DNA binding domain alone was capable of activating Top-Flash in the presence β-catenin. In support of our hypotheis, the DNA binding domain of Lef1, which lacks the second β-catenin binding domain, was unable to activate the Lef1-responsive promoter. We showed by immunoprecipitation that Lef1ΔN and β-catenin physically interact with each other. Based on these results, the third aim of this thesis is map the region of Lef1ΔN that interacts with β-catenin. This goal was achieved by creating a series of Lef1ΔN constructs with progressive deletions spanning from the start of Lef1ΔN to the DNA binding domain. These Lef1ΔN deletion constructs were used in immunoprecipitation experiments with β-catenin to determine the region of Lef1ΔN that associates with β-catenin. We performed transcription assays with the various Lef1ΔN deletion constructs on Top-Flash in the presence β-catenin to determine the region of Lef1ΔN capable of interacting with β-catenin to activate the Lef1-responsive promoter.

Table 1: Summary of Bone Phenotypes in Genetic Models of Altered Wnt Signaling

Gene	Role in Wnt pathway(s)	Bone Phenotype(s)*	References
APC	Downregulates of β-catenin signaling	CKO: Increased BMD	Holmen, 2005 Ahmed, 2002
Axin2	Downregulates of β-catenin signaling	CKO: Craniosynostosis; Increased BMD	Ikeda, 1998 Yu, 2005
β-Catenin	Interacts with Lef1/Tcfs to regulate gene expression	CKO: Ectopic formation of chondrocytes Tg: Increased ossification, suppression of chondrocytes	Hu, 2005 Hill, 2005 Day, 2005 Glass, 2005 Rodda, 2006
Dkk1	Inhibits β-catenin signaling	Het: High bone mass Tg: Low bone mass	Li, 2006 Ai, 2005
Dkk2	Inhibits β-catenin signaling	KO: Low bone mass Het: Normal	Li, 2005
Fzd9	Receptor for Wnts	KO: Decreased bone formation; Osteopenic	Albers, 2008
GSK3β	Inhibits β-catenin signaling by targeting β-catenin for degradation	Het: Increased bone formation Pharmacological inhibition: Increases BMD	Kugimiya, 2007 Clement-Lacroix, 2005
Kremin 1/2	Potentiate capacity of Dkks to inhibit β-catenin signaling	CKO: Increases BMD	MacDonald, 2007
Lrp5	Co-receptor for Wnts	KO: Decreased BMD CKO (Osteoblasts): Normal GOF: Increased BMD	Kato, 2002 Yadav, 2008 Little, 2002 Boyden, 2002
Lrp6	Co-receptor for Wnts	CKO: Decreased BMD	Zylstra, 2008
Sfrp1	Secreted inhibitor of β-catenin signaling	KO: Increased BMD	Kawano, 2003
SOST	Inhibits β-catenin signaling	KO: Increased BMD Tg: Osteopenic	Balemans, 2002 Li, 2008
Tcf7 (Tcf1)	Transcription factor activated through interaction with β-catenin	DKO ($Tcf7^{-/-}Lef1^{-/-}$): Similar to $Wnt3a^{-/-}$ phenotype KO: Low bone mass, increase in bone resorption	Galceran, 1999 Glass, 2005
Wnt3a	Activates β-catenin pathway	Het: No change in BMD	Takada, 2007
Wnt5a	Activates β-catenin and noncanonical pathways	Het: Reduced BMD, increased adipogenesis	Takada, 2007
Wnt10b	Activates β-catenin	KO: Reduced BMD, increased adipogenesis Tg: Increased BMD	Bennett, 2007 Bennett, 2005

* BMD: bone mineral density; CKO: conditional knockout mouse; KO: global knockout; DKO: double global knockout; Tg: transgenic; GOF: gain of function

Figure 1: The structure of long bone. This illustration depicts the general structure of long bones in the mature human skeleton. Long bones consist of a hollow shaft, called the diaphysis, which widens at the ends to form the metaphyses (regions below the growth plate) and the epiphyses (regions above the growth plate). Cortical bone lines the outer cortex of bone and surrounds cancellous, or trabecular, bone. The fibrous outer coating of bone is called the periosteum, and the lining of the endocortical surfaces is termed the endosteum.

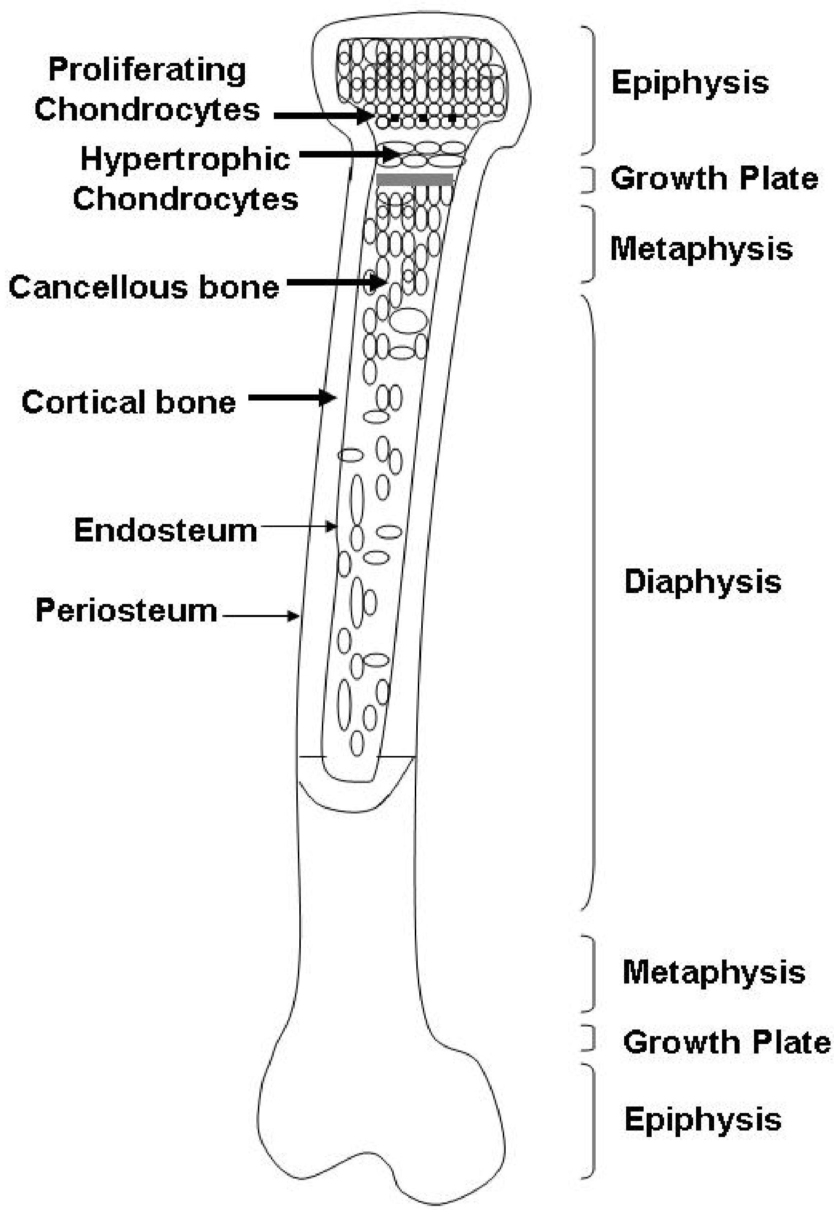
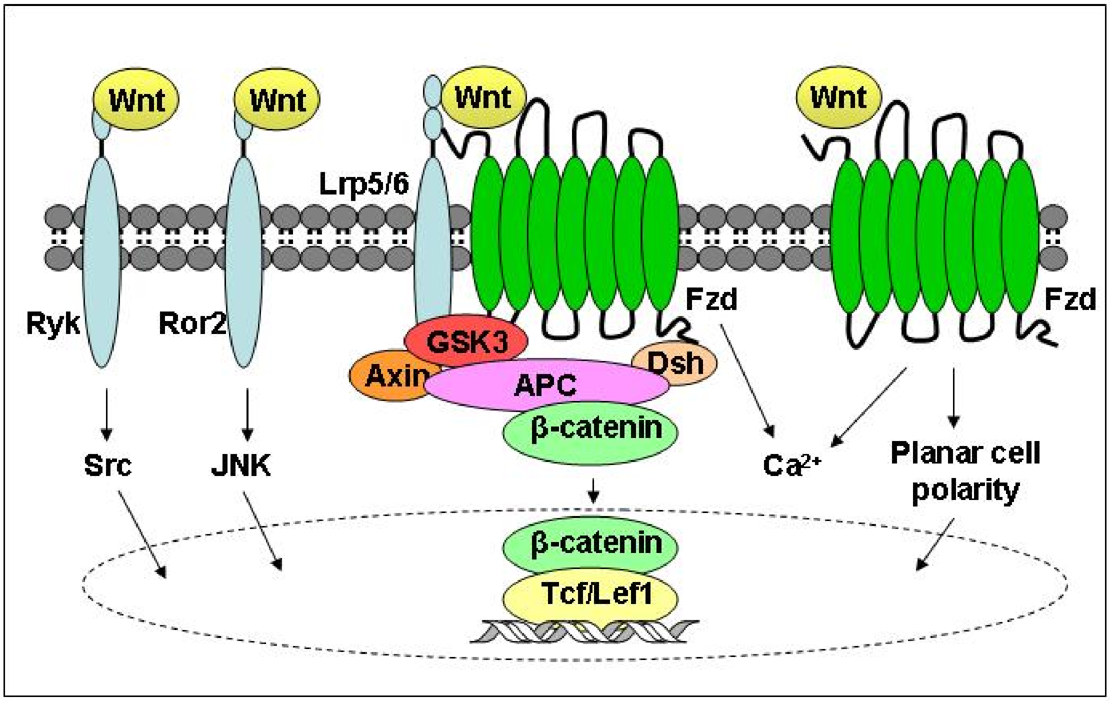

Proliferating
Chondrocytes
Hypertrophic
Chondrocytes
Cancellous bone
Cortical bone
Endosteum
Periosteum
Epiphysis
Growth Plate
Metaphysis
Diaphysis
Metaphysis
Growth Plate
Epiphysis

Figure 2: Current model of Wnt signaling. Wnt ligand binding to a Fzd receptor and LRP5 or LRP6 coreceptor leads to activation of Disheveled (Dsh), which inhibits a cytoplasmic complex composed of glycogen synthase kinase 3β (GSK3β), Axin, and APC and prevents GSK3β from targeting β-catenin for ubiquitination and degradation by the proteosome. Cytoplasmic β-catenin levels increase and β-catenin translocates to the nucleus where it associates with Tcf/Lef transcription factors to regulate gene expression. During non-canonical Wnt signaling, Wnts bind to a Fzd receptor, but the downstream signaling events do not involve GSK3β or β-catenin. At least two non-canonical Wnt signaling cascades have been identified: 1) Wnt/calcium signaling which results in an increase of intracellular calcium and activation of protein kinase C and calcium/calmodulin-dependent kinase and 2) Wnt/planar cell polarity pathway which signals through Rho/Rac GTPases and C-Jun N-terminal kinase to modulate cytoskeletal elements and gene expression. Wnts bind to receptor tyrosine kinases Ryk and Ror2 to activate Src and JNK signaling, respectively, through currently unknown β-catenin-independent mechanisms.


Wnt
Wnt
Wnt
Wnt
Lrp5/6
Ryk
Ror2
Fzd
Fzd
GSK3
Axin
APC
Dsh
β-catenin
Src
JNK
Ca2+
Planar cell polarity
β-catenin
Tcf/Lef1

Chapter 2

Runx2 and Bone Morphogenic Protein 2 Regulate the Expression of an Alternative Lef1 Transcript During Osteoblast Maturation

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Lymphoid Enhancer Binding Factor (Lef) 1 is a transcriptional effector of the Wnt/Lrp5/β-catenin signaling cascade, which regulates osteoblast differentiation, bone density and skeletal strength. In this study, we describe the expression and function of an alternative Lef1 isoform in osseous cells. Lef1ΔN is a naturally occurring isoform driven by a promoter (p2) within the intron between exons 3 and 4 of *Lef1*. Lef1ΔN is induced during late osteoblast differentiation. This is converse to the expression pattern of the full-length Lef1 protein, which as we previously showed, decreases during differentiation. Agonists of osteoblast maturation differentially affected Lef1ΔN expression. BMP2 stimulated Lef1ΔN expression, whereas Wnt3a blocked basal and BMP2-induced expression of Lef1ΔN transcripts during osteoblast differentiation. We determined that the Lef1ΔN p2 promoter is active in osteoblasts and Runx2 regulates its activity. Stable overexpression of Lef1ΔN in differentiating osteoblasts induced the expression of osteoblast differentiation genes, osteocalcin and type 1 collagen. Taken together, our results suggest Lef1ΔN is a crucial regulator of terminal differentiation in osseous cells.

Introduction

The canonical Wnt pathway dictates osteoblast specification from osteo/chondro-progenitors, stimulates osteoblast proliferation, regulates synthesis of matrix proteins, enhances osteoblast and osteocyte survival, and transmits mechanical loading signals to bone lining cells (reviewed in (Bonewald and Johnson, 2008; Khosla et al., 2008)). Canonical Wnt signaling is initiated when Wnts bind to receptor complexes consisting of Lrp5/6 and Frizzled proteins. This stabilizes β–catenin and induces its translocation to the nucleus where it displaces co-repressors from Tcf7 and Lef1 transcription factors, leading to the activation of genes involved in cell proliferation, including cyclin D and c-myc (Westendorf et al., 2004). If unchecked or rampant, the Wnt pathway is often dangerous and oncogenic because of this pro-proliferative effect (Polakis, 2007).

Lef1 is one of four transcription factors (the others are Tcf7 (a.k.a. Tcf1), Tcf7L1 (a.k.a. Tcf3) and Tcf7L2 (a.k.a. Tcf4)) that bind β–catenin and regulate gene expression (Arce et al., 2006; Hoppler and Kavanagh, 2007). First discovered in lymphocytes, Lef1 binds one of the most common motifs in human promoters, CTTTGT (Xie et al., 2005), and is a context-dependent regulator of gene expression. When Lef1 interacts with DNA, it induces a sharp 130^{o} bend in the double helix and alters the DNA binding of other transcription factors to regulate gene transcription (Carlsson et al., 1993; Giese et al., 1991; Giese and Grosschedl, 1993; Giese et al., 1995; Giese et al., 1997; Love et al., 1995; van de Wetering and Clevers, 1992). In the absence of nuclear β–catenin, Lef1 interacts weakly with chromatin (Tutter et al., 2001) and associates with transcriptional co-repressors (*e.g.* CtBP, TLE and histone deacetylases) (Billin et al., 2000; Brannon et al., 1999; Brantjes et al., 2001; Levanon et al., 1998). Nuclear β–catenin displaces these co-repressors and recruits co-activators (*e.g.* p300, Bcl9, and Pygopus) to facilitate chromatin binding and gene expression (Behrens et al., 1996; Billin et al., 2000; Daniels and Weis, 2005; Hecht et al., 2000; Hsu et al., 1998; Huber et al., 1996; Kramps et al., 2002; Miyagishi et al., 2000; Sun et al., 2000; Tutter et al., 2001). Interestingly, in some contexts Wnt signaling and β–catenin facilitate Lef1/Tcf7-mediated repression through poorly defined mechanisms (Baker et al., 1999; Cadigan et al., 1998; Cadigan et al., 2002; Jamora et al., 2003; Kahler and Westendorf, 2003; Payre et al., 1999; Piepenburg et al., 2000; Willert et al., 2002; Yang et al., 2000).

Lef1 and Tcf7 proteins share many molecular and biochemical characteristics; however, functional diversity exists between Tcf7/Lef factors during development (Arce et al., 2006; Brugmann et al., 2007; Hoppler and Kavanagh, 2007; Schroeder et al., 2004). In skeletal structures of mouse embryos (E14.5), *Lef1* is detectable in tail prevertebrae, osteogenic cells of the hipbone, and the mesenchymal cells around the cochlea. By comparison, *Tcf7 (previously referred to as Tcf1)* is found in the pre-cartilagenous cells of the palate, maxilla, mandible, nasal and basioccipital bones, the thoracic pre-vertebrae and ribs at E14.5 (Oosterwegel et al., 1993). Knockout mice also reveal specific functions for Lef1 and Tcf7. *Lef1*-deficient mice are smaller than normal littermates, display multiple defects in tissues formed by epithelial and mesenchymal interactions (e.g. they lack teeth, body hair and whiskers), and die within two weeks of birth (Oosterwegel et al., 1993). A recent report examining the role of the Lef1 in the regulation of bone mass found that *Lef1*$^{+/-}$ female mice had decreased trabecular bone mass due to reduced osteoblast activity (Noh et al., 2009). *Tcf7*-deficient animals are viable, but they lack early thymocyte progenitors (Verbeek et al., 1995) and have low bone mass caused by decreased OPG expression and increased osteoclastogenesis (Glass et al., 2005). This bone loss is enhanced when *Tcf7*-null mice are crossed with *β–catenin*-deficient mice (Glass et al., 2005). Animals lacking both *Lef1* and *Tcf7* most resemble *Wnt3a*$^{-/-}$ mice (Galceran et al., 1999). Thus, Lef1 and Tcf7 act in concert during development to mediate canonical Wnt3a signaling.

In adults, the expression of the Lef1/Tcf7 transcription factors is generally restricted to mitotically active cells in renewable tissues. Examples of such tissues include lymphoid follicles, skin, hair follicles, colon, intestine, testis and human tumors (Mayer et al., 1997; Porfiri et al., 1997). Bone is also a renewable tissue, and accordingly, Lef1/Tcf7 activity is increased in areas of bone remodeling and regeneration, and in proliferating osteogenic cells (de Jong et al., 2002; Hadjiargyrou et al., 2002; Kahler and Westendorf, 2003; Kato et al., 2002; Qi et al., 2003). Lef1/Tcf7 expression is usually downregulated as cells stop proliferating and is lost in terminally differentiated cells (Hadjiargyrou et al., 2002; Kato et al., 2002; Kratochwil et al., 1996; Mariadason et al., 2001; Oosterwegel et al., 1993; Shibamoto et al., 2004; Travis et al., 1991; Zhou et al., 1995) including osteoblasts (Kahler et al., 2006).

Each of the four Lef1/Tcf7 genes generate functional diversity by encoding multiple isoforms through differential promoter usage and alternative splicing (Hovanes et al., 2001; Hovanes et al., 2000; Van de Wetering et al., 1996). Isoforms lacking the N-terminal high affinity β−catenin binding domain sometimes act as competitive inhibitors of full-length Lef1/Tcf7 proteins (Arce et al., 2006; Hoppler and Kavanagh, 2007). Lef1ΔN lacks the first 113 amino acids found in Lef1. Its 2.3 kb transcript is driven by a promoter (p2) within the intron between exons 3 and 4 (Arce et al., 2006; Hovanes et al., 2001). Differential expression of the full-length and shorter ΔN transcripts is now extensively documented in lymphocytes, myeloid cells, tumors, skin and the intestine (Merrill et al., 2001; Niemann et al., 2002; Skokowa et al., 2006; Takeda et al., 2006; Wang et al., 2005). In this report, we demonstrate that Lef1ΔN is induced in osteoblasts during the terminal differentiation process and accelerates the expression of genes involved in osteoblast differentiation when introduced into pre-osteoblasts.

Materials and Methods

Cell lines, primary calvarial osteoblast isolation, and osteoblast differentiation. C2C12 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 200 mM L-glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin. C3H10T1/2, MC3T3-E1, ROS 17/2.8, and primary osteoblast cells were cultured in Minimal Essential Medium (MEM) containing the supplements listed above. Primary calvarial osteoblasts were isolated from C57Bl/6 mice as previously described (Schroeder et al., 2005). To induce osteogenic differentiation, cells were cultured in α-MEM supplemented with 10% FBS, 50 μg/ml ascorbic acid and 10 mM β-glycerolphosphate after reaching confluency. Where indicated, media were supplemented with 300 ng/ml BMP2 (Sofamor Danek) and 67 ng/ml Wnt3a (R&D Biosystems). Media were replaced every three days. Transient transfections were performed in 12 well plates with Lipofectamine (Invitrogen) according to the manufacturer's instructions.

RNA Isolation, Northern Blotting and Reverse Transcriptase-PCR. RNA was harvested from cells using Trizol Regent (Invitrogen). RNA (northern) blotting was performed as described by Kahler et al (Kahler et al., 2006). Quantitative RT-PCR was performed using the QuantiTech SYBR Green RT-PCR kit (Qiagen) and reactions were run on an iCycler (BioRad). RNA (10 ng) was used in a 20 μl reaction with QuantiTech SYBR Green RT mastermix, QuantiTech RT mix, and 0.5 pmol/μl of each of the primers for mouse Lef1

(Primer Pair #1), forward: GAT CCC CTT CAA GGA CGA AG, reverse: GGC TTG TCT GAC CAC CTC AT; mouse Lef1 + Lef1ΔN (Primer Pair #2), forward: TCA CTG TCA GGC GAC ACT TC, reverse: TGA GGC TTC ACG TGC ATT AG; mouse alkaline phosphatase, forward: TGT TGA CAA GGC AGA CAA GC, reverse: CAG GAC CGT TGC CGT ATA GT; mouse osteocalcin, forward: AAG CAG GAG GGC AAT AAG GT, reverse: TTT GTA GGC GGT CTT CAA GC; mouse actin, forward: AAG GAA GGC TGG AAA AGA GC, reverse: GCT ACA GCT TCA CCA CCA CA. Data were normalized to mouse actin levels. The reverse transcriptase reaction to convert the RNA to cDNA was performed at 50°C for 30 minutes. Initial denaturation was made at 95°C for 15 minutes followed by 40 cycles of three-step PCR: 95°C denaturation for 1 minute, 57°C (59.1°C for Lef1 primers) annealing for 1 minute, and 72°C elongation for 1 minute. The Lef1 primer pairs were used for differential quantitative RT-PCR. The efficiencies of Lef1 primer pairs 1 and 2 were determined by amplifying serial dilutions of Lef1 plasmid DNA. The efficiency (E) of each primer pair was taken into account when calculating the relative amounts of cDNA amplified through the equation, $E^{-\Delta\Delta Ct}$ (Pfaffl, 2001).

Electrophoretic Mobility Shift Assays (EMSA) and Chromatin Immunoprecipitation (ChIP). EMSA were performed with a double stranded probe with a 24 bp sequence from p2 that surrounds and includes the Runx2 binding element as previously described (Kahler and Westendorf, 2003). ChIP analyses for Runx2 binding were performed on lysates from ROS17/2.8 cells as previously described (Schroeder et al., 2004) using antibodies against Runx2 (Santa Cruz, M-70) or normal rabbit IgG (Santa Cruz). PCR was performed using the following primers: 5' GGC CTT GCA TGT TAA CAC CT and 5' GGC TCA ACA GCA AAC CAG AT.

For histone and RNA polymerase II ChIP analyses, lysates were collected from differentiated MC3T3 cells as previously described (Lambert and Nordeen, 2003), except the cultures were treated with collagenase type 2 (Worthington; 1 mg/ml)/BSA (Sigma Aldrich; 1 mg/ml) at 37°C for 1 hour (to remove extracellular collagen that accumulates in osteoblast cultures) before adding 1% formaldehyde to crosslink intracellular proteins and DNA. Fractions of the lysate (20%) from a 10 cm plate were incubated with antibodies (2 μg) recognizing histone H3 (Upstate, 05-928), phosphorylated RNA polymerase II (GeneTex, GTX44758) or normal rabbit IgG (Santa Cruz). After reversing the crosslinks, DNA was purified and analyzed for enrichment of the Lef1 p2 promoter with real-time PCR (see above). Primers used to amplify the Lef1 P2 promoter were the “proximal” primer set: 5’ TGCTGTTAAAGCCATTGAGG 3’ and 5’ AGACAGCCCAGACTTTACGC 3’, and with the “distal” primer set: 5’ GTCCTCTCAGGAGCCCTACC 3’ and 5’ GTATGTGGGGAAGTCTTGCAT 3’. Nonspecific background was eliminated in data by subtracting the relative amount of product detected in the control IgG immunoprecipitates from that detected in the H3 and RNA polymerase II immunoprecipitates.

Promoter/Reporter Plasmids. The Lef1 p2 promoter (-447-+35) was amplified from Balb/c mouse genomic DNA using gene specific oligonucleotides (primer sequences available upon request) and PCR. PCR products were cloned into the TOPO vector (Invitrogen) and then subcloned into the pGL2 plasmid (Promega) using *Hind*III restriction sites. The Lef1 promoter 1 (p1) was obtained from Dr. Marian Waterman. The murine osteocalcin gene (mOG) 2-luciferase plasmid (Montecino et al., 1996) was obtained from Dr. Jane Lian. The proximal Runx2 site within mOG2 was replaced with a GAL4 site, UAS (CGGAGTACTGT CCTCCG), with overlapping PCR.

Transcription Assays. Cells were transfected with Lipofectamine (Invitrogen) in 12-well plates with the indicated amounts of luciferase reporter plasmids and 50 ng pRL-null. pCMV-Runx2 expression plasmids (300 ng unless otherwise specified) were added as indicated. pcDNA3.1 was added to transfections to maintain a uniform amount of total DNA per transfection. Luciferase activity was measured 24 or 48 hours after transfection using the dual-luciferase assay system (Promega). Each transfection was performed in triplicate, and normalized to Renilla-luciferase activity.

Retroviral Subcloning and Transduction. To produce retroviruses, 293T cells were co-transfected with 5 μg of pMSCV-puro-Lef1-Flag or pMSCV-puro-Lef1ΔN-Flag and 5 μg of pCL2 using calcium phosphate precipitation. Virus-containing supernatants were collected after 48 and 72 hours, filtered, and added to C2C12 cells in the presence of 8 μg/ml polybrene. The transduction was repeated 8 hours and 24 hours later. Transduced cells were selected with 5 μg/ml puromycin for a minimum of 3 days.

Immunoblotting. Cells were treated with proteasome inhibitor MG132 (Sigma; 10 μM) for 4 hours prior to lysis. Cells were washed twice with PBS. Whole cell lysates were collected by incubating cells with modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.25% NaDOC, protease inhibitors). Nuclear lysates were obtained from indicated cells by scraping the cells off the plate in 1 ml of PBS, centrifuging at 2000 rpm for 5 minutes, suspending the pellet in 100 μl Iso-Hi Buffer (10 mM Tris-HCl, pH 7.8, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40, protease inhibitors), incubating on ice for 5 minutes, centrifuging at 5000 rpm for 5 minutes at 4°C, and suspending the pellet in modified RIPA buffer. Lysates were sonicated and insoluble material was removed by centrifugation. Equal amounts of protein lysates were resolved by SDS–12% PAGE. Proteins were transferred to PVDF membrane (Immobilon-P, Millipore). Membranes were blotted with the indicated antibody diluted 1:1000 in 3% non-fat dried milk in TBST (TBS + 0.04% Tween-20). The antibodies used are: anti-Lef1 (Cell Signaling, C18A7) and anti-Lamin B (Santa Cruz).

Results

Osteoblasts Express Multiple Lef1 mRNA Transcripts. Lef1 mRNA expression levels were analyzed during MC3T3 osteoblast maturation by northern blotting. A 3.6 kb transcript was induced during the differentiation time course (Figure 1A). This transcript encodes a full-length isoform of Lef1 containing the high-affinity β–catenin binding domain at the N-terminus and the DNA binding domain at the C-terminus (Hovanes et al., 2000). A 2.3 kb transcript was also detected MC3T3 cells after six days of differentiation. This transcript appeared before the osteocalcin transcript (Figure 1A) and was only detected with a probe directed to the 3' region of the Lef1 gene (Figure 1B), whereas a probe recognizing the 5' region of *Lef1* detected only the full-length 3.6 kb transcript (Figure 1B). These results indicate that maturing osteoblasts produce an alternative Lef1 transcript that resembles one previously described in T lymphocytes (Hovanes et al., 2001).

Based on the results of Hovanes and colleagues (Hovanes et al., 2001), we hypothesized that the 2.3 kb transcript encodes a truncated protein that lacks the N-terminal β–catenin binding domain, but retains the DNA binding domain (Figure 2A). To test this and verify the northern blotting analysis, we developed a differential PCR approach, similar to ones described by others (Wang et al., 2005; Willinger et al., 2006), to examine the relative amount of the shorter transcript in cells. Two primers pairs were synthesized (Figure 2A) and their relative efficiencies in amplifying Lef1 cDNA were calculated. Primer pair 1 amplifies sequences only in the 3.6 kb transcript, whereas primer pair 2 amplifies both the 3.6 and 2.3 kb mRNAs. The difference between the two products is the amount of the shorter transcript in the cells. Using this differential PCR approach, results similar to those obtained by northern blotting were obtained (Figure 2B). Thus, the longer transcript, Lef1 (recognized by primer pair 1), did not change significantly during the first 7 days of differentiation. However, the amount of product recognized by primer pair 2 increased more than 20 fold by day 7. No changes were observed at days 2 (Figure 2B) or 5 (data not shown). We derived the amount of the transcript only recognized by primer pair 2 by subtracting primer pair 1 products from primer pair 2 amplicons and called this Lef1ΔN transcript.

We verified the induction of Lef1ΔN in mouse primary calvarial cells. As in MC3T3 cells, Lef1ΔN transcripts (Figure 2C) were detected prior to the time when osteocalcin transcripts (Figure 2D) were detectable in these cultures (days 28 and 34, respectively). Due to the longer differentiation period required for primary osteoblasts, Lef1ΔN levels peaked at 28 days, while osteocalcin levels continued to rise during days 28 to 34.

BMP2 and Wnt3a Differentially Affect Lef1ΔN Expression. BMP2 and Wnt3a are agonists of osteoblast proliferation. BMP2 binds receptor complexes and activates numerous signaling pathways to regulate osteoblast gene expression, proliferation and differentiation (Katagiri et al., 1990; Katagiri et al., 1994; Wang et al., 1990; Yamaguchi et al., 1991). Wnt3a initiates canonical Wnt signaling by binding to receptor complexes consisting of Lrp5/6 and Frizzled on the cell surface, which results in nuclear translocation of β-catenin and activation of Lef1/Tcf transcription factors (Bhanot et al., 1996; Billin et al., 2000; Cadigan et al., 1998; Hecht et al., 2000; Shtutman et al., 1999; Sun et al., 2000; Wehrli et al., 2000; Yang-Snyder et al., 1996). Using differential quantitative PCR to evaluate Lef1ΔN transcript levels, we found that BMP2 is a very potent inducer of Lef1ΔN expression during osteoblast differentiation (Figure 3A). Thus, while Lef1ΔN was induced 35-fold by osteogenic medium lacking exogenous BMP2 (open boxes), it was stimulated $5x10^5$ fold by BMP2 (closed boxes). Conversely, Wnt3a repressed basal and BMP2-induced Lef1ΔN expression (circles, Figure 3A). Alkaline phosphatase (AP) and osteocalcin transcripts were measured to monitor the differentiation process. AP, a relatively early marker of osteoblast differentiation, was induced by BMP2, while Wnt3a repressed basal and BMP2-induced alkaline phosphatase expression (Figure 3B). Basal expression of osteocalcin, a late osteoblast differentiation marker, was also upregulated by BMP2 and repressed by Wnt3a (Figure 3C). However, Wnt3a was unable to repress BMP2-induced expression of osteocalcin (Figure 3C). These results are consistent with other reports showing that Wnt3a blocks early osteogenesis of mesenchymal progenitor cells (Boland et al., 2004). To confirm the induction of Lef1ΔN during osteoblast differentiation, immunoblotting was preformed on lysates collected during osteoblast differentiation in the presence of BMP2. Consistent with the increase of Lef1ΔN transcript levels, Lef1ΔN protein levels increased during osteoblast differentiation in the presence of BMP2 (Figure 3D).

The Lef1ΔN Promoter, p2, is Active in Osteoblasts. The temporal and inducible expression of Lef1ΔN indicates that it is important for late osteoblast differentiation. To understand how expression of Lef1ΔN is regulated, we examined the murine Lef1ΔN promoter, referred to as p2. In human cells, the transcriptional start site is ten base pairs 5' of exon 4, and translation begins with a methionine in the middle of exon 4 (Hovanes et al., 2001). To determine whether p2 is active in murine osteoblasts, we used chromatin immunoprecipitation (ChIP) to evaluate levels of histone 3 (H3) and phosphorylated RNA polymerase II at distal and proximal regions of the p2 promoter (Figure 4A). We first examined the levels of post-translationally modified H3 at the promoter. Surprisingly, levels of both acetylated (an activation mark) and methylated (a repressive mark) H3 were reduced (data not shown). Thus, we hypothesized that nucleosomes in this region were being disassembled. We tested this possibility by investigating total H3 levels. During the first 1 to 2 days of osteoblast differentiation, the amount of total H3 present at the distal and proximal regions of the p2 promoter decreased and remained low throughout the first 5 days of osteoblast differentiation (Figures 4B & 4D). This loss of total H3 suggests nucleosome disassembly and is expected to correspond with increased accessibility for transcriptional machinery. To determine if the promoter was being actively transcribed, we monitored the recruitment of phosphorylated RNA polymerase II to the p2 promoter in differentiating osteoblasts. From day 0 to day 2 of osteoblast differentiation, phosphorylated RNA polymerase II levels at the promoter decreased. However, between days 2-5 of differentiation, levels of phosphorylated RNA polymerase II steadily increased suggesting that the Lef1ΔN p2 promoter was becoming active at both the distal and proximal regions (Figure 4C & 4E). Our observations that total H3 levels were low and that phosphorylated RNA polymerase II levels were increasing at distal and proximal regions of p2 during the first 5 days of osteoblast differentiation correspond with our findings that Lef1ΔN message becomes detectable at days 6 to 7 of osteoblast differentiation (Figure 1A & 2B) and indicate that the Lef1 p2 promoter is activated in differentiating osteoblasts.

Runx2 Regulates p2 Activity. To understand how Lef1 p2 is regulated in osteoblasts, we scanned the sequence for transcription factor binding sites common in osteoblast promoters. Runx2 is an essential transcription factor for osteoblast development and regulates the expression of numerous osteoblast genes. We identified a potential Runx2 binding element in the p2 promoter at position -215 (Figure 5A). Runx2 bound a double-stranded probe containing this sequence of the promoter in electrophoretic mobility shift assays (Figure 5B, lane 2). Formation of the labeled DNA-Runx2 complex was specifically competed with an unlabeled double-stranded oligonucleotide containing a wildtype Runx2 binding element, but not by an unlabeled oligonucleotide containing a mutant Runx2 binding site (Figure 5B, lanes 3 and 4). Moreover, the complex was supershifted by a Runx2 antibody (Figure 5B, lane 5). Chromatin immunoprecipitation assays in ROS17/2.8 osseous cells demonstrated that Runx2 bound the Lef1 p2 promoter in vivo (Figure 5C). To determine the effects of Runx2 on the promoter, we amplified the p2 promoter (-447-+35) and placed it in a vector containing a luciferase reporter gene. This p2 reporter was as active as the Lef1 p1 promoter in C3H10T1/2 cells when subcloned in the forward (F) but not reverse (R) orientation (Figure 5D). Runx2 activated the reporter in a concentration-dependent fashion (Figure 5E); however, Runx2 did not activate a mutant p2 construct lacking a functional Runx2 binding site (Figure 5F). To determine whether Runx2 is required for Lef1 p2 activity, we differentiated primary calvarial cells from wildtype and *Runx2*$^{-/-}$ mice in osteoblast differentiation medium and evaluated Lef1ΔN transcript levels by differential qPCR. As previously shown (Figure 2C), Lef1ΔN rose in wildtype primary osteoblasts during late osteoblast differentiation (days 28 and 34). In contrast, Lef1ΔN levels did not change in the *Runx2*-deficient cells (Figure 5G). These data demonstrate that Runx2 binds the Lef1 p2 promoter and positively regulates its expression.

Overexpression of Lef1ΔN Induces Expression of Late Osteoblast Differentiation Genes. Given that the Lef1 p2 promoter is active in maturing osteoblasts and regulated by Runx2, we sought to understand the role of Lef1ΔN in osteoblast differentiation. To this end, we stably overexpressed Lef1ΔN in C2C12 myo-osteoblast progenitor cells using retroviral transduction and induced the cells to undergo osteoblast differentiation by culturing them in osteogenic medium containing BMP2. We verified exogenous Lef1ΔN protein and transcript expression by immunoblotting (Figure 6A) and differential qPCR (data not shown), respectively. To elucidate the role of Lef1ΔN in osteoblast differentiation, we evaluated the effects of Lef1ΔN overexpression on markers of osteoblast differentiation. Exogenous Lef1ΔN augmented the expression of osteocalcin and type 1 collagen in cells cultured in osteogenic medium (Figure 6B and 6C). Thus, overexpression of Lef1ΔN in myo-osteoblast progenitor cells accelerated the expression of genes involved in osteoblast differentiation.

Lef1, but not Lef1ΔN, inhibits Runx2 activation of the osteocalcin promoter. We previously demonstrated that the full length isoform of Lef1 blocks Runx2-dependent activation of the osteocalcin promoter, which contains a Lef1 binding site just 4 base pairs away from the proximal Runx2 element (Kahler and Westendorf, 2003). To further understand the role of Lef1ΔN in osteoblast differentiation, we evaluated whether Lef1ΔN was also capable of blocking Runx2-dependent activation of the osteocalcin promoter. We found that Lef1ΔN, which lacks the β-catenin binding domain, does not repress Runx2-mediated osteocalcin expression, even though it retains a functional DNA binding domain (Figure 6D). Lef1ΔC, which lacks the DNA binding domain, also did not repress Runx2 and in fact activated the osteocalcin promoter (Figure 6D). To determine whether Lef1 could sterically block the access of Runx2 to the neighboring sequence in the osteocalcin promoter, we changed the proximal Runx2 site to a GAL4 binding site. VP16 strongly activated this mutant promoter (Figure 6E). Full-length Lef1 repressed VP16-driven activation of the osteocalcin promoter, but neither Lef1ΔN nor Lef1ΔC repressed VP16 activation (Figure 6E). These data suggest that full-length Lef1 can sterically block assess of transcription factors, specifically Runx2, to adjacent regions on the osteocalcin promoter and that the N-terminus of Lef1 is necessary for this repression.

Discussion and Conclusions

The Human Genome Project revealed that phenotypic variability is the result of genome complexity and versatility that creates many alternative gene products, rather than the presence of more genes (Tress et al., 2007). In this report, we show that an alternative Lef1 transcript appears during osteoblast maturation just prior to osteocalcin gene expression. This transcript is induced by BMP2 and Runx2, but is suppressed by Wnt3a. To the best of our knowledge, this is the first time that alternate Lef1 transcripts have been described in osseous cells. Lef1 was identified as a differentially regulated gene in several osteoblast microarray studies (James et al., 2006; Vaes et al., 2002), but it is not known which transcript(s) was affected because probes on microarray chips are heavily weighted to 3' regions of genes, which would detect both transcripts.

The four Lef1/Tcf7 genes encode multiple isoforms through differential promoter usage and alternative splicing (Hovanes et al., 2001; Hovanes et al., 2000; Van de Wetering et al., 1996). Lef1ΔN lacks the first 113 amino acids found in Lef1. Its 2.3 kb transcript is driven by a promoter (p2) within the intron between exons 3 and 4 (Hovanes et al., 2001; Li et al., 2006). Differential expression of the full-length Lef1 and short Lef1ΔN transcripts is well documented in lymphocytes, myeloid cells, tumors, skin and the intestine (Merrill et al., 2001; Niemann et al., 2002; Skokowa et al., 2006; Takeda et al., 2006; Wang et al., 2005). In T cells, both Lef1 isoforms are high in resting cells, but the short transcript is downregulated upon activation and proliferation (Willinger et al., 2006). In murine epidermal stem cells, Lef1ΔN suppresses hair cell differentiation, but stimulates sebocyte differentiation, epidermal cysts and skin tumors (Merrill et al., 2001; Niemann et al., 2002); moreover, point mutations in the N-terminus of Lef1 that prevent beta-catenin binding are found in human sebaceous tumors (Takeda et al., 2006). Interestingly, Lef1ΔN is sufficient to rescue the differentiation block induced by *Lef1*-deficiency in granulocytes (Skokowa et al., 2006). Thus, Lef1ΔN acts as an inhibitor of proliferative signals emanating from the canonical Wnt pathway in some cells, but it promotes the differentiation of other cells and is sufficient to rescue Lef1 functions in certain tissues. In this report, we demonstrate that Lef1ΔN is induced in osteoblasts during the terminal differentiation process and can increase the expression of osteoblast genes (Figure 6).

We previously reported that the full length isoform of Lef1 blocks Runx2-dependent activation of the osteocalcin promoter (Kahler and Westendorf, 2003). Here, we demonstrate that Lef1ΔN, unlike the full-length isoform of Lef1, does not to repress Runx2-mediated activation of the osteocalcin promoter. Correspondingly, Lef1ΔN exogenously expressed in differentiating osteoblasts induces the expression of osteocalcin. We also previously showed that Lef1 protein levels decrease during osteoblast differentiation (Kahler et al., 2006), but the current results indicate mRNA levels encoding the full length protein do not change and might increase during osteoblast maturation. These data and those within Kahler et al. (Kahler et al., 2006) support the notion that Lef1 levels are regulated posttranscriptionally. The discordance between Lef1 mRNA and protein levels was previously observed in differentiating cells at the hair follicle bases (DasGupta and Fuchs, 1999; Jimenez et al., 2005). The mechanisms regulating Lef1 protein levels in osteoblasts are unclear. However, Nrarp (Notch-regulated ankyrin repeat protein) has been shown to block Lef1 ubiquitination and stabilize Lef1 protein (Ishitani et al., 2005). We have found that Nrarp is expressed in osteoblasts (unpublished data). Taken together, these data suggest that Lef1 isoforms have distinguishable activities during osteoblast maturation, particularly with regards to regulation of the osteocalcin gene, and their regulation is complex.

BMP2 induced expression of Lef1ΔN, as well as alkaline phosphatase and osteocalcin, two markers of osteoblast differentiation. Interestingly, Wnt3a suppressed basal and BMP2-induced Lef1ΔN and alkaline phosphatase expression. These data are consistent with a previous study demonstrating that Wnt3a blocks early osteogenesis of mesenchymal progenitor cells (Boland et al., 2004). The inability of Wnt3a to suppress BMP2-induced osteocalcin expression indicates that Wnt3a might not suppress late osteoblast differentiation genes as potently as those expressed earlier.

Regulation of the Lef1ΔN promoter (p2) closely paralleled the observed timeframe of Lef1ΔN mRNA expression. Thus, the Lef1ΔN transcript (Figures 1 & 2) and protein (Figure 3D) become detectable at days 6 to 7 of osteoblast differentiation, while total histone H3 levels decreased and phosphorylated RNA polymerase II levels increased at distal and proximal regions of the Lef1 p2 promoter within the first 5 days of osteoblast differentiation. The short delay between detection of p2 activity and detection of Lef1ΔN transcript suggests that cofactors required for Lef1ΔN expression must be recruited. We identified a Runx2 binding element in p2 and demonstrated that Runx2 positively regulates the expression of Lef1ΔN. Others have demonstrated that Lef/Tcf binding sites exist within p2; however, overexpressed β-catenin and Tcf only weakly activated p2 due to a repressor element within the promoter (Li et al., 2006). A database search for putative transcription factor binding sites within the p2 promoter revealed that cofactors such as AP-1, c-myc, CREB, and Sp1 may be recruited to the p2 promoter to regulate transcription of Lef1ΔN.

In summary, we describe the expression pattern and function of an alternative Lef1 isoform in osteoblasts. Lef1ΔN transcription and translation increases during osteoblast maturation and is induced by Runx2 and BMP2. Lef1ΔN overexpression increases osteocalcin expression, a measure of osteoblast maturation. This contrasts the effect of full-length Lef1, which represses Runx2-dependent osteocalcin expression. We conclude that Lef1ΔN is an induced regulator of osteoblast maturation.

Figure 1. Northern Blot analysis for Lef1 transcripts in differentiating MC3T3 osteoblasts.

A. Northern blot analysis of MC3T3 cells differentiated in ascorbic acid and β-glycerolphosphate for 0 to 15 days. Blots were incubated with probes recognizing the 3' region of the Lef1 gene, osteocalcin (Ocn) or cyclophilin (Cyp). The 3.6 and 2.3 kb bands are indicated by closed and open arrows, respectively.

B. Northern blot analysis of MC3T3 cells that had been differentiated for 9 days. Blots were incubated with probes recognizing the 5' or 3' regions of the Lef1 gene as indicated. (Credit: Rachel Kahler, Ph.D.)

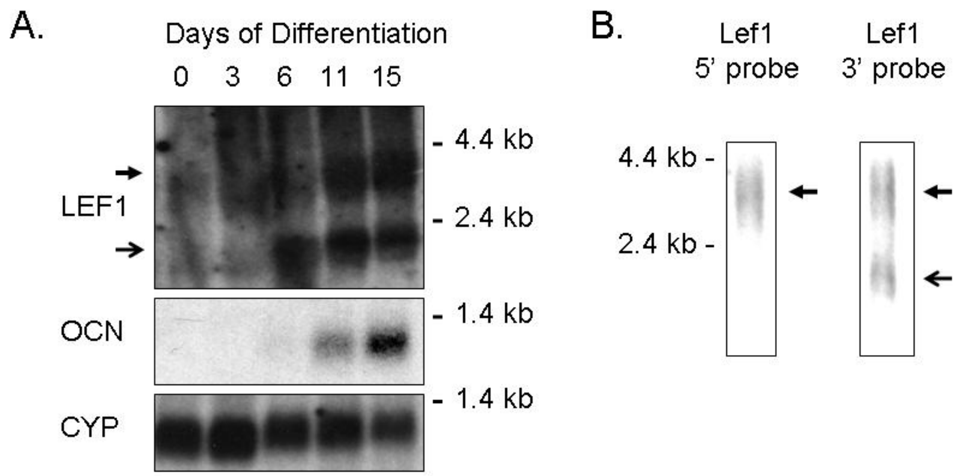

A.
Days of Differentiation
0 3 6 11 15
LEF1
OCN
CYP
4.4 kb
2.4 kb
1.4 kb
1.4 kb
B.
Lef1
5' probe
Lef1
3' probe
4.4 kb
2.4 kb

Figure 2. Detection of short Lef1ΔN transcripts by PCR in osteoblasts.

A. Schematic of the relative location of primer pairs for differential PCR. **B.** Results of differential quantitative RT-PCR assays. Primer pair 1 exclusively detects full-length Lef1, whereas primer pair 2 amplifies both full-length Lef1 and Lef1ΔN. To calculate the relative amount of Lef1ΔN present, yield of primer pair 1 product is subtracted from yield of primer pair 2 product. **C-D.** RNA was isolated from mouse primary calvarial cells after the indicated number of days in culture. Lef1ΔN was measured using differential quantitative real-time PCR (C), while osteocalcin was quantitated using standard gene-specific quantitative RT-PCR (D). Data were normalized to actin products. As in MC3T3 cells, Lef1ΔN transcripts appear immediately prior to osteocalcin transcripts.

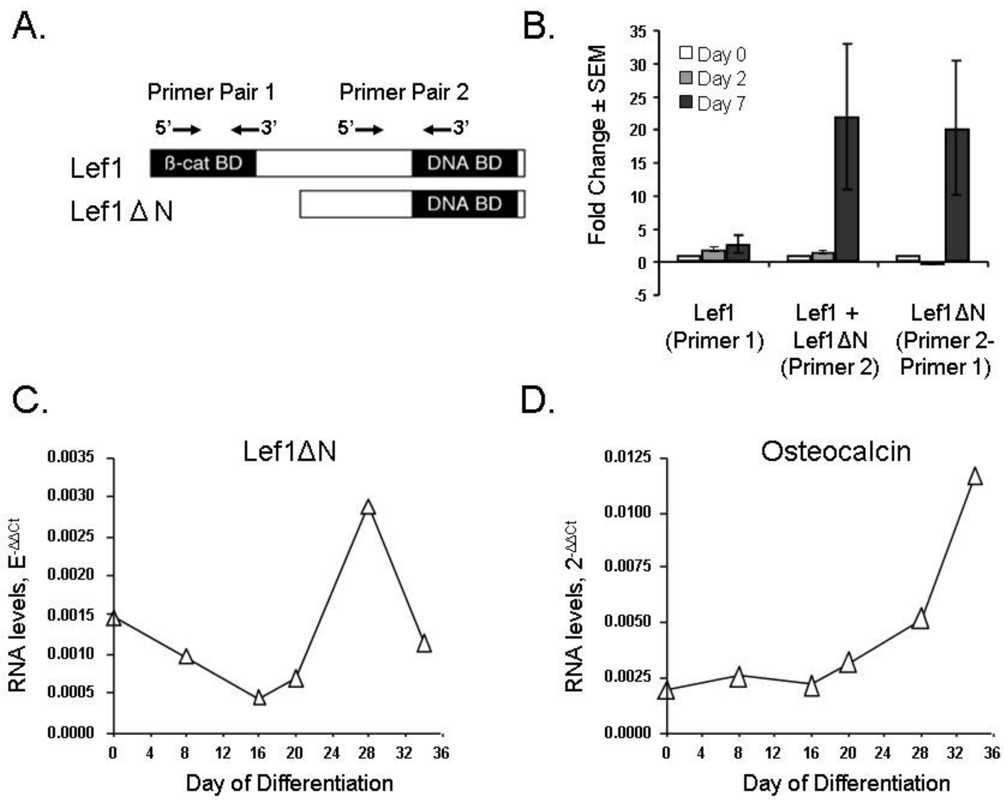

A.
Primer Pair 1
Primer Pair 2
5'
3'
5'
3'
Lef1
ß-cat BD
DNA BD
Lef1 Δ N
DNA BD
B.
Day 0
Day 2
Day 7
Fold Change ± SEM
35
30
25
20
15
10
5
0
-5
Lef1 (Primer 1)
Lef1 + Lef1ΔN (Primer 2)
Lef1ΔN (Primer 2- Primer 1)
C.
Lef1ΔN
RNA levels, E-ΔΔCt
0.0035
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
0 4 8 12 16 20 24 28 32 36
Day of Differentiation
D.
Osteocalcin
RNA levels, 2-ΔΔCt
0.0125
0.0100
0.0075
0.0050
0.0025
0.0000
0 4 8 12 16 20 24 28 32 36
Day of Differentiation

Figure 3. BMP2 and Wnt3a affect Lef1ΔN transcript expression. A-C. MC3T3 cells were differentiated in osteogenic media for 12 days in the presence of BMP2, Wnt3a, Wnt3a and BMP2, or endogenous cytokines. RNA was collected at regular intervals for quantitative PCR. All data were normalized to murine actin. Lef1ΔN transcript levels were determined by differential quantitative real-time PCR as described in Figure 2. **D.** MC3T3 cells were differentiated in osteogenic media containing BMP2 for 6 days. Nuclear protein lysates were collected at the indicated days, separated by SDS-PAGE electrophoresis, transferred to Immobilon-P membrane, probed with anti-Lef1, and reprobed with anti-Lamin B.

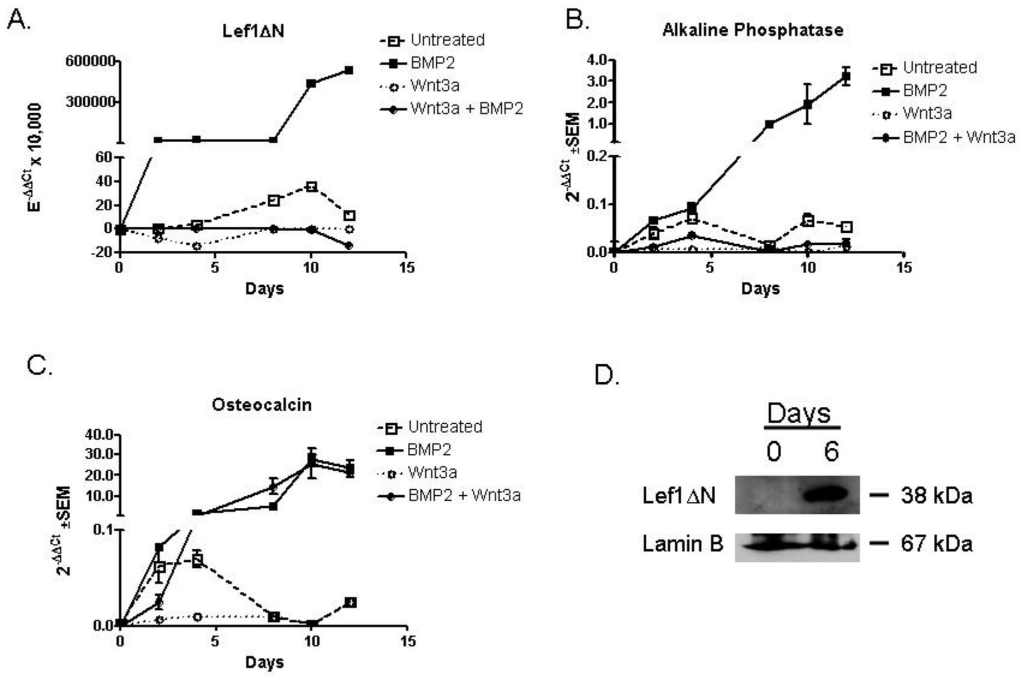
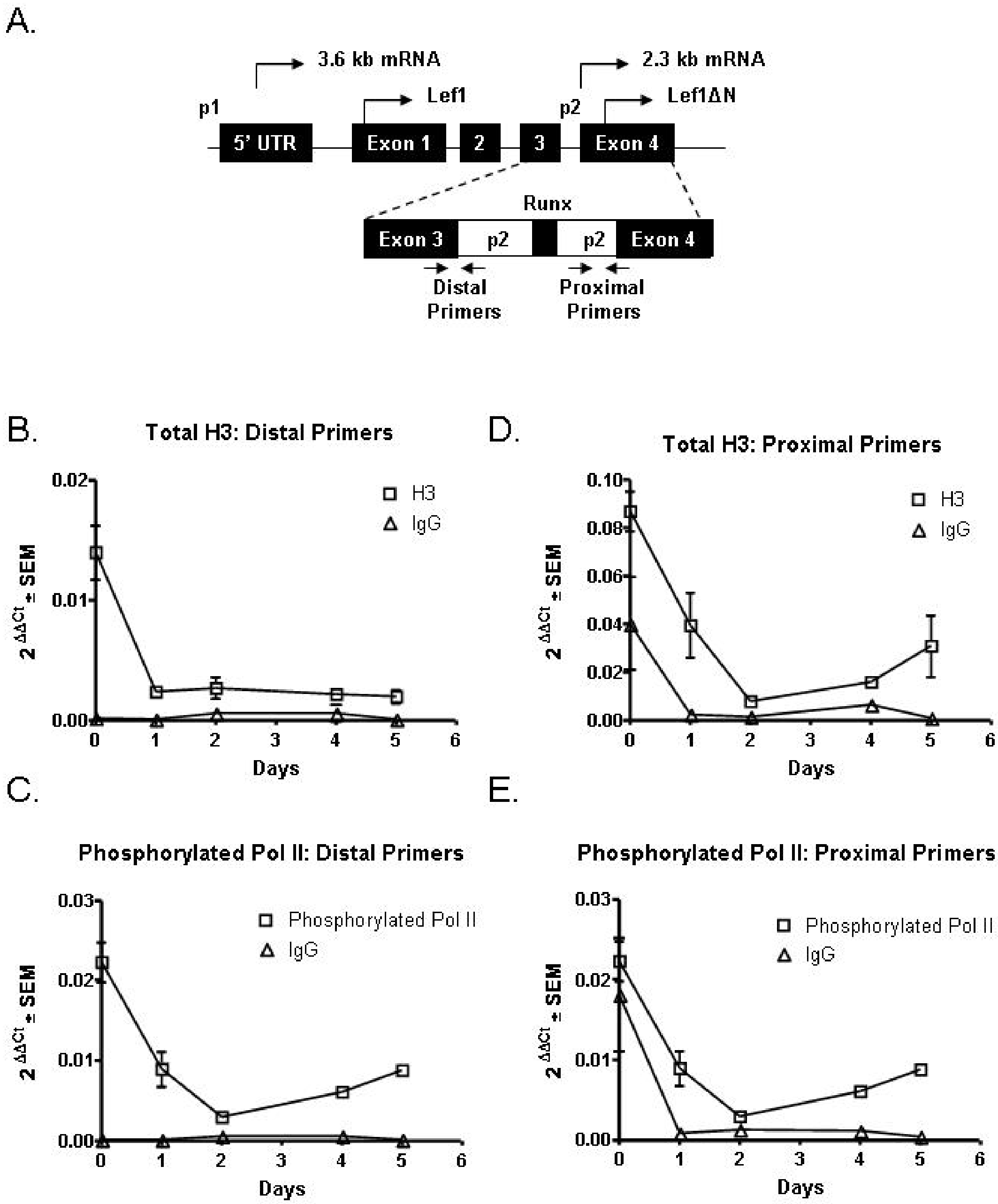

A.
Lef1ΔN
E-ΔΔCt x 10,000
Days
Untreated
BMP2
Wnt3a
Wnt3a + BMP2
B.
Alkaline Phosphatase
2-ΔΔCt ±SEM
Days
Untreated
BMP2
Wnt3a
BMP2 + Wnt3a
C.
Osteocalcin
2-ΔΔCt ±SEM
Days
Untreated
BMP2
Wnt3a
BMP2 + Wnt3a
D.
Days
0
6
Lef1ΔN
38 kDa
Lamin B
67 kDa

Figure 4. Identification of active elements in the murine p2 promoter. A. Schematic of the relative location of distal and proximal primers used for chromatin immunoprecipitation. **B-E.** Lysates were collected from differentiated MC3T3 cells, intracellular proteins and DNA were crosslinked and incubated with antibodies recognizing Histone H3, phosphorylated RNA polymerase II, or IgG. After reversing the crosslinks, DNA was purified and used for qPCR with the indicated primer pairs. Nonspecific background was eliminated in data by subtracting the relative amount of product detected in the control IgG immunoprecipitates from that detected in the H3 and RNA polymerase II immunoprecipitates. (Credit: Frank Secreto, Ph.D.)



A.
3.6 kb mRNA
2.3 kb mRNA
Lef1
Lef1ΔN
p1
p2
5' UTR
Exon 1
2
3
Exon 4
Runx
Exon 3
p2
p2
Exon 4
Distal Primers
Proximal Primers
B.
Total H3: Distal Primers
H3
IgG
2 ΔΔCt ± SEM
Days
D.
Total H3: Proximal Primers
H3
IgG
2 ΔΔCt ± SEM
Days
C.
Phosphorylated Pol II: Distal Primers
Phosphorylated Pol II
IgG
2 ΔΔCt ± SEM
Days
E.
Phosphorylated Pol II: Proximal Primers
Phosphorylated Pol II
IgG
2 ΔΔCt ± SEM
Days

Figure 5. Runx2 regulates the Lef1 p2 promoter. A. Schematic of the 5' portion of murine Lef1. Wildtype (wt) and a mutant (mt) p2 promoter wherein the Runx2 site is eliminated were cloned into the pGL2 luciferase plasmid and used in D-E. Transcriptional and translational start sites are indicated by the thin and thick arrows, respectively. **B-C.** Runx2 binds a consensus-binding element in the Lef1 p2 promoter as determined by EMSA (B) and chromatin immunoprecipitation (C). **D.** The p2 promoter (-447-+35) is active in C3H10T1/2 cells when placed in the forward (F) but not reverse (R) orientation. **E.** Runx2 activates the p2 promoter in a concentration-dependent manner in C3H10T1/2 cells. **F.** Mutating the Runx2 binding element prevents Runx2-dependent activation of the p2 promoter. **G.** Primary osteoblasts derived from wildtype (WT) and Runx2$^{-/-}$ mice were differentiated in osteogenic media for 34 days. RNA was collected for quantitative PCR. All data were normalized to murine actin. Lef1ΔN transcript levels were determined by differential quantitative real-time PCR as described in Figure 2. (Credit: Xiaodong Li, Eric Jensen, Ph.D.)

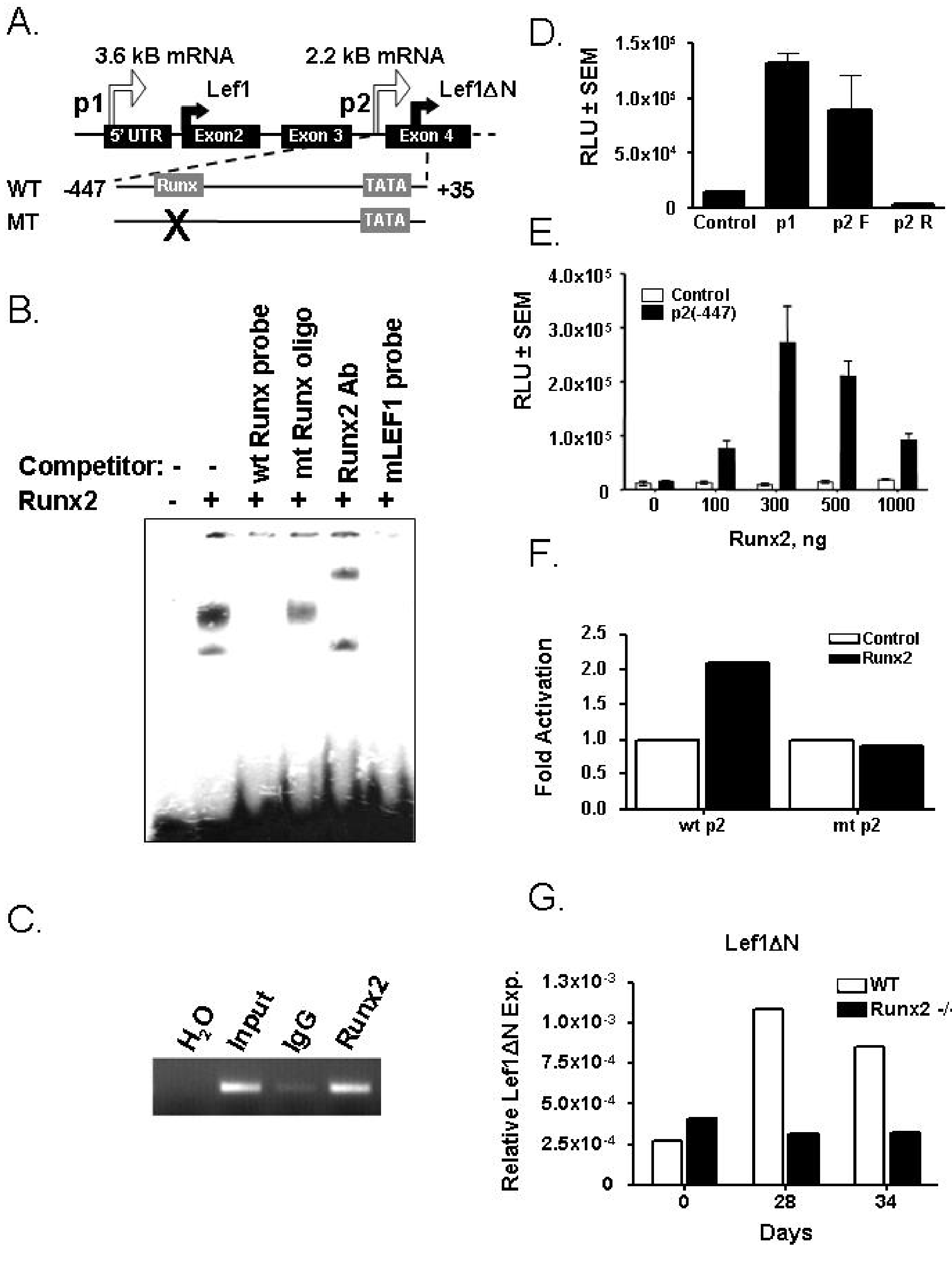
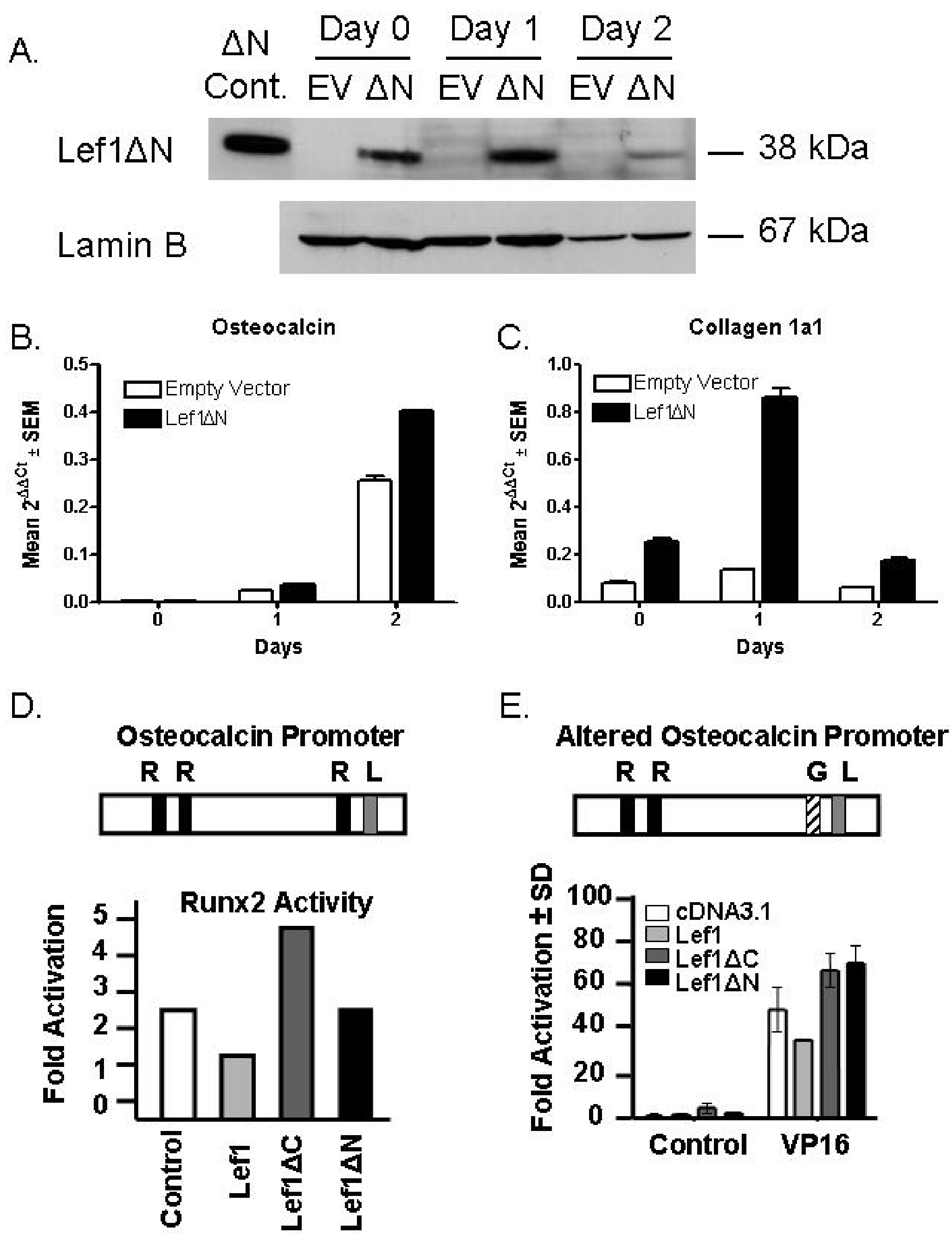

A.
3.6 kB mRNA
2.2 kB mRNA
p1
Lef1
p2
Lef1ΔN
5' UTR
Exon2
Exon 3
Exon 4
WT
-447
Runx
TATA
+35
MT
X
TATA
B.
Competitor: - -
+ wt Runx probe
+ mt Runx oligo
+ Runx2 Ab
+ mLEF1 probe
Runx2 - +
C.
H2O
Input
IgG
Runx2
D.
RLU ± SEM
1.5x10⁵
1.0x10⁵
5.0x10⁴
0
Control
p1
p2 F
p2 R
E.
RLU ± SEM
4.0x10⁵
3.0x10⁵
2.0x10⁵
1.0x10⁵
0
Control
p2(-447)
0
100
300
500
1000
Runx2, ng
F.
Fold Activation
2.5
2.0
1.5
1.0
0.5
0.0
Control
Runx2
wt p2
mt p2
G.
Lef1ΔN
Relative Lef1ΔN Exp.
1.3x10-3
1.0x10-3
7.5x10-4
5.0x10-4
2.5x10-4
0
WT
Runx2 -/-
0
28
34
Days

Figure 6: Overexpression of Lef1ΔN in C2C12 cells. A-C. C2C12 cells were transduced with retrovirus containing supernatants derived from 293T cells transfected with MSCV-Lef1ΔN or empty MSCV vector. Lef1ΔN and empty vector (EV) cells were differentiated in osteogenic medium in the presence of BMP2. **A.** Protein lysates were collected after the indicated days in culture, separated by SDS-PAGE electrophoresis, transferred to Immobilon-P membrane, probed with anti-Lef1, and reprobed with anti-Lamin B. **B-C.** RNA was collected at regular intervals for quantitative PCR. All data were normalized to murine actin. **D-E.** Schematics of the osteocalcin promoters show the location of Runx2 (R), Lef1 (L), and GAL4 (G) binding elements. C2C12 cells were transfected with luciferase reporter plasmids driven by osteocalcin promoters, Runx2 (D) or VP16 (E), and pcDNA3.1 (control), Lef1, Lef1ΔC, or Lef1ΔN as indicated. The proximal Runx2 binding element in the osteocalcin promoter was mutated to a GAL4 binding site (E). Luciferase activity was measured 48 hours after transfection.


A.
ΔN Cont.
Day 0
Day 1
Day 2
EV ΔN EV ΔN EV ΔN
Lef1ΔN
— 38 kDa
Lamin B
— 67 kDa
B.
Osteocalcin
Empty Vector
Lef1ΔN
Mean 2^ΔΔCt ± SEM
Days
C.
Collagen 1a1
Empty Vector
Lef1ΔN
Mean 2^ΔΔCt ± SEM
Days
D.
Osteocalcin Promoter
R R R L
Runx2 Activity
Fold Activation
Control
Lef1
Lef1ΔC
Lef1ΔN
E.
Altered Osteocalcin Promoter
R R G L
Fold Activation ± SD
cDNA3.1
Lef1
Lef1ΔC
Lef1ΔN
Control
VP16

Chapter 3

Lef1ΔN Binds β-catenin via a Domain Necessary for Osteoblast Differentiation

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Lymphoid Enhancer Binding Factor (Lef) 1 is a transcriptional effector of the Wnt/Lrp5/6/β-catenin signaling pathway and regulates osteoblast differentiation and bone formation. We recently showed that a naturally-occurring, truncated Lef1 isoform, Lef1ΔN, is induced during osteoblast maturation and enhances osteoblast differentiation *in vitro*. Lef1ΔN lacks the first 113 amino acids of Lef1 and a well-characterized β-catenin binding domain. In this study, we demonstrate that despite the absence of the N-terminal β-catenin binding domain, Lef1ΔN retains the ability to physically and functionally interact with β-catenin in osseous cells. In contrast to its ability to competitively inhibit Lef1-driven gene expression in T lymphocytes, Lef1ΔN activated a Wnt-responsive promoter in the absence of exogenous β-catenin and cooperated with constitutively active β-catenin to further stimulate transcription in mesenchymal and osteoblastic cells. An element within first 61 amino acids of Lef1ΔN was required to enhance transcription. These 61 residues were also necessary for β-catenin binding and enhancing expression of osteocalcin and type 1 collagen during osteoblast differentiation. Thus, Lef1ΔN retains the ability to bind β-catenin during terminal osteoblast differentiation.

Introduction

The canonical Wnt signaling pathway has emerged as an important regulator of bone formation and regeneration (Bonewald and Johnson, 2008; Hoeppner et al., 2009b; Khosla et al., 2008). Activation of canonical Wnt signaling is initiated upon binding of Wnt ligands to receptor complexes comprised of Frizzled and Lrp5/6 proteins, resulting in β-catenin translocation to the nucleus and subsequent displacement of transcriptional co-repressors (i.e. CtBP, TLE, and histone deacetylases) from Tcf7 and Lef1 transcription factors (Billin et al., 2000; Brannon et al., 1999; Brantjes et al., 2001; Levanon et al., 1998). Tcf7 and Lef1 recruit co-activators (i.e. p300, Bcl9, and Pygopus) (Behrens et al., 1996; Billin et al., 2000; Daniels and Weis, 2005; Hecht et al., 2000; Hsu et al., 1998; Huber et al., 1996; Kramps et al., 2002; Miyagishi et al., 2000; Sun et al., 2000; Tutter et al., 2001) leading to chromatin relaxation and the transcription of genes involved proliferation, including cyclin D and c-myc (Westendorf et al., 2004). However, in some contexts, Wnt signaling and β-catenin facilitate Lef1/Tcf7-mediated repression through poorly understood mechanisms (Baker et al., 1999; Cadigan et al., 1998; Cadigan et al., 2002; Jamora et al., 2003; Kahler and Westendorf, 2003; Payre et al., 1999; Piepenburg et al., 2000; Willert et al., 2002; Yang et al., 2000). In the absence of nuclear β-catenin, Lef1/Tcf7 remains associated with transcriptional co-repressors and weakly interacts with chromatin (Tutter et al., 2001). Genetic studies indicate that Lef1 and Tcf7 play different roles in bone cells. *Tcf7*-deficient animals are viable, but have low bone mass caused by decreased OPG expression and increased osteoclastogenesis (Glass et al., 2005). This bone loss is enhanced when *Tcf7*-null mice are crossed with *β-catenin*-deficient mice (Glass et al., 2005). *Lef1* deficiency is postnatally lethal (van Genderen et al., 1994); however, *Lef1* haploinsufficiency decreased trabecular bone mass in female mice by reducing osteoblast activity (Noh et al., 2009). Animals lacking both *Lef1* and *Tcf7* most resemble *Wnt3a*$^{-/-}$ mice (Galceran et al., 1999). Thus, there is strong evidence that Lef1 and Tcf7 act in concert during development to mediate canonical Wnt3a signaling.

Lef1 contains a well-characterized N-terminal β-catenin binding domain and a C-terminal high mobility group (HMG) domain that associates with DNA. The DNA binding domain interacts one of the most common motifs in human promoters, CTTTGT (Xie et al., 2005), and is a context-dependent regulator of gene expression. A context-dependent activation (CAD) domain mediates interaction with co-factors, such as Groucho (Arce et al., 2009) and PITX2 (Amen et al., 2007), and is located between the β-catenin binding domain and the DNA binding domain (Giese and Grosschedl, 1993). Lef1ΔN is a naturally occurring Lef1 isoform that is transcribed from a promoter (p2) within the intron between exons 3 and 4 in mice (exons 2 and 3 in humans) and is induced by Runx2 and Bmp2 (Arce et al., 2006; Hovanes et al., 2001). The 2.3 kb transcript of Lef1ΔN lacks the first 113 amino acids of Lef1, the well characterized β-catenin binding domain, and a portion the of CAD domain, but retains the DNA binding motif and the nuclear localization signal.

Lef1/Tcf expression is usually restricted to mitotic cells in developing or renewable tissues (Mayer et al., 1997; Porfiri et al., 1997), including proliferating osteoblasts and bone (de Jong et al., 2002; Hadjiargyrou et al., 2002; Kahler and Westendorf, 2003; Kato et al., 2002; Qi et al., 2003). Lef1/Tcf7 activity is increased in areas of bone remodeling and regeneration (reviewed in (Hoeppner et al., 2009b; Secreto et al., 2009)). As cells stop proliferating, Lef1/Tcf expression is typically downregulated and is lost as cells become terminally differentiated (Hadjiargyrou et al., 2002; Kratochwil et al., 1996; Mariadason et al., 2001; Oosterwegel et al., 1993; Shibamoto et al., 2004; Travis et al., 1991; Zhou et al., 1995). Accordingly, we demonstrated that Lef1 protein expression decreases during osteoblast differentiation (Kahler et al., 2006) and Lef1ΔN is induced during late osteoblast differentiation (Hoeppner et al., 2009a). Differential expression of the Lef1 full-length and ΔN transcripts was also reported in lymphocytes, myeloid cells, tumors, skin and the intestine (Merrill et al., 2001; Niemann et al., 2002; Skokowa et al., 2006; Takeda et al., 2006; Wang et al., 2005). Lef1ΔN is an example of the functional diversity the four Lef1/Tcf7 genes generate through the use of differential promoters and alternative splicing (Hovanes et al., 2001; Hovanes et al., 2000; Van de Wetering et al., 1996). Likewise, a Tcf7 isoform is transcribed from an alternative promoter in the second intron that produces a truncated mRNA lacking the coding sequences for the β-catenin binding domain (Van de Wetering et al., 1996). Lef1ΔN and Tcf7ΔN were shown to act as competitive inhibitors of their full-length counterparts, and since β-catenin/Lef1/Tcf7 complexes drive cell proliferation, ΔN isoforms may modulate their proliferation-promoting activities (Arce et al., 2006; Hovanes et al., 2001; Weerkamp et al., 2006). Previous work from our lab suggests Lef1ΔN is a crucial regulator of terminal differentiation in osseous cells (Hoeppner et al., 2009a). Given that Lef1ΔN was found to be a competitive inhibitor of the full-length isoform that drives proliferation in other cell types, we sought to determine how Lef1ΔN induces terminal differentiation in osseous cells during the late stages of differentiation. Interestingly, Lef1ΔN does not act as a competitive inhibitor of β-catenin-mediated activation of a Lef1-responsive promoter, but rather enhances activation in osteogenic cells. Our results suggest Lef1ΔN interacts with β-catenin through a second β-catenin binding domain. In this report, we functionally define this second β-catenin binding domain in osseous cells.

Materials and Methods

Cell lines and osteoblast differentiation. C2C12 and Cos-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 200 mM L-glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin. Jurkat cells were cultured in RPMI-1640 medium containing the aforementioned supplements. C3H10T1/2 and MC3T3-E1 cells were cultured in Minimal Essential Medium (MEM) containing 1x nonessential amino acids and the supplements listed above. To induce osteogenic differentiation, confluent C2C12 cell cultures were incubated in DMEM supplemented with 10% FBS, 50 μg/ml ascorbic acid,10 mM β-glycerolphosphate, and 300 ng/ml BMP2 (Sofamor Danek) for the indicated number of days.

RNA isolation and reverse transcriptase-PCR. Total RNA was harvested from cells using Trizol Reagent (Invitrogen). Quantitative RT-PCR was performed using the QuantiTech SYBR Green RT-PCR kit (Qiagen) and reactions were run on an iCycler (BioRad). Thus, RNA (10 ng) was added to 20 μl reactions with QuantiTech SYBR Green RT mastermix, QuantiTech RT mix, and 0.5 pmol/μl of each of the primers for murine osteocalcin (forward: AAG CAG GAG GGC AAT AAG GT, reverse: TTT GTA GGC GGT CTT CAA GC), murine type I collagen (forward: GAA ACC CGA GGT ATG CTT GA, reverse: GGG TCC CTC GAC TCC TAC AT), or actin (forward: AAG GAA GGC TGG AAA AGA GC, reverse: GCT ACA GCT TCA CCA CCA CA). Within this mix, the reverse transcriptase reaction to convert the RNA to cDNA was performed at 50°C for 30 minutes. Initial denaturation was made at 95°C for 15 minutes followed by 40 cycles of three-step PCR: 95°C denaturation for 1 minute, 57°C annealing for 1 minute, and 72°C elongation for 1 minute. Data from osteocalcin and type 1 collagen reactions were normalized to mouse actin levels as described previously (Pfaffl, 2001).

Construction of Lef1ΔN Deletion Plasmids. Expression plasmids producing truncated Lef1 proteins (Lef1 (128-397), (150-397), (175-397), (252-397), and (282-397)) were generated by PCR from pCMV-Lef1ΔN. Restriction sites and an ATG start codon were introduced using PCR. The resulting amplicons were digested with KpnI and EcoRI and ligated into a pCMV-Flag plasmid. Note that the residue numbers coincide with amino acids within Lef1. Within this number scheme, Lef1ΔN is Lef1(114-397).

Transcription assays. Cells were transfected with Lipofectamine (Invitrogen) in 12-well plates with the indicated amounts of luciferase reporter plasmids and 10 ng pRL-null. Lef1ΔN and constitutively active β-catenin expression plasmids (300 ng unless otherwise specified) were added as indicated. pcDNA3.1 was added to transfections to maintain a uniform amount of total DNA per transfection. Luciferase activity was measured 24 or 48 hours after transfection using the dual-luciferase assay system (Promega). Each transfection was performed in triplicate, and firefly luciferase activity was normalized to renilla-luciferase activity.

Retroviral subcloning and transduction. To produce retroviruses, HEK 293T cells were co-transfected with 5 μg of pCL2 and 5 μg of pMSCV-puro-Lef1ΔN, pMSCV-puro-Lef1 (175-397), pMSCV-puro-Lef1 (252-397), or empty pMSCV-puro vector using calcium phosphate precipitation. Virus-containing supernatants were collected after 48 and 72 hours, filtered, and added to C2C12 cells in the presence of 8 μg/ml polybrene. The transduction was repeated 8 hours and 24 hours later. Transduced cells were selected with 5 μg/ml puromycin for a minimum of 3 days.

Immunoblotting and immunoprecipitation. Immunoblotting was performed as previously described (Hoeppner et al., 2009a) using 1:1000 dilutions of Lef1 (clone C18A7, Cell Signaling) and Lamin B (Santa Cruz Biotechnology) antibodies. For immunoprecipitations, transfected Cos-7 cells were harvested in lysis buffer (50 mM Tris HCl, 75 mM NaCl, 1 mM EDTA, 0.25% Triton X, 1 mM DTT, and 10% glycerol) containing protease inhibitors (Roche). Equal fractions of the lysates were incubated with 1 μg of Lef1 antibody (clone C18A7, Cell Signaling) overnight at 4°C. The immune complexes were collected with Protein G Dynabeads (Invitrogen, 100.03D). Proteins were resolved by SDS-PAGE, transferred to PVDF membrane (Immobilon-P, Millipore) and immunoblotted with Myc-tag antibody (clone 9B11, Cell Signaling) to detect constitutively active β-catenin. Correspondingly whole cell lystate fractions were immunoblotted as described (Hoeppner et al., 2009a) with anti-Myc and anti-Lef1 to detect overall constitutively active β-catenin and Lef1 protein expression.

Results

We previously demonstrated that Lef1 protein expression declines during the course of osteoblast differentiation (Kahler et al., 2006), while the shorter isoform, Lef1ΔN, is induced during late osteoblast differentiation (Hoeppner et al., 2009a). Stable expression of Lef1ΔN in preosteoblasts accelerated the expression of genes involved in late osteoblast differentiation and suggested Lef1ΔN contributes to the terminal differentiation progression of osseous cells (Hoeppner et al., 2009a). Lef1ΔN lacks the well defined β-catenin domain present in the full-length isoform of Lef1 (Hovanes et al., 2001), and therefore, mechanistically Lef1ΔN may induce terminal differentiation by acting as an antagonist of β-catenin-mediated expression of genes involved in osteoblast proliferation. In support of this mechanism, overexpression of Lef1ΔN repressed constitutively active β-catenin-induced activation of a Lef1-responsive promoter in human T lymphocyte Jurkat cells (Figure 1A); a result that is consistent with previous reports (Hovanes et al., 2001). We hypothesized that Lef1ΔN might also repress β-catenin-dependent transcriptional activation in osseous cells as a means preventing cell proliferation and inducing terminal differentiation. To test this hypothesis, we transiently transfected MC3T3, C3H10T1/2, and C2C12 cells with increasing amounts of Lef1ΔN in the absence or presence of constitutively active β-catenin and the Lef1-responsive reporter, Top-Flash. Surprisingly, Lef1ΔN enhanced the activation of Top-Flash and cooperated with constitutively active β-catenin to further enhance reporter expression in every osteoblast cell line evaluated (Figure 1B-D).

In 2005, Daniels and Weis reported a novel second β-catenin binding domain within Lef1 in a region retained by the Lef1ΔN isoform and N-terminal to the DNA-binding domain; however, the functional action of this domain was not described (Daniels and Weis, 2005). We hypothesized that Lef1ΔN enhances activation of a Lef1-responsive promoter by interacting with β-catenin through this second β-catenin binding domain in osteoblasts. To test this hypothesis, we transfected C2C12 cells with Lef1, Lef1ΔN or Lef1 (282-397), which contains the DNA binding domain and nuclear localization signal (Figure 2A). As expected, Lef1 and β-catenin were potent co-stimulators of transcription (Figure 2B). Lef1ΔN also cooperated with β-catenin to synergistically activate the Top-Flash reporter. Although Lef1ΔN was not as effective as Lef1, it still augmented transcription approximately 200-fold in the presence of constitutively active β-catenin. However, Lef1 (282-397) was unable to activate the Lef1-responsive promoter, and modestly repressed basal activity in the presence of β-catenin (Figure 2B). These data suggest that Lef1 residues 114 through 282 were necessary for β-catenin responsiveness of Lef1ΔN.

To determine if Lef1ΔN physically interacts with β-catenin, immunoprecipitations were performed. Cos-7 cells were transiently co-transfected with constitutively active, myc-tagged β-catenin and full-length Lef1 or Lef1ΔN. As expected, both Lef1 and Lef1ΔN interacted with β-catenin (Figure 2C, lanes 1 & 2). To define the region of Lef1ΔN that contains the second β-catenin binding domain, we created a series of Lef1ΔN deletion constructs by progressively truncating Lef1ΔN from the N-terminus until only the DNA binding domain (Lef1 (282-397)) remained (Figure 2A). Lef1 (128-397) and (150-397) associated with constitutively active β-catenin, whereas Lef1 (175-397) and (252-397) did not (Figure 2C). These results suggest that Lef1 residues 150 to 175 are necessary for Lef1ΔN to interact with β-catenin.

We next asked whether Lef1 (150-175) was necessary for β-catenin-mediated activation of Top-Flash. As expected, Lef1 (114-397) enhanced reporter expression and augmented β-catenin-dependent activation of Top-Flash (Figure 3A). Lef1 (150-397) also stimulated gene transcription and cooperated with β-catenin to further enhance reporter expression. However, Lef1 (175-397) and Lef1 (252-397) had modest effects on basal Top-Flash activity and failed to significantly augment Top-Flash activation beyond that observed with β-catenin alone (Figure 3A). To determine if Lef1 (175-397) and Lef1 (252-397) were competitive inhibitors of β-catenin-mediated activation of the Lef1-responsive promoter, we transfected cells with increasing amounts (100, 300 or 600 ng) of Lef1 (175-397) or Lef1 (252-397). Similar to results shown in Figure 1, Lef1ΔN activated Top-Flash in a concentration dependent manner. Neither Lef1 (175-397) nor Lef1 (252-397) augmented β-catenin activation; rather both repressed β-catenin-dependent activation of Top-Flash, with Lef1 (252-397) acting as a potent competitive inhibitor at high concentrations (Figure 3B). These results were similar to those observed with Lef1 (282-397) (Figure 2B).

We previously demonstrated that stable expression of Lef1ΔN in osteoprogenitors accelerated expression of osteocalcin and type I collagen, genes associated with osteoblast differentiation (Hoeppner et al., 2009a). To determine if the β-catenin binding region of Lef1ΔN contributed to this phenotype, we stably transduced C2C12 cells (a myo-osteoblast progenitor cell line) with Lef1ΔN, Lef1 (175-397), Lef1 (252-397) or a corresponding empty vector. The stably transduced cells were differentiated in osteogenic medium for seven days. All Lef1 proteins were produced in the C2C12 cells before differentiation was induced (Figure 4A) and expression remained throughout the 7 day culture period (data not shown). While Lef1ΔN enhanced osteocalcin and type I collagen expression as previously reported (Hoeppner et al., 2009a), Lef1 (175-397) and Lef1 (252-397) failed to induce their expression and in fact repressed expression of these late osteoblast differentiation genes below that observed in control cells transduced with the empty retroviral vector (Figure 4B-C). Together, our results suggest that Lef1ΔN induces osteocalcin and type I collagen expression by interacting with β-catenin through a second β-catenin binding located between residues 150 and 175 of Lef1.

Discussion

Lef1ΔN and Lef1 transcription factors play distinct functional roles in many cell types and tend to be temporally expressed. We observed that the full-length Lef1 protein is expressed early and declines as osteoblast differentiation proceeds (Kahler et al., 2006), whereas the ΔN isoform is undetectable in proliferating cells, but is expressed during late osteoblast differentiation (Hoeppner et al., 2009a). We sought to determine how Lef1ΔN induces terminal differentiation in osseous cells during the late stages of differentiation. Here, we report that Lef1ΔN interacts with β-catenin through a second β-catenin binding domain resulting in the expression of genes involved in osteoblast terminal differentiation. We physically and functionally mapped the second β-catenin binding domain to amino acids 114-175 of Lef1 through immunoprecipitation and transcription assays (Figures 2 and 3). Correspondingly, we found that overexpression of Lef1ΔN (114-397) in differentiating osteoblasts induced genes (i.e. type I collagen and osteocalcin) involved in terminal differentiation, whereas Lef1 175-397 and Lef1 252-397 failed to induce expression of these genes (Figure 5B-C). Lef1 (150-397) interacted with β-catenin and activated Top-Flash, while conversely, Lef1 (175-397) and larger N-terminal truncations did not. Therefore, our data indicate that the 150-175 region of Lef1 is the minimum element required to interact with β-catenin.

Lef1 and *Tcf7* genes use alterative promoters within introns to transcribe ΔN isoforms, which lack the primary β-catenin binding domain (Van de Wetering et al., 1996). Differential expression of the full-length and Lef1ΔN isoforms has been reported in tumors, the intestine, lymphocytes, skin, and myeloid cells (Hovanes et al., 2001; Li et al., 2006; Merrill et al., 2001; Niemann et al., 2002; Skokowa et al., 2006; Takeda et al., 2006). Full-length Lef1 is aberrantly expressed in 80% of colon tumors, and interestingly, the Lef1ΔN promoter (p2) is completely silenced in these same tumors (Hovanes et al., 2001; Li et al., 2006). Both Lef1 isoforms are high in resting T lymphocytes, but the short isoform is downregulated upon activation and proliferation (Willinger et al., 2006). In murine epidermal stem cells, Lef1ΔN suppresses hair cell differentiation, but stimulates sebocyte differentiation and spontaneous skin tumors (Merrill et al., 2001; Niemann et al., 2002). Furthermore, inactivating point mutations in the β-catenin binding domain of Lef1 occur in human sebaceous tumors and basically create a ΔN-like competitive inhibitor, which represses Wnt target gene expression and promotes differentiation (Takeda et al., 2006). Finally, Lef1ΔN is sufficient to rescue the differentiation block induced by *Lef1*-deficiency in granulocytes (Skokowa et al., 2006). Thus, Lef1ΔN generally acts as an inhibitor of proliferative signals emanating from the canonical Wnt pathway and promotes differentiation. In this report, we demonstrate that Lef1ΔN is not a competitive inhibitor of β-catenin-dependent transcription, but retains the ability to bind β-catenin and induce terminal differentiation of osteoblasts (Figure 5).

The second β-catenin binding domain that enables Lef1ΔN to bind β-catenin was first described by Daniels and Weis in 2005 (Daniels and Weis, 2005). Their work demonstrated that β-catenin displaced the repressive element Groucho/TLE from Lef1 by binding to this novel, second β-catenin binding site

(Daniels and Weis, 2005). Our results indicate that the alterative β-catenin binding site is within a region encompassing residues 150 and 175 of Lef1 and in osteoblasts interacts with β-catenin to enhance activation Lef1-responsive promoters thereby inducing genes associated with terminal differentiation. Daniels and Weis (Daniels and Weis, 2005) suggested a specific interaction with the DNA binding domain of Lef1 is not required for β-catenin to bind Lef1ΔN, which corresponds with our functional data showing the DNA-binding domain of Lef1 does not augment β-catenin-mediated activation of a Lef1-responsive promoter in osteogenic cells (Figure 2B). This was the first piece of evidence indicating that the alternative β-catenin binding domain exists between the beginning of Lef1ΔN and the DNA-binding domain. However, Daniels and Weis reported that β-catenin bound to Lef1 (252-397) in GST affinity purification experiments in the presence of DNA (Daniels and Weis, 2005). While TLE-GST bound to Lef1 (252-397)-DNA in the micromolar range of concentrations, β-catenin-GST failed to bind and only bound Lef1 (252-397)-DNA at millimolar concentrations (Daniels and Weis, 2005). Furthermore, deletion constructs containing Lef1 amino acid sequence N-terminal to Lef1 (252-397) were not tested in the affinity purification assays or in functional experiments. Our studies were conducted in osteogenic cells where adapter molecules and cofactors are available, whereas the GST pulldown system used purified proteins. Lef1ΔN retains a large portion of the CAD domain, which may be sufficient to interact with important cofactors, such as Groucho (Arce et al., 2009) and PITX2 (Amen et al., 2007). In osteoblasts and T lymphocytes, Lef1ΔN in the presence β-catenin has opposite effects on a Lef1-responsive promoter ((Hovanes et al., 2001), Figure 1), which suggests cell-type specific factors may contribute to Lef1ΔN/β-catenin interactions and effects. Finally, we provide physical and functional evidence that β-catenin interacts with an element within the first 61 amino acids of Lef1ΔN, whereas Daniels and Weis (Daniels and Weis, 2005) define the alternative β-catenin binding domain solely through biochemical association studies with purified proteins.

We showed that an element within the first 61 amino acids of Lef1ΔN is required to enhance the expression of osteocalcin and type 1 collagen during terminal differentiation in osseous cells. Our previous work indicates that Lef1ΔN becomes expressed after about 6 to 7 days of osteoblast differentiation in MC3T3 cells and just prior to the onset of osteocalcin expression (Hoeppner et al., 2009a). Together, our results suggest that Lef1ΔN expression helps induce terminal differentiation in proliferating osteoblasts. To date, the effect of Lef1ΔN overexpression or deletion has not been directly studied *in vivo*. In both the *Lef1*-null and *Lef1*$^{+/-}$ mice, a C-terminal Lef1 exon was disrupted; therefore, both Lef1 and the shorter Lef1ΔN isoform were ablated. *Lef1* haploinsufficiency decreased trabecular bone mass in female mice by reducing osteoblast activity. We recently created Lef1ΔN transgenic mice that express Lef1ΔN in committed osteoblasts using the 2.3 kB fragment of the type 1 collagen promoter. These mice exhibited increased bone formation, mineral apposition, and osteocalcin expression (Secreto, unpublished data), which supports the notion that Lef1ΔN plays a role in osteoblast terminal differentiation.

In this study, we aimed to answer how Lef1ΔN induces terminal differentiation in osseous cells. We found Lef1ΔN enhances β-catenin-mediated activation of a Lef1-responsive promoter in osseous cells, despite observations that Lef1ΔN has the opposite effect in other cell types (Figure 1; (Hovanes et al., 2001)). We used truncated Lef1ΔN proteins in transcription assays and immmunoprecipitations to define the 150-175 region of Lef1 as the minimum element required to interact with β-catenin, although it is possible that other regions of Lef1ΔN come in contact with β-catenin during this interaction. Taken together, we conclude that Lef1ΔN physically and functionally interacts with β-catenin in mature osteoblasts and this interaction regulates the transcription of osteoblast differentiation genes.

Figure 1: Lef1ΔN enhances β-catenin-mediated activation in osteogenic cells, but represses activation in T lymphocytes. A-D. Jurkat (A), MC3T3 (B), CH310T1/2 (C), and C2C12 (D) cells were transfected with the Lef1 luciferase reporter plasmid Top Flash (200 ng), Lef1ΔN as indicated, constitutively active β-catenin (300 ng) as indicated, and pcDNA3.1 (control). Luciferase activity was measured 24 h after transfection. Firefly luciferase values were normalized to renilla controls. Reactions were performed in triplicate and data are representative of at least three experiments. The single asterisk indicates p values <0.05 and the double asterisk indicates p values <0.01.

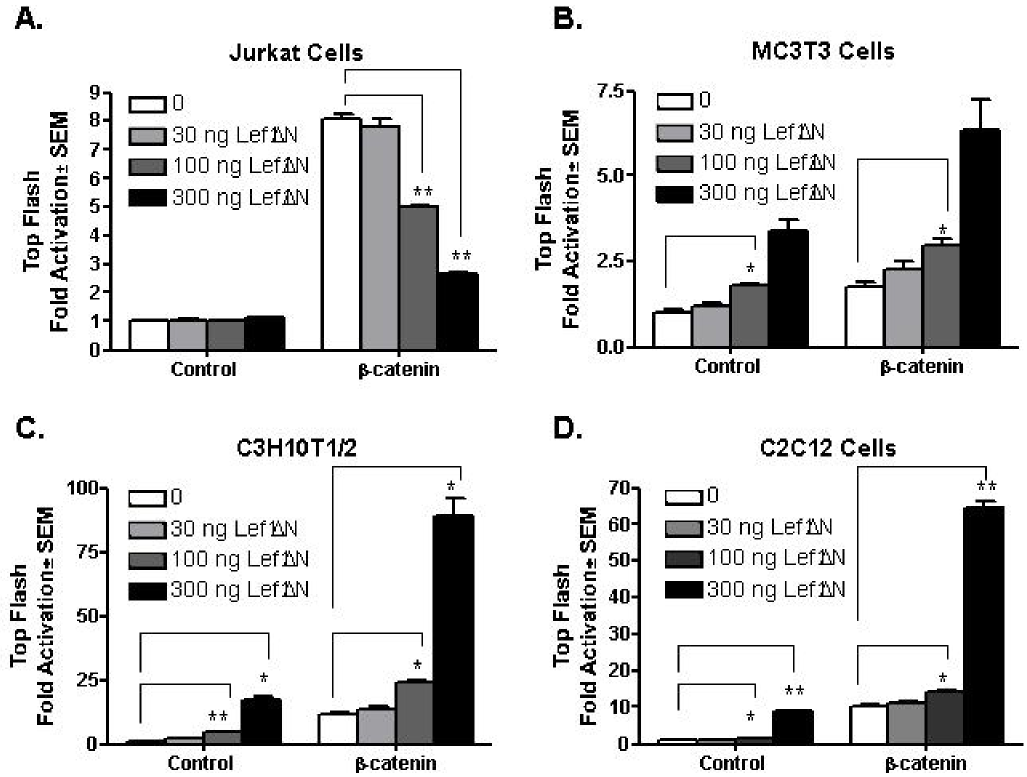

A.
Jurkat Cells
Top Flash
Fold Activation± SEM
0
30 ng Lef1ΔN
100 ng Lef1ΔN
300 ng Lef1ΔN
**
**
Control
β-catenin
B.
MC3T3 Cells
Top Flash
Fold Activation± SEM
0
30 ng Lef1ΔN
100 ng Lef1ΔN
300 ng Lef1ΔN
*
*
Control
β-catenin
C.
C3H10T1/2
Top Flash
Fold Activation± SEM
0
30 ng Lef1ΔN
100 ng Lef1ΔN
300 ng Lef1ΔN
**
*
*
*
Control
β-catenin
D.
C2C12 Cells
Top Flash
Fold Activation± SEM
0
30 ng Lef1ΔN
100 ng Lef1ΔN
300 ng Lef1ΔN
*
**
*
**
Control
β-catenin

Figure 2: Amino acids 114 to 175 of Lef1 interact with β-catenin. A. The Lef1 constructs used with amino acids in parentheses and a summary indicating whether each Lef1 construct enhanced β-catenin-mediated activation of Top Flash and interacted with β-catenin through immunoprecipitation. Note that the residue numbers coincide with amino acids within Lef1. Within this number scheme, Lef1ΔN is Lef1(114-397). ND indicates that a particular construct was not tested in the indicated assay. **B.** C2C12 cells were transfected with Top-Flash (200 ng), constitutively active β-catenin (300 ng) as indicated, pcDNA3.1 (control) and full-length Lef1 (300 ng), Lef1ΔN (300 ng), or Lef1 DNA binding domain (300 ng). Luciferase activity was measured 24 h after transfection. Firefly luciferase values were normalized to renilla controls. Reactions were performed in triplicate and data are representative of at least three experiments. **C.** β-catenin immunoprecipitates with full-length Lef1 (positive control), Lef1ΔN (114-397), Lef1 (128-397), and Lef1 (150-397), but not with Lef1 (175-397) and Lef1 (252-397). Cos-7 cells were transfected with myc-tagged constitutively active β-catenin and each of the Lef1 constructs indicated. Whole cell lysates (WCL) were immunoprecipitated with anti-Lef1. Proteins were transferred to membranes and detected by immunoblotting with anti-myc. WCL fractions were immunoblotted with anti-myc or anti-Lef1 to demonstrate expression levels of β-catenin and the various Lef1 constructs.

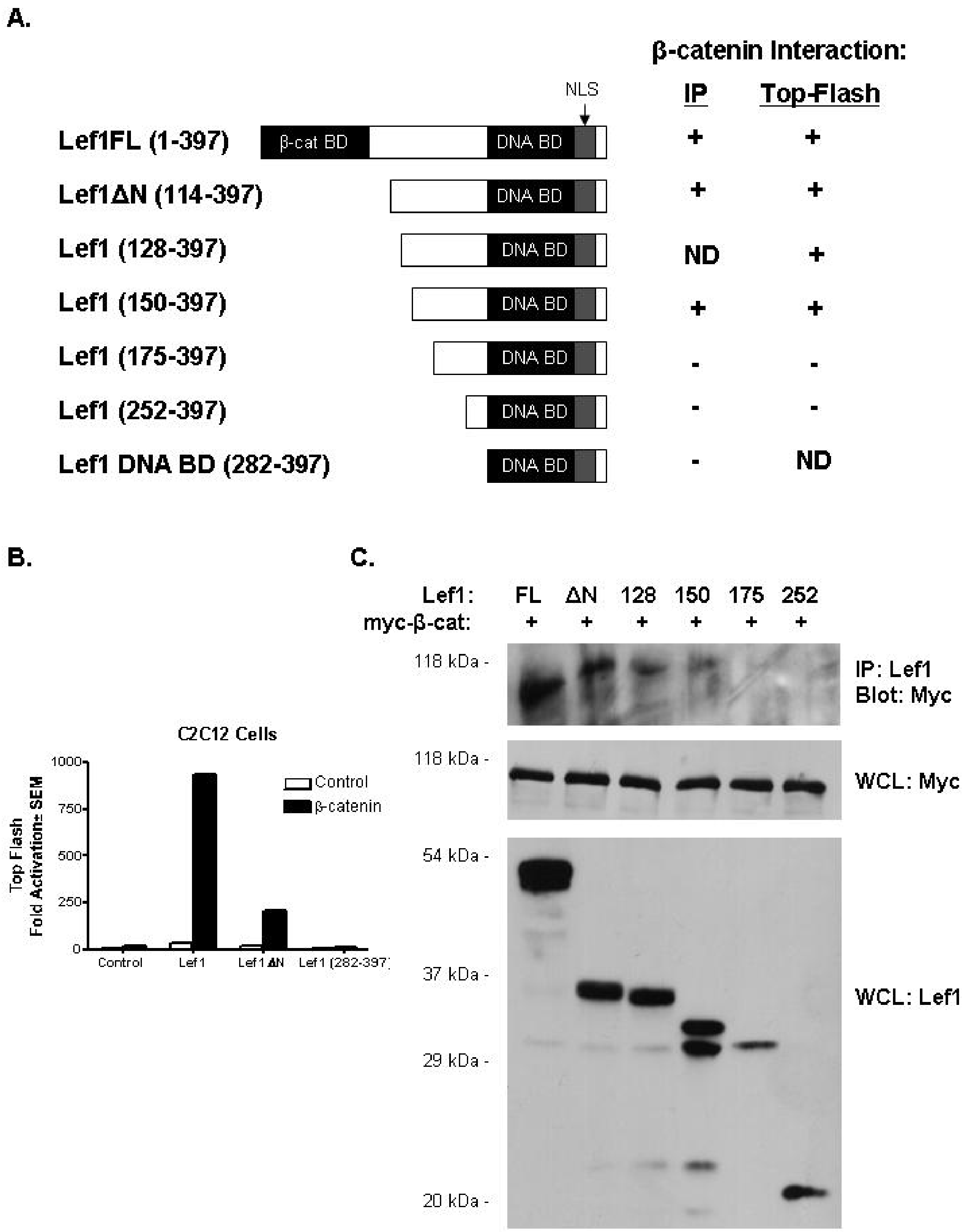
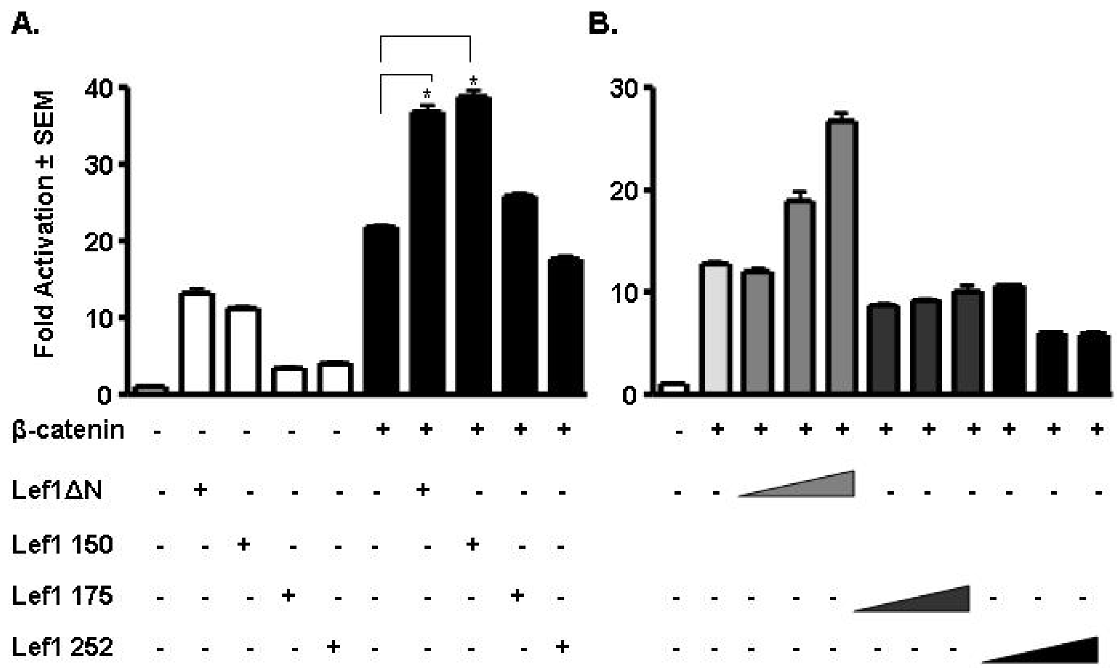
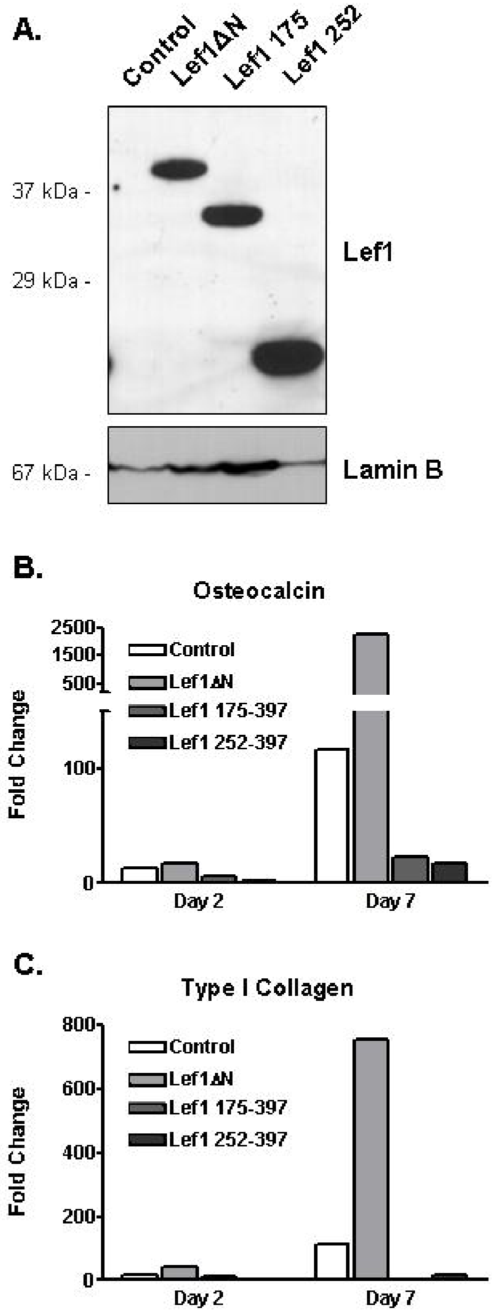

A.
β-catenin Interaction:
IP
Top-Flash
NLS
Lef1FL (1-397)
β-cat BD
DNA BD
Lef1ΔN (114-397)
Lef1 (128-397)
Lef1 (150-397)
Lef1 (175-397)
Lef1 (252-397)
Lef1 DNA BD (282-397)
ND
B.
C2C12 Cells
Top Flash
Fold Activation± SEM
1000
750
500
250
0
Control
β-catenin
Control
Lef1
Lef1 ΔN
Lef1 (282-397)
C.
Lef1: FL ΔN 128 150 175 252
myc-β-cat: + + + + + +
118 kDa -
IP: Lef1
Blot: Myc
118 kDa -
WCL: Myc
54 kDa -
37 kDa -
29 kDa -
20 kDa -
WCL: Lef1

Figure 3: Amino acids 114 to 175 of Lef1 enhance β-catenin-mediated activation in osteogenic cells. A. C2C12 cells were transfected with the Lef1 luciferase reporter plasmid Top-Flash (200 ng), constitutively active β-catenin (300 ng) as indicated, pcDNA3.1 (control), and Lef1ΔN (114-397) (300 ng), Lef1 (150-397) (300 ng), Lef1 (175-397) (300 ng), or Lef1 (252-397) (300 ng) as indicated. The asterisk indicates p values <0.005. **B.** C2C12 cells were transfected with the Lef1 luciferase reporter plasmid Top Flash (200 ng), constitutively active β-catenin (300 ng) as indicated, pcDNA3.1 (control), and increasing amounts (100 ng, 300 ng, and 600 ng) of Lef1ΔN (114-397), Lef1 (175-397), or Lef1 (252-397) as indicated. **A-B.** Luciferase activity was measured 24 h after transfection.



A.
B.
Fold Activation ± SEM
β-catenin
Lef1ΔN
Lef1 150
Lef1 175
Lef1 252

Figure 4: Amino acids 114-175 of Lef1 are required for induction of osteocalcin and type 1 in differentiating osteoblasts. A-C. C2C12 cells were transduced with retrovirus containing supernatants derived from 293T cells transfected with MSCV-Lef1ΔN, MSCV-Lef1 (175-397), MSCV-Lef1 (252-397), and empty MSCV vector (control). The various transduced C2C12 cell lines were differentiated in osteogenic media in the presence of BMP2. **A.** Protein lysates were collected from undifferentiated cells, separated by SDS-PAGE electrophoresis, transferred to Immobilon-P membrane, probed with anti-Lef1, and reprobed with anti-Lamin B. **B-C.** RNA was collected after 2 and 7 days of osteogenic differentiation for quantitative PCR. All data were normalized to murine actin. Fold change was calculated by normalizing values from each differentiated cell line to values from the corresponding cells prior to induction of differentiation.


A.
Control
Lef1ΔN
Lef1 175
Lef1 252
37 kDa -
29 kDa -
Lef1
67 kDa -
Lamin B
B.
Osteocalcin
2500
1500
500
100
0
Control
Lef1ΔN
Lef1 175-397
Lef1 252-397
Fold Change
Day 2
Day 7
C.
Type I Collagen
800
600
400
200
0
Control
Lef1ΔN
Lef1 175-397
Lef1 252-397
Fold Change
Day 2
Day 7

Chapter 4

Discussion, Conclusions and Future Discussions

Since the role of Wnt signaling in bone formation gained recognition in 2001, numerous studies have shown the importance of Wnt pathway components in bone formation, regeneration and repair. Cumulatively, this work has demonstrated that activation of canonical Wnt signaling generally promotes osteoblast proliferation and enhances bone mass, while suppression of Wnt signaling results in bone loss. Extensive basic and translational studies have resulted in a comprehensive understanding of the Wnt pathway in general, and yet, much remains to be accomplished concerning the impact of Wnt-dependent transcription factors on the skeleton. *Tcf7*-deficient mice have low bone mass due to decreased OPG expression and increased osteoclastogenesis (Glass et al., 2005), whereas female *Lef1* haploinsufficient animals exhibit decreased bone mass caused by reduced osteoblast activity (Noh et al., 2009). Gaining a better understanding of Wnt signaling in the context of skeletal metabolism is important because current skeletal regeneration and repair treatments have limitations, anti-resorptive therapies have unknown long-term health consequences and the demand for therapies is rising as our population ages. About 44 million Americans have osteoporosis, and it is estimated that half of women and a quarter of men over the age of 50 years old will experience an osteoporosis-related fracture in their lifetime (Bonura, 2009). Cancer survival rates drop drastically in patients suffering from bone metastases and the prevalence of metastatic bone lesions will increase as our population ages. A complete, molecular understanding of the effects of Wnt-dependent transcription factors on gene expression may provide a means of monitoring the outcomes of translational Wnt-based therapies aimed at inducing bone formation.

One focus of our lab is to understand the role of Lef1, one of the four Wnt-dependent transcription factors, on osteoblast maturation. We previously demonstrated that Lef1 represses Runx2-dependent activation of the late osteoblast differentiation gene, osteocalcin (Kahler and Westendorf, 2003), and that Lef1 protein levels decline throughout osteoblast differentiation (Kahler et al., 2006). Manipulation of Lef1 expression levels demonstrated that Lef1 inhibits terminal differentiation of osteoblasts and acts to delay the onset of osteoblast maturation (Kahler et al., 2006). During osteoblast differentiation, we investigated Lef1 mRNA expression by northern blot and observed that the expression of the 3.6 kb Lef1 transcript remained unchanged throughout differentiation. However, a 2.3 kb mRNA product appeared following 6 days of differentiation, and was undetectable by northern analysis using a probe directed to the 5' region of the Lef1 gene. These results indicate that during the course of osteoblast maturation, these cells express an alternative Lef1 isoform referred to as Lef1ΔN, which is transcribed from intronic promoter (p2) within Lef1 and was first described in T lymphocytes (Hovanes et al., 2001).

The second chapter of this thesis examines the role of Lef1ΔN in osteoblast maturation. We defined the temporal expression of Lef1ΔN during osteoblast differentiation and demonstrated that Lef1ΔN is positively regulated by Runx2 and BMP2, while Wnt3a represses its expression. Using differential, quantitative RT-PCR, we showed that Lef1ΔN mRNA began to be expressed around day seven in differentiating MC3T3 osteoblasts, which corresponded to our northern blot results. We also demonstrated that Lef1ΔN transcript becomes detectable prior to the expression of the late osteoblast differentiation gene osteocalcin in differentiating MC3T3 cells and primary osteoblasts. Through chromatin immunoprecipitation at distal and proximal regions of the Lef1ΔN p2 promoter, we found that histone 3 levels were low throughout five days of osteoblast differentiation and that phosphorylated RNA polymerase II levels steadily increased during days two to five of osteoblast differentiation. These results suggested nucleosome disassembly and recruitment of transcriptional machinery to p2 by the fifth day of osteoblast differentiation, which supported our previous work demonstrating that Lef1ΔN message becomes detectable around days six to seven of osteoblast differentiation. While successful at deciphering the pattern of Lef1 and Lef1ΔN mRNA expression during osteoblast differentiation, we have been unable to consistently monitor Lef1ΔN protein levels during these same studies. Unfortunately, none of the six commercially available Lef1 antibodies we tested were capable of detecting endogenous Lef1ΔN in untreated differentiating osteoblasts. We found BMP2 strongly promoted Lef1ΔN mRNA expression during osteoblast differentiation, and we detected endogenous Lef1ΔN protein in BMP2-treated differentiating osteoblasts following six days of differentiation. Our laboratory is in the process of localizing Lef1ΔN in differentiated osteoblasts using the same rabbit monoclonal Lef1 antibody we used to identify Lef1ΔN by western analysis. We could also use this antibody in immunohistochemistry experiments to attempt to detect Lef1ΔN protein in wildtype murine bone samples, which could illustrate at which stage of *in vivo* osteoblast development Lef1ΔN is being expressed. The fact that this Lef1 antibody recognizes both Lef1ΔN and the full-length isoform is problematic for these applications because unlike immunoblotting, the two isoforms cannot be distinguished based on their size. To circumvent this challenge, we could use another commercially available rabbit monoclonal Lef1 antibody that recognizes an N-terminal epitope. In our hands, this antibody only detects the full-length Lef1 isoform (western blot analysis). Serial sections stained with the antibody recognizing both Lef isoforms or only the full-length isoform could then be compared in order to determine the prevalence of each isoform in a particular region of tissue.

In addition to our observation that BMP2 increased Lef1ΔN expression, we found Lef1ΔN expression was induced by Runx2, while downregulated by Wnt3a. Wnt3a stimulates the canonical pathway and acts to suppress both basal and BMP2-induced Lef1ΔN and alkaline phosphate expression. This is consistent with a previous study in which Wnt3a blocked early osteogenesis and decreased alkaline phosphate levels in mesenchymal progenitor cells (Boland et al., 2004). Our laboratory is in the process of examining whether other canonical Wnt ligands (i.e. Wnt 1, Wnt 8, Wnt 10b) affect Lef1ΔN expression in a fashion similar to Wnt3a. Genetic manipulation of Wnt expression levels can result in skeletal phenotypes. Although bone mineral density was unchanged in *Wnt3a* haploinsufficient mice (Takada et al., 2007), *Wnt10b* null mice had reduced bone mineral density (Bennett et al., 2005; Bennett et al., 2007).

Unlike Wnt3a, Runx2 positively regulates Lef1ΔN. We demonstrated that Runx2 binds to the Lef1ΔN p2 promoter, and the expression of Lef1ΔN message is inhibited in *Runx2*$^{-/-}$ primary osteoblasts during differentiation. Other transcription factors such as AP-1, c-myc, CREB, and Sp1 have putative binding sites within p2. We could determine if any of these transcription factors regulate Lef1ΔN by testing their activity on a p2 reporter in transcription assays. If we see an effect, we could mutate the transcription factor binding site(s) within the p2 reporter and attempt to negate the effect to show the given transcription factor is indeed regulating p2. Electrophoretic mobility shift assays and chromatin immunoprecipitations could be performed to demonstrate that the given transcription factor binds p2 promoter DNA.

Our laboratory has clearly demonstrated that Lef isoforms are expressed in a differentiation-dependent fashion in murine osteoblasts. Previous *in vitro* manipulation of Lef1 isoform expression levels in differentiating osteoblasts shows that Lef1 and Lef1ΔN elicit unique patterns of gene regulation. Therefore, we sought to identify genes regulated by Lef1ΔN to more fully understand the role this transcription factor plays during osteoblast maturation. We found that Lef1ΔN overexpression in differentiating C2C12 osteoblast progenitor cells enhanced the expression of type 1 collagen and osteocalcin, two genes associated with the later stages of osteoblast differentiation. Not surprisingly, Lef1ΔN failed to inhibit Runx2-mediated activation of the osteocalcin promoter. In contrast, our previous observations showed that Lef1 blocks activation of the same promoter, which highlights the distinct roles of Lef1 and Lef1ΔN during osteoblast maturation (summarized in Figure 1). Our results suggest that Lef1ΔN promotes terminal osteoblast differentiation; therefore, it would be interesting to determine other gene expression changes mediated by Lef1ΔN. To that end, we could perform microarray analyses to compare gene expression in differentiated Lef1ΔN tranduced C2C12 cells versus differentiated control C2C12 cells tranduced with the corresponding empty retroviral vector. The identication of additional genes regulated by Lef1ΔN will help define its role in osteoblast maturation.

Given that Lef1ΔN enhanced the expression of genes involved in terminal osteoblast differentiation, we explored the mechanism through which Lef1ΔN regulates osteoblast maturation. Lef1ΔN may prevent osteoblast proliferation and promote terminal differentiation by acting as an antagonist of β-catenin-dependent expression of genes involved in osteoblast proliferation because it lacks the primary β-catenin binding domain present in the full-length isoform. In T lympocytes, Lef1ΔN acts in this fashion by competively inhibiting β-catenin-mediated activation of a Lef1-responsive promoter. Surprisingly, we found that Lef1ΔN enhanced the activation of Top-Flash in the presence of β-catenin in all the osteoblast cells tested. The opposite effects of Lef1ΔN in T lymphocytes and osteoblasts suggest that cell-type specific factors may contribute to Lef1ΔN/β-catenin interactions and effects. We hypothesized that Lef1ΔN augments activation of a Lef1-responsive promoter in osteoblasts though a second β-catenin binding domain retained by Lef1ΔN. The third chapter of this thesis focuses on defining the region of Lef1ΔN that interacts with β-catenin.

First, we demonstrated that Lef1ΔN physically interacts with β-catenin through immunoprecipitation. To define the element of Lef1ΔN that binds β-catenin, we created a series of Lef1ΔN deletion constructs by progressively truncating Lef1ΔN until only the DNA binding domain (Lef1 (282-397)) remained. Using these deletion plasmids in transcription assays and immunoprecipitation experiments, we found that the 150-175 region of Lef1 was the minimum element required to functionally and physicallly interact with β-catenin. In the second chapter, we showed that Lef1ΔN induced the expression of late osteoblast differentiation genes, osteocalcin and type 1 collagen. To determine whether the enhanced expression of these genes was dependent upon an interaction between Lef1ΔN and β-catenin, we transduced osteoblast progenitor cells with Lef1ΔN (114-397), Lef1 (175-397), Lef1 (252-397) or a corresponding control retroviral empty vector and induced osteoblast differentiation in these cells. Lef1ΔN (114-397) enhanced the expression of osteocalcin and type 1 collagen compared to the control, whereas Lef1 (175-397) and Lef1 (252-397) slightly repressed expression of the late osteoblast differentiation genes. These results suggest that β-catenin interacts with the 114 to 175 region of Lef1 to promote osteocalcin and type 1 collagen expression during osteoblast maturation (summarized in Figure 2).

Since Lef1 (175-397) and Lef1 (252-397) repressed type 1 collagen and osteocalcin, we sought determine whether they were acting as competitive inhibitors. Transcription assays using increasing amounts of Lef1 (175-397) or

Lef1 (252-397) in the presence of constitutively active β-catenin on a Lef1-responsive promoter revealed that Lef1 (252-397) was acting as a competitive inhibitor at high concentrations. Lef1 (252-397) retains the DNA-binding domain and has the ability to interact with corepressors while remaining bound to a Lef1-responsive promoter, thus potentially inhibiting Lef1ΔN-mediated activation of the promoter. Binding sites for Groucho/TLE and PITX2 were identified within the Lef1 DNA-binding domain (Amen et al., 2007; Arce et al., 2009). Studies demonstrated that Groucho/TLE mediates histone deacetylase-dependent repression of Lef1/Tcf factors (Arce et al., 2009) and PITX2 decreases Lef1ΔN expression (Amen et al., 2007). We could perform immunoprecipitation experiments with cells transfected with Lef1 (252-397) to determine whether endogenous Groucho/TLE, PITX2 or another known Lef1 corepressor (i.e. CtBP, histone deacetylases) interacts with the Lef1 (252-397). If we fail to detect interactions, we could try cotransfecting cells with Lef1 (252-397) and exogenous corepressors. Upon identification of a corepressor that interacts with Lef1 (252-397), we could monitor whether increasing amounts of the corepressor augment repression of a Lef1-responsive promoter in the presence of Lef1 (252-397) and constitutively active β-catenin.

The temporal expression pattern and function of Lef1ΔN differs according to cell type. In murine epidermal stem cells, Lef1ΔN blocks hair cell differentiation, but promotes sebocyte differentiation (Merrill et al., 2001; Niemann et al., 2002). Lef1ΔN rescues a differentiation block induced by *Lef1*-deficiency in granulocytes (Skokowa et al., 2006). In both T lymphocytes and osteoblasts, Lef1ΔN expression is low during proliferation. Despite this similarity, in osteoblasts Lef1ΔN enhances β-catenin mediated activation of a Lef1-responsive promoter, while Lef1ΔN represses this activation in T lymphocytes. T lympocytes and osteoblast cells express different cofactor proteins, which likely contributes to the contrasting function of Lef1ΔN in these cells. Furthermore, the Jurkat T lymphocytes used in our experiments had been immortalized and thus unable to differentiate, whereas the osteoblast cell lines maintained the ability to differentiate. This disparity in differentiation potential could also contribute to the opposite transcriptional function of Lef1ΔN in T lymphocytes and osseous cells.

Our characterization of the Lef1 second β-catenin binding was based largely upon data generated using Lef1ΔN truncation proteins. All of these Lef1ΔN deletion constructs retain the nuclear localization signal and should be able to enter the nucleus to act as transcriptional effectors. Yet, it is important to validate the nuclear location because failure of a Lef1ΔN deletion protein to enter the nucleus would alter our results. Immunofluorescence studies examining cells transfected with Flag-tagged Lef1ΔN (114-397), Lef1 (175-397), Lef1 (252-397), or a corresponding negative control are currently being conducted. We are using Flag antibody to detect the exogenous Lef1 proteins and Hoechst stain to idenfity nuclei. We also propose repeating the immunoprecipitation experiments using nuclear fractions (rather than whole cell lysates) to demonstrate whether Lef1ΔN deletion proteins and β-catenin are associating in the nucleus.

We described a region of Lef1ΔN that cooperates with β-catenin to regulate osteoblast maturation and demonstrated that β-catenin can be pulled down in complex including Lef1ΔN through immunoprecipitation experiments. To test for a direct association, we propose performing GST-pulldown experiments with purified Lef1ΔN-GST and β-catenin-GST proteins. The lysate-free GST system would eliminate the possibility that cofactors aid in the interaction of Lef1ΔN and β-catenin and enable us to conclude whether their interaction is direct.

We showed that Lef1ΔN regulates genes involved in osteoblast maturation by interacting with β-catenin. Our studies were conducted *in vitro*, primarily through transcription assays, immunoprecipitations and overexpression studies. To strengthen our results, we propose examining whether Lef1ΔN cooperates with β-catenin in an *in vivo* setting to regulate osteoblast maturation. We recently created Lef1ΔN transgenic mice that express Lef1ΔN in committed osteoblasts using the 2.3 kB fragment of the type 1 collagen promoter. These mice exhibited increased bone formation, mineral apposition, and osteocalcin expression, which supports the notion that Lef1ΔN plays a role in osteoblast terminal differentiation (Secreto et al, unpublished data). Previous reports indicate that conditional ablation of *β-catenin* in osteoblast progenitor cells caused a block in osteoblast differentiation and led to osteopenia (Day et al., 2005; Glass et al., 2005; Hill et al., 2005; Hu et al., 2005). To determine whether Lef1ΔN interacts with β-catenin *in vivo*, we could cross our Lef1ΔN transgenic mice to osteoblast progenitor cell-specific *β-catenin* null mice. If Lef1ΔN interacts with β-catenin to promote osteoblast maturation, we would expect to observe a decrease in the high bone formation, mineral apposition and osteocalcin expression seen in the Lef1ΔN transgenic mice.

We demonstrated that Lef1ΔN becomes expressed during late osteoblast differentiation, and its expression is positively regulated by Runx2 and Bmp2 and downregulated by Wnt3a. Lef1ΔN overexpression in differentiating osteoblasts induced osteocalcin and type 1 collagen expression, which suggests Lef1ΔN is a crucial regulator of terminal differentiation in osseous cells. We sought to mechanistically determine how Lef1ΔN regulates osteobast maturation. We found Lef1ΔN interacts with β-catenin to activate a Lef1-responsive promoter and stimulate the transcription of genes involved in late osteoblast differentiation. We mapped the region of Lef1ΔN that associates with β-catenin to an element within the first 61 amino acids of Lef1ΔN and showed this region was required to induce type 1 collagen and osteocalcin expression during osteoblast maturation. Taken together, Lef1ΔN interacts with β-catenin to regulate terminal differentiation in osseous cells.

Prior to this thesis work, the temporal expression pattern and role of Lef1ΔN in osteoblast maturation had not been defined. This research has a laid a foundation for investigating Lef1 isoforms in the context of bone development and highlights the complexities of Lef1 isoform expression, regulation, and differing roles in various cell types. Although canonical Wnt signaling promotes bone formation, transcription factors involved in this pathway, such as Lef1 isoforms, cannot be readily manipulated as potential bone anabolic agents. However, inhibition of natural secreted antagonists of the canonical Wnt signaling cascade has emerged as a promising therapeutic pursuit. Neutralizing antibodies to secreted Wnt pathway inhibitors, including Dickkopf 1, secreted Frizzled-related protein 1, and sclerostin, increased bone formation in various preclinical and clinical studies (reviewed in (Hoeppner et al., 2009b)). A complete, molecular understanding of the effects of Wnt-dependent transcription factors on gene expression will provide a means of monitoring the outcomes of such translational Wnt-based therapies aimed at inducing bone formation. Furthermore, knowledge of Lef1-dependent actions during osteoblast maturation could be used translationally as genetic markers for assessing bone health.

Figure 1: Role of Lef1 and Lef1ΔN During Osteoblast Maturation. Full-length Lef1 protein is expressed early during the proliferative phase of osteoblast differentiation. Lef1 blocks Runx2-mediated activation of the osteocalcin promoter. Lef1ΔN expression occurs late in differentiation when osteoblasts are undergoing terminal differentiation. Runx2-mediated activation of the osteocalcin promoter is not inhibited by Lef1ΔN, which enables osteocalcin to be transcribed during late osteoblast differentiation.

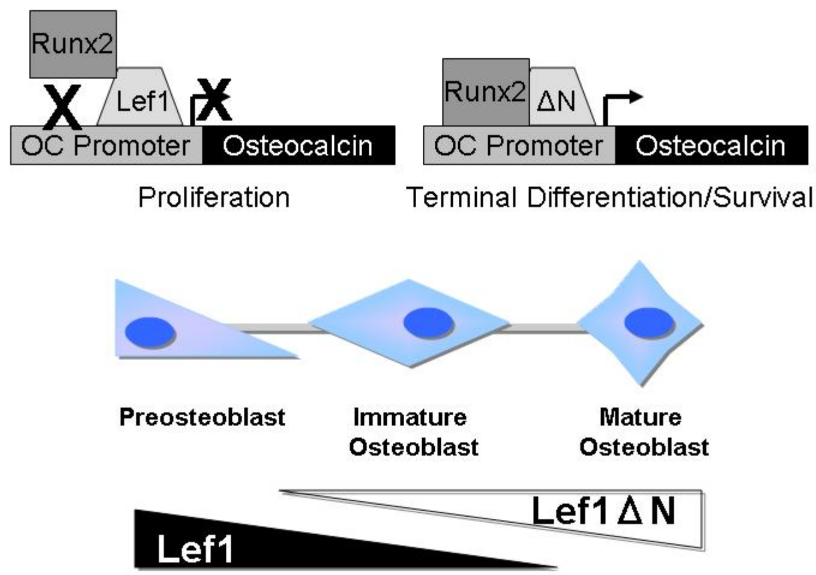
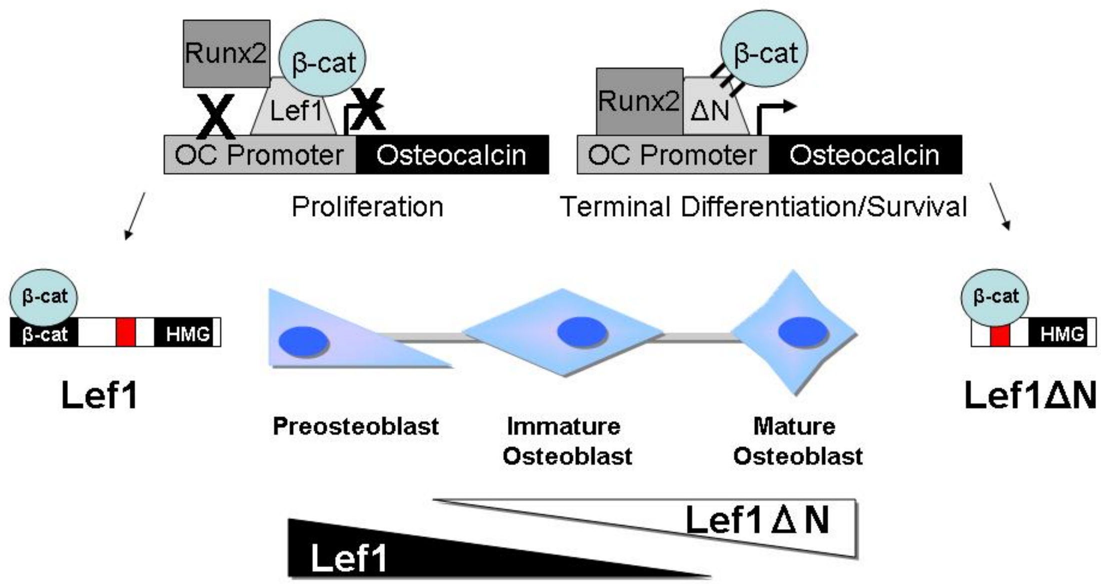

Runx2
Lef1
OC Promoter
Osteocalcin
Proliferation
Runx2
ΔN
OC Promoter
Osteocalcin
Terminal Differentiation/Survival
Preosteoblast
Immature Osteoblast
Mature Osteoblast
Lef1 Δ N
Lef1

Figure 2: Interactions of Lef1 and Lef1ΔN with β-catenin. Full-length Lef1 protein is expressed early during the proliferative phase of osteoblast differentiation and regulates gene expression by interacting with β-catenin through its primary β-catenin binding domain. When Lef1ΔN is expressed during late osteoblast differentiation, Lef1ΔN interacts with β-catenin through a second β-catenin binding domain to regulate genes involved in terminal differentiation, such as osteocalcin.


Runx2
β-cat
Lef1
OC Promoter
Osteocalcin
Runx2
β-cat
ΔN
OC Promoter
Osteocalcin
Proliferation
Terminal Differentiation/Survival
β-cat
β-cat
HMG
Lef1
Preosteoblast
Immature Osteoblast
Mature Osteoblast
β-cat
HMG
Lef1ΔN
Lef1 Δ N
Lef1

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