EFFECT OF NF-κB HAPLOINSUFFICIENCY ON CALVARIAL BONE HEALING

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ABSTRACT

RUIWEI LIANG: EFFECT OF NF-κB HAPLOINSUFFICIENCY ON CRANIAL BONE HEALING
(Under the direction of Lyndon F. Cooper)

Bone defect is a major and challenging health concern. The treatment for bone defect aims to enhance bone regeneration, which is highly regulated by many molecular signaling pathways. Growing evidence suggested that proper inflammatory signaling was crucial for bone regeneration. Previous study showed that treatment of MSCs with expression of NF-κB increased MSCs engraftment in damaged tissue. Previous work in our lab indicated a role of NF-κB on osteoblast differentiation during physiological bone development. The present study was designed to study role of NF-κB signaling in bone healing using genetically-modified mouse with haploinsufficiency of p65 in osteoblasts. Here, we showed that mice with osteoprogenitor-specific NF-κB haploinsufficiency displayed reduced calvarial defect bone repair manifested by micro-CT and histological analysis. The progenitor cells from p65 haploinsufficient mice demonstrated fewer CFU-OB colonies and decreased osteoblastic markers expression (*Sp7*, *Alp* and *Bsp*) in response to rhBMP2. Furthermore, rhBMP2 mediated Smad phosphorylation was disrupted in the absence of sufficient p65 signal. Therefore, we concluded that NF-κB haploinsufficiency impairs bone repair by downregulation of BMP2 mediated canonical Smads signaling and osteogenic differentiation. The effect of inflammatory mediators on bone formation was

complex and yet to be elucidated. Based on our findings, we proposed a direct regulatory role of NF-κB in rhBMP2 mediated bone repair, suggesting that sufficient inflammatory cues are essential for bone regeneration. Uncovering the function of NF-κB in MSC-mediated repair will improve understanding of bone regeneration mechanism and provide a clue for bone regenerative therapy for treatment of bony defect.

To my family

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LIST OF ABBREVIATIONS

ACS	Absorbable collagen sponge
ALP	Alkaline phosphatase
BGLAP	Bone gamma-carboxyglutamic acid-containing protein
BMD	Bone mineral density
BMP	Bone morphogenetic protein
BMP-R	BMP receptor
BMSC	Bone marrow stromal cell
BSA	Bovine serum albumin
BSP	Bone sialoprotein
C2C12	Mouse embryonic premyoblast cell line
C3H10T1/2	Mouse embryonic fibroblastic cell line
cDNA	Complementary DNA
CFU-F	Fibroblast colony forming unit
CFU-OB	Osteoblast colony forming unit
COL1A1	Alpha chain of type 1 collagen
COX2	Cyclooxygenase 2

CRE	Cre recombinase
Ct	Threshold cycle
DLX5	Distal-less homeobox 5
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde-3-pohophate dehydrogenase
GFP	Green fluorescent protein
GO	Gene oncology
GPM6BA	Glycoprotein m6b
H&E	Hematoxylin and Eosin
IκB	Protein inhibitor of NF-κB
IKK	IκB kinase
IL	Interleukin

LRP5	Low-density lipoprotein receptor-related protein 5
LOXP	DNA sequence of locus of X-over P1 as a target of Cre
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MC3T3	Preosteoblastic cell line from mouse calvaria
M-CSF	Macrophage-colony-stimulating factor
MSC	Mesenchymal stem cell
NFATC1	Nuclear factor of activated T cells, cytoplasmic 1
NF-κB	Nuclear factor kappa B
NIK	NF-κB inducing kinase
NSAIDs	Non-steroidal anti-inflammatory drugs
OCN	Osteocalcin
OPG	Osteoprotegerin
OPN	Osteopontin
OSX	Osterix, Sp7
p65 fl/fl	p65 gene flanked with loxP DNA sequence
PBS	Phosphate buffer saline

PDTC	Pyrrolidine dithiocarbamate
PDVF	Polyvinylidene fluoride
PGE2	Prostaglandin E2
PPARɣ	Peroxisome proliferative activated receptor gamma
PRX-1	Paired-related homeobox
PTH	Parathyroid hormone
RANK	Receptor activator of NF-κB
RANKL	Ligand of receptor activator of NF-κB
RHBMP2	Recombinant human BMP2
RHD	Rel homology domain
RIN	RNA integrity number
RIPA buffer	Radioimmunoprecipitation assay buffer
RT-PCR	Reverse transcriptase-polymerase chain reaction
RUNX2	Runt-related transcription factor 2
SATB2	Special AT-rich sequence-binding protein
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SIBLING	Small integrin-binding ligand N-linked glycoprotein
SMADs	Mothers against decapentaplegic (MAD) homolog
STAT1	Signal transducers and activators transcription factor 1
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
TNFR	Receptor for tumor necrosis factor
TRAP	Tartrate resistant acid phosphatase
TWIST1	Twist-related protein 1
WNT	Wingless-type MMTV integration site family
VEGF	Vascular endothelial growth factors

CHAPTER 1

INTRODUCTION

Bone defects and tissue regeneration

Bony defect, a condition affecting the integrity of bone function, is a major and challenging problem for dental and orthopedic surgeons. Patients with bony defect suffer from detrimental impact to both physiological and psychological aspects of their lives. This condition represents a great economic burden, in the United States, as over 2.5 billion dollars are used for the treatment and care of bone defect patients each year. Moreover, this number is expected to double by 2020 (Amini et al., 2012; Baroli, 2009). Thus, there is high demand for affordable therapeutic options that can effectively treat this clinical condition. Current research aims to develop techniques that will enhance bone regeneration and promote bone repair.

Bone is a dynamic tissue with an intrinsic capability to regenerate and remodel both during development and throughout life (Dimitriou et al., 2011; Einhorn, 1998). However, the self-repair capability of bone following injury is limited, and dependent on various factors such as age, gender, and injury severity. Previous literature demonstrates that successful spontaneous bone regeneration only occurs in children younger than 2 years old (Aghaloo et al., 2010). In many other clinical settings, where large bone defects caused by trauma, infections or surgery exist, the bone regeneration process is often compromised or

delayed (Szpalski et al., 2010). In such cases, strategies to restore bone regeneration and function are required. Understanding the bone regeneration process may help to identify the key factors that control bone healing, so that they may be targeted in future clinical applications.

Bone healing is a complex but well-organized process, involving many cellular and molecular cascades. The process of bone healing resembles natural bone development. It has been described as three sequential and overlapping phases.

Phase 1: Inflammatory phase

The inflammation phase occurs immediately upon injury. This acute inflammatory response lasts for five to seven days in a limited, temporal manner. The onset of injury stimulates a series of signaling cascades, leading to a remarkable production of pro-inflammatory cytokines (Mountziaris and Mikos, 2008; Mountziaris et al., 2011) that are secreted by inflammatory cells, macrophages, and cells of mesenchymal origin on the periosteum (Kon et al., 2001). The tremendous induction of cytokines suggests that the contributing cells include not only the local residents within sites of trauma, but also recruited cells from other distinct locations. The expression levels of interleukin-1 (IL-1), IL-6, and tumor necrosis factor α (TNFα) peak at 24 hours following skeletal injury and then return to baseline at 72 hours (Kon et al., 2001). Other inflammatory cytokines observed in the initial inflammation phase include IL-11, IL-18, osteoprotegerin (OPG), receptor activator of NF-kB ligand (RANKL), and macrophage colony–stimulating factor (M-CSF) (Ai-

Aql et al., 2008; Rundle et al., 2006). RANKL, OPG and M-CSF are vital regulators for osteocalstogenesis and osteoclast activity.

The inflammation phase of bone healing is crucial for bone regeneration as it is responsible for initiating the healing cascades (Schmidt-Bleek et al., 2012). The pro-inflammatory cytokines help to recruit mesenchymal stem cells and promote angiogenesis (Schmidt-Bleek et al., 2012). The transforming growth factor-beta (TGF-β) and vascular endothelial growth factors (VEGFs) synthesized by recruited MSCs and osteoblasts further stimulate stem cell proliferation and differentiation (Ai-Aql et al., 2008).

The acute inflammation phase is critical for early bone repair and thus greatly affects the outcome of healing. Previous studies based on animal models suggest that the healing process is impaired in the absence of proper inflammatory signals (Gerstenfeld et al., 2003a; Katavic et al., 2003; Zhang et al., 2002). Anti-inflammatory medications, such as nonsteroidal anti-inflammatory drugs (NSAIDs), increase the risk of nonunion, malunion, and infections in patients treated for long bone fracture (Bhattacharyya et al., 2005; Jeffcoach et al., 2014).

Phase 2: Bone formation phase

A combination of intramembranous ossification and endochondral ossification processes occur subsequently from 7-14 days following injury. This phase is associated with elevated level of TGFs and BMPs, which promote bone regeneration *via* the differentiation and growth of recruited progenitor cells. VEGFs rise in accordance with enhanced angiogenesis and vascularization. Mesenchymal stem cells differentiate into an osteoblastic

lineage that produces bone matrix. Large amounts of extracellular matrix are deposited (Collagen, Aggrecan, OPN, BSP, etc.) at bone defects (Gerstenfeld et al., 2003b). The inflammatory response is absent during this phase.

Phase 3: Bone remodeling phase

Bone remodeling begins approximately 10 days, and can last anywhere from 28 days to 2 years, following injury (Ai-Aql et al., 2008; Khan et al., 2008; Kon et al., 2001). Pro-inflammatory cytokines (IL-1, IL-6, TNFα) produced primarily by osteoblasts and hypertrophic chondrocytes reach a second peak. Along with factors that regulate osteoclast function (RANKL, OPG, M-CSF), these cytokines participate in replacing woven bone with mature lamellar bone.

The major goal for treatment of bony defects is to induce and accelerate bone regeneration. Successful bone regeneration is achieved through bone formation by osteoblasts and bone resorption by osteoclasts. The differentiation of primitive mesenchymal stem cells into mature osteoblasts is referred to as osteoblastogenesis. It represents an essential component of bone healing and occurs in both bone development and post-natal bone repair. Bone regeneration shares a similar ossification mechanism with sequential expression of critical bone markers resembling the natural bone development. Understanding the process of bone development and identifying the signaling pathways involved in lineage specification of bone precursors will help to treat bone disorders that require bone regeneration.

Bone development and homeostasis

Bone is one of the hardest tissues in the human body and provides mechanical support for the body and protection to the vital organs (brain, heart, etc.). In addition, it serves as a mineral reservoir for calcium and phosphorus. Bone is composed of organic components (bone matrix) including type I collagen and proteoglycans; and inorganic mineral composed of primarily hydroxyapatite (Allori et al., 2008b; Boskey and Posner, 1984). At the cellular level, bone formation is accomplished by highly regulated cooperation of bone forming cells (osteoblasts), bone resorbing cells (osteoclasts) and osteocytes (Nakahama, 2010).

Bone development initiates during the embryonic state through two distinct mechanisms: endochondral and intramembranous ossification. Endochondral ossification starts with a cartilaginous template formed by chondrocytes followed by replacement with mineralized matrix synthesized by osteoblasts. The majority of bone tissue, including long bones of both the axial and appendicular skeleton, are derived from this process (Javed et al., 2010). During intramembranous ossification, mesenchymal stem cells (MSCs) from condensed mesenchyme differentiate into osteoprogenitors and then develop into mature osteoblasts. Craniofacial bones (the flat bones of the skull and mandible) primarily develop from this mechanism (Franz-Odendaal, 2011; Javed et al., 2010). The maintenance of bone mass evolves through bone modeling and remodeling controlled by strictly regulated cooperation of osteoblasts and osteoclasts. Osteoblasts are bone forming cells that produce bone matrix (osteoid) and are responsible for matrix mineralization. In response to specific stimuli, bone marrow stromal cells or connective tissue mesenchymal stem cells can differentiate into osteoblasts postnatally (Tuan et al., 2003).

Bone undergoes continuous turnover, with a balance between bone formation by osteoblasts and bone resorption by osteoclasts. Osteoclasts are multi-nucleated cells that function in bone resorption. Osteoclasts are differentiated from hematopoietic cells of the monocyte/macrophage lineage. They differ from osteoblasts, which are developed from mesenchymal stem cells. Characterized by the expression of tartrate-resistant acid phosphatase (TRAP), calcitonin receptors, p60c-src and vitronectin receptors, mature osteoclasts have the capacity to degrade bone tissue (Suda et al., 1997). Previous evidence has shown that the maturation of osteoclasts from the progenitors requires osteoblasts (Owens et al., 1996). Further investigation found that several factors secreted by osteoblasts are essential for osteoclast differentiation (Suda et al., 1999). These factors include M-CSF and RANKL. By association with their respective receptors on osteoclast precursors, M-CSF and RANKL promote osteoclastogenesis. This represents a positive regulatory role of osteoblast in osteoclast differentiation. In contrast, osteoblasts also produce OPG, a decoy receptor for RANK, thus inhibiting the RANKL-RANK binding (Hsu et al., 1999). In this way, osteoblast negatively regulates osteoclast differentiation. The modulation of osteoclast differentiation by osteoblast indirectly affects bone resorption and homeostasis.

The process of osteoblast differentiation can be characterized into three stages: 1) commitment of progenitors into the osteoblastic lineage; 2) proliferation of osteoblast precursors; and 3) bone matrix deposition, maturation and mineralization (Stein et al., 1990). It is a well-orchestrated and highly regulated process, with stage-specific expression of many transcription factors. In bone tissue, multipotential mesenchymal stem cells

differentiate into osteochondroprogenitors that express *Prx1* and *Twist1* (ten Berge et al., 1998; Yu et al., 2003). These early skeletal progenitors have the capacity to develop into either chondrogenic or osteoblastic lineages. Sox9 is the key transcription factor that drives chondrogenic differentiation (Mori-Akiyama et al., 2003). On the other hand, runx2 and osterix primarily direct the cell commitment into osteoblastic lineage (Harada and Rodan, 2003). The key transcription factors and several important osteogenic proteins involved in osteoblastogenesis are summarized below (Figure 1.1).

Runx2

Progressive osteoblast differentiation from early mesenchymal precursors requires tight modulation of transcription factors. Runx2 is the earliest transcriptional regulator, followed by osterix. Runx2 belongs to the Runx family, containing a runt DNA binding domain which is homologous with the Drosophila pair-rule gene runt (Komori, 2005). Runx2 is widely accepted as the master regulator for osteoblast differentiation and plays a pivotal role during bone development. Runx2-null mice exhibit complete absence of both endochondral and intramembranous bone as a result of the lack of mature osteoblasts, and altered chondrocyte maturation as a result of hypertrophic chondrocytes. (Komori et al., 1997; Otto et al., 1997).

Runx2 expressing osteoprogenitors are able to differentiate into either osteoblasts or chondrocytes. Further differentiation from osteoprogenitors into functional mature osteoblasts that produce bone matrix requires osterix expression.

Osterix

Osterix is a zinc finger-containing transcription factor belong to the Sp/XKLF family. Osterix functions immediately downstream of Runx2 (Javed et al., 2010). Previous literature demonstrates that osterix-deficient mice display normal Runx2 expression, while Runx2-null mice demonstrate no expression of osterix (Nakashima et al., 2002).

Osterix is also essential for osteoblast differentiation and is expressed in all the osteoblasts throughout the embryonic bone development. *Sp7* -/- mice show a similar bone phenotype to the *Runx2* -/- mice, with a lack of mature differentiated osteoblasts in either intramembranous or endochondral ossification. However, different from *Runx2* -/- mice, the osterix-null mice develop normal cartilage formation as well as terminally differentiated hypertrophic chondrocytes (Nakashima et al., 2002). This observation highlights a specific role of osterix in osteoblastic differentiation.

Alkaline phosphatase (ALP)

Once committed into osteoblastic cell lineages, the osteoprogenitors then go through a proliferative period before they differentiate into osteoblasts that synthesize extracellular matrix. Alkaline phosphatase is a membrane-bound enzyme and is expressed immediately after the proliferation period by osteoblasts. ALP is an early marker for osteoblasts during matrix maturation. Its expression is associated with maximal production of type I collagen (Javed et al., 2010).

ALP also plays a pivotal role in the initiation of bone mineralization. The expression of ALP is regulated by many osteogenic signals including the BMP2-Runx2 axis as well as parathyroid hormone (PTH) and Wnt signaling pathways (Rawadi et al., 2003).

Bone sialoprotein (BSP)

Osteoblasts are responsible for ECM production, maturation and mineralization. The ECM proteins in bone include mainly type I collagen, proteoglycans and other non-collagenous proteins (bone sialoprotein, osteopontin, osteocalcin, etc.). These matrix proteins regulate osteoblast proliferation, differentiation and survival (Rodan and Noda, 1991). BSP is a glycoprotein that belongs to the SIBLING (Small Integrin-binding Ligand N-linked Glycoprotein) family. It is widely expressed by osteoblasts, hypertrophic chondrocytes and osteoclasts.

BSP was shown to influence osteoblast differentiation and induce bone mineralization (Gordon et al., 2007). Mice lacking BSP displayed delayed long bone growth, mineralization and a decrease of bone markers including osteopontin (OPN) and osteocalcin (OCN) (Bouleftour et al., 2014). BSP deficient mice also demonstrated impaired bone healing, implicating a role for BSP in bone regeneration via bone formation and osteoclast activity (Malaval et al., 2009; Monfoulet et al., 2010).

Osteocalcin (OCN)

Osteocalcin, a vitamin K and vitamin D dependent non-collagenous protein, is also secreted by osteoblasts. It is not expressed in the early stages of osteoblast differentiation and, in fact, is the marker for the late phase of osteoblastogenesis (Bellows et al., 1999). *Bgalap*-expressing osteoblasts are usually recognized as mature osteoblasts.

These osteogenic factors are essential for lineage specification and osteoblast differentiation. As a result, they are commonly referred to as bone markers. The

overlapping expression of bone markers directs the commitment, proliferation and differentiation of stem cells into osteoblasts. Evaluation of bone marker expression is often used to assess the differentiation stage of progenitor cells. The progression of stem cells into each differentiation stage requires many extracellular signals including growth factors (BMPs, FGFs, etc.), hormones (PTH, estrogen, etc.) and cytokines (interleukins) (Allori et al., 2008a). In this sense, these osteogenic signals function in a stage-specific manner with a defined temporal expression profile.

BMP2 in tissue engineering

For the past several decades, tremendous efforts have been taken by scientists to develop effective treatment for critical-sized defects. In such cases where a large amount of bone is missing, spontaneous bone repair cannot occur. Therefore, the goal for treatment is to restore function by providing a local environment favorable for bone regeneration.

Autografts have been utilized as a golden standard for treating bony defects in the clinical settings. Autografts are collected from patients' own bone, primarily ileum or iliac crest, and then implanted into sites of bone defect. This technique yields a high possibility of success for bone regeneration due to high osteoconductivity, osteoinductivity and non-immunogenic properties of autografts. However, it is always associated with considerable risks including donor site pain, post-surgery patient morbidity and high cost. (Amini et al., 2012; Blokhuis et al., 2013). A common alternative to autografting is the application of allografting, which utilizes human cadaver bone. This method eliminates the surgical bone graft procedure that is necessary for autografting. The disadvantages associated with

allografts include the potential transmission of disease, immunogenic reaction and limited availability of cadaver bones. Due to the limitations of autografts and allografts, there is an increasing demand for the discovery of graft substitutes, which could replace, and resemble the form and properties of, natural bone (Giannoudis et al., 2005).

In recent decades, researchers have focused on developing synthetic bone graft substitutes. An optimal substitute material is expected to provide adequate strength in response to mechanical load, and should gradually be absorbed during the process of new bone formation. The key properties that will determine the regenerative outcome of graft substitutes are: 1) a biocompatible and degradable scaffold carrier that serves as a template for bone cells to adhere, proliferate and differentiate (osteoconduction); 2) growth factors that stimulate progenitor differentiation into osteoblastic lineages (osteoinduction); and 3) existence of host cells or implanted cells to assist with bone repair (osteogenesis). However, an optimal material that fulfills all of the above requirements has yet to be developed.

Due to this complexity of bone repair, an improved strategy using a combination of synthetic scaffolds, growth factors and cells holds the most promise as a tissue engineering application (Agarwal et al., 2009; Rahman et al., 2014). As researchers continue to gain a better understanding of the molecular pathways involved in bone healing, many relevant key factors have already been investigated in the lab and applied in clinical settings. Among the factors studied, bone morphogenetic proteins (BMPs), which belong to the transforming growth factor β (TGF-β) superfamily, have been heavily studied due to their strong osteoinductive capability. The TGF-β superfamily is a group of approximately 50 structurally and functionally related proteins. They participate in many biological processes such as

organogenesis, embryogenesis and wound healing (Attisano and Wrana, 2002; Miyazono, 2000a). BMPs greatly impact bone development and healing. In 1965, BMPs were first identified as osteoinductive agents in demineralized bone matrix, and were later termed bone morphogenetic proteins by Urist (Urist, 1965; Urist and Strates, 1971). Currently, at least 20 BMPs have been recognized and described in humans as well as other species (Carreira et al., 2014).

BMP2 transduces signals through direct binding to its serine-threonine kinase receptor complex (type I and type II receptors, BMPRI and BMPRII). This ligand-receptor interaction catalyzes the phosphorylation of downstream signaling transducers and activates the intracellular cascades (Carreira et al., 2014). In the canonical Smads pathway, receptor specific Smad proteins (R-Smads, Smad 1/5/8) are phosphorylated. Activated R-Smads are then released from the cell membrane into the cytoplasm where they interact with Smads such as co-Smad and Smad 4. (Attisano and Wrana, 2002). The binding of R-Smads with Smad4 enables the exposure of nucleus-imported sequences, allowing for the nuclear translocation of the R-Smads-Smad 4 complex and initiating transcriptional regulation (Kawabata et al., 1999; Miyazono, 2000b) (Figure 1.2).

In the nucleus, the Smad heteromer modulates the transcription of major osteogenic genes including *Runx2*, *Dlx5*, *Sp7*, *Alp* and *Bglap* (Chen et al., 2012; Lee et al., 2003; Luu et al., 2007; Nakashima et al., 2002). Microarray based gene profiling revealed that osteogenic BMP2 signaling induces activation of over 200 genes responsible for pre-osteoblast proliferation and osteoblastic differentiation (Peng et al., 2003). This represents an important mechanism by which BMP2 regulates osteoblasts differentiation, bone

development and regeneration. Moreover, BMP2 is also a central mediator that can control the expression of other several other BMPs. (Edgar et al., 2007).

Elevated BMP2 expression was observed as soon as 24 hours following bone injury, suggesting that it plays a role in the initiation of the bone healing process (Farhadieh et al., 2004; Khanal et al., 2008; Kugimiya et al., 2005). BMP2 is found actively involved throughout the bone regeneration phase for at least 21 days after bone fracture (Bais et al., 2009). The Smad 1/5/8 complex shares a similar expression pattern and localization with BMP2, as shown by immunohistochemical staining after osteotomy (Khanal et al., 2008).

The importance of BMP2 in bone healing was addressed by the finding that mice with impaired BMP2 expression display normal bone development but defective bone healing (Tsuji et al., 2006). BMP2 is produced by osteoprogenitors, mesenchymal cells, chondrocytes and osteoblasts that either reside at, or are recruited to, the sites of injury. BMP2 induction upon bone injury was suggested to promote bone repair by: 1) initiating healing cascades and chemotaxis, and recruiting of mesenchymal stems cells; 2) activating other BMPs; and 3) activating many growth factors that control angiogenesis, cell proliferation and differentiation (Dimitriou et al., 2005).

In the late 1980s, with the robust advancement of the recombinant DNA technique, human BMPs became available in large quantities (Wang et al., 1988). In 2002, the United States Food and Drug Administration (FDA) licensed rhBMP2 (InFuse Bone Graft, Medtronic Sofamor Danec USA, Inc.) as a clinical treatment of long bone open fractures, non-union fractures and spinal fusion. Since then, rhBMP2 has been extensively investigated for tissue

engineering applications in both experimental and clinical scenarios. For optimal effect, rhBMP2 is often delivered by carriers, which not only prolong protein retention, but also provide a platform for progenitor adhesion, proliferation and differentiation. Due to the desirable properties (biocompatibility, biodegradability, safety and being favorable for cell adhesion), absorbable collagen sponges (ACS) have become attractive carriers for tissue engineering.

Local delivery of BMP2 by absorbable collagen carriers enhances bone healing and regeneration in both experimental and clinical scenarios (Kim et al., 2005; Lin et al., 2013; Simpson et al., 2006). The beneficial effect of rhBMP2/ACS has been applied to many clinical cases and has been reported to bridge critical-sized defects, promote fracture healing, induce spinal fusion and promote dental and craniofacial reconstruction (Chenard et al., 2012; Geiger et al., 2003). Robust release of several pro-inflammatory cytokines (IL-1, IL-6, TNFα, etc.) is observed during both natural bone healing as well as rhBMP2 mediated bone regeneration (Gerstenfeld et al., 2003a; Lu et al., 2013). Interestingly, elevation of BMP2 expression occurs and peaks in parallel with pro-inflammatory cytokines, at 24 hours post injury, and is sustained throughout the acute inflammation phase (Kon et al., 2001; Mountziaris and Mikos, 2008). BMP2 enhances the early inflammation phase, acting as a chemoattractant for immune cells, such as lymphocytes and macrophages (Cunningham et al., 1992). Moreover, BMP2 induces the expression of inflammatory mediators in various cell types including preosteoblasts (Akeel et al., 2012; Helbing et al., 2011).

Inflammatory signals positively affect bone regeneration, representing one mechanism by which BMP2 participates in bone healing. This is supported by previous

findings that the osteoinductive capability of BMP2 is impaired in the absence of proper inflammatory signals (Katavic et al., 2003; Zhang et al., 2002). In addition, proinflammatory factors are proven to stimulate osteogenic differentiation of stem cells by increasing BMP2 production (Fukui et al., 2003; Hess et al., 2009; Rifas, 2006). This evidence, taken together, implies a tight connection between the osteoinductivity of BMP2 and the inflammatory signaling pathway. On the other hand, this connection between BMP2 and inflammation is complex and not well understood, as many cases demonstrate a negative regenerative effect of rhBMP2. Excessive inflammation caused by systemic inflammatory disease or local infection impairs bone repair (Huang et al., 2014). High doses of rhBMP2 could cause adverse inflammatory responses such as soft tissue swelling, thereby diminishing the efficacy of rhBMP2-mediated repair (Lee et al., 2011). The exact role of inflammation on the osteoinductive capability of BMP2 remains unclear. Further understanding of this interaction will improve the current therapeutic strategy for skeletal repair.

Although promising bone regenerative effects have been achieved using the rhBMP2 based tissue engineering approach in the past several decades, an optimal healing process that mimics all of the healing cascades of natural bone repair has yet to be developed.

NF-κB pathway and its involvement in bone biology

The nuclear factor kappa B (NF-κB) transcription factor family is well known as a major component of innate immunity and the inflammatory response (Clohisy et al., 2004). In mammals, there are five NF-κB members including RelA (p65), RelB, c-Rel, p105/p50 (NF-κB1) and p100/p52 (NF-κB2), which share an N-terminal Rel homology domain (RHD)

responsible for dimerization and DNA binding. The five monomers can form homodimers or heterodimers that function in gene expression regulation. Among those transcriptionally active dimers, the p65-p50 heterodimer is the most common form of NF-κB (Oeckinghaus and Ghosh, 2009; Vallabhapurapu and Karin, 2009).

The NF-κB signaling is activated through two major pathways: the classical (canonical) pathway and alternative (non-canonical) pathway (Figure 1.3). These two pathways are different in many aspects including the participating NF-κB dimers activating signals as well as the biological functions. The classical pathway is induced by many extracellular cues such as receptor activator of the NF-κB ligand (RANKL) and TNFα. In an unstimulated state, NF-κB complexes are sequestered in the cytoplasm via association with IκB proteins (IκBα, IκBβ, and IκBε). The classical NF-κB signaling pathway is initiated upon activation of the IκB kinase complex (IKKα, IKKβ, IKKγ). The IKK catalytic complex phosphorylate IκB proteins for polyubiquitination and degradation in proteasomes, releasing the NF-κB dimer for nuclear translocation. p65-p50 and cRel-p50 heterodimers mainly signal through the classical pathway (Hayden and Ghosh, 2008; Oeckinghaus and Ghosh, 2009). On the other hand, the alternative pathway is stimulated by only a subset of the TNF superfamily and is mainly involved in lymphoid development. NF-κB inducing kinase (NIK) is the key regulatory point which activates IKKα, leading to the processing of p100 into p52. The p52-RelB heterodimer is the primary component in the alternative NF-κB signaling pathway (Bonizzi and Karin, 2004).

NF-κB signaling is essential for embryogenesis and has an important role in endochondral ossification during bone development. The effect of NF-κB on osteoclasts and

related inflammatory bone disorders has been extensively investigated. Studies using a p50 and p52 double knockout animal model demonstrated that these animals display osteopetrosis, with retarded growth and a significant decrease in osteoclast numbers (Iotsova et al., 1997). Other transgenic mice models with manipulated NF-κB elements also suggest the requirement of NF-κB signaling during osteoclastogenesis (Soysa and Alles, 2009). Further investigation has determined the importance of NF-κB in osteoclast differentiation, survival and function by transcriptional regulation of osteoclast master genes including c-Fos, NFATc1, tartrate-resistant acid phosphatase (TRAP) and cathepsin K (Novack, 2011; Soysa and Alles, 2009).

The role of NF-κB on osteoclasts is well defined. However, the effect of NF-κB on bone forming cells (osteoblasts) is relatively less explored. A role of NF-κB in osteoblast differentiation was suggested when Hess et al. reported that TNFα treatment/ p65 activation positively affects osteogenic differentiation in human mesenchymal stromal cells through regulation of BMP2 expression (Hess et al., 2009). As a potent NF-κB activator, TNFα was also reported to induce osteogenic differentiation of human dental pulp stem cells, mesenchymal stem cells and human stem cells from apical papilla respectively through NF-κB signaling pathway (Cho et al., 2010; Feng et al., 2013; Li et al., 2014). In addition, NF-κB/p65 signaling activation facilitates BMP2-mediated chondrogenic differentiation during endochondral ossification (Caron et al., 2012). Two putative NF-κB response elements were found located in the promoter region of the BMP2 gene, implicating a direct regulatory mechanism of NF-κB in the BMP2 pathway (Feng et al.,

2003). These findings indicate crosstalk between the NF-κB pathway and BMP2-mediated osteogenesis.

In an attempt to investigate the precise role of NF-κB in osteogenesis, a transgenic animal model characterized by manipulation of the NF-κB function has been employed. Global knockout of p65 (RelA) leads to embryonic lethality of mice due to liver degeneration at 15-16 days of gestation caused by liver cell apoptosis (Beg et al., 1995). To circumvent the early lethality, strategies to generate conditional NF-κB knockout animals enabled the further investigation of NF-κB function on bone *in vivo*. A Cre-LoxP system has been widely used for tissue-specific elimination of target gene expression in mouse models. In mice harboring both the Cre transgene and a floxed target gene, Cre-recombinase mediates the gene deletion by cleaving the two specific 34-base LoxP sites that flank the target gene. A genetically modified Cre-recombinase transgene under the control of a tissue-specific promoter allows for deletion of a target gene in a specific tissue (Chambers, 1994).

bglap2-IKK-DN mice with dominant negative IKKγ function in mature osteoblasts exhibit increased bone mineral density (BMD) and BV/TV at 2 and 4 weeks old (Chang et al., 2009). In another study, *Coll2*-IKK2ca mice, in which IKKβ is constitutively active in chondrocytes, display impaired bone phenotype (Swarnkar et al., 2014). The promoter of *bglap2* and *Coll2* allows for the modulation of NF-κB signaling to occur specifically in mature osteoblasts and early skeletal progenitors, respectively. These two animal models both modulate NF-κB function by manipulating NF-κB regulatory elements (IKK complex). These findings suggest a negative role for NF-κB in bone formation, however, several other groups have suggested a positive effect of NF-κB on osteogenic differentiation of stem cells for

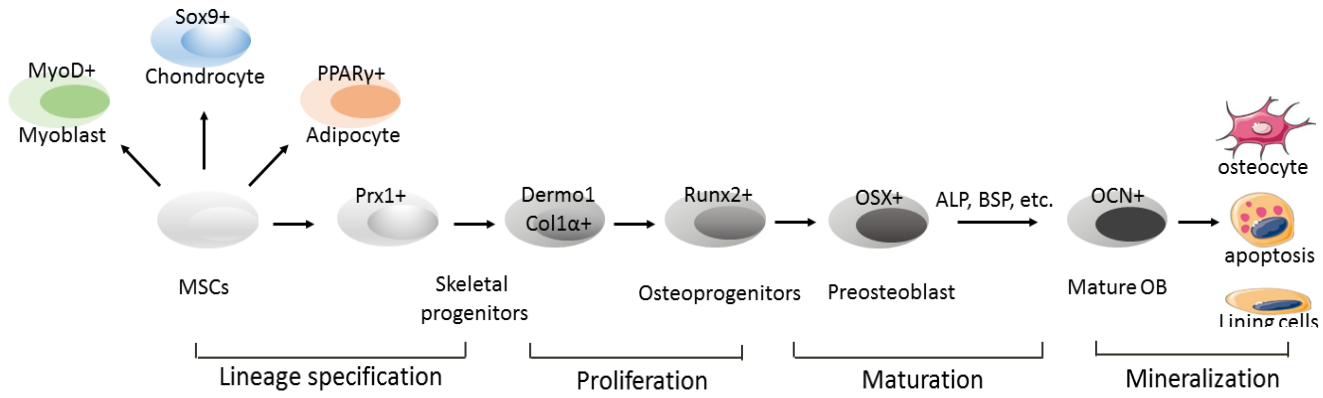
bone formation (Caron et al., 2012; Cho et al., 2010; Feng et al., 2013; Hess et al., 2009; Li et al., 2014). The current findings regarding the function of NF-κB on osteogenesis are controversial.

The discrepancy of the results may lie in the difference of approaches that have been applied. One issue that comes with the transgenic mouse with manipulated NF-κB regulators is the difficulty to interpret the results. Since many NF-κB subunits share the same regulators, the indirect effect from other NF-κB components may contribute to the observed outcome. The strategies employed in previous studies include the local application of NF-κB inhibitors or activators and generation of virus expressing dominant negative or constitutive active NF-κB elements (Hess et al., 2009). Since NF-κB is a major inflammatory mediator, expressed by many cell types and not limited to bone cells, the treatment of NF-κB inhibitors and/or activators may affect other cells, indirectly influencing the outcome. An improved approach for investigating the direct role of NF-κB on osteogenesis will greatly improve the current knowledge and enhance accuracy and interpretation of future experiments.

In sum, accumulating evidence has suggested a role for NF-κB in osteoblast function, in addition to its important role in osteocalstogenesis and bone resorption. A controlled balance between bone formation and resorption is critical for bone homeostasis as well as bone regeneration. Previous studies have shown controversial results and the exact role of NF-κB in osteoblastogenesis remains unclear. It was previously indicated that NF-κB may contribute to BMP2 mediated bone formation. As discussed earlier, inflammatory signals positively affect bone healing (Katavic et al., 2003; Zhang et al., 2002). There is a demanding

need to elucidate the precise function of NF-κB in bone homeostasis and healing, particularly as it relates to bone cells (osteoblasts). Deciphering the mechanism by which NF-κB affects bone healing in conjunction with BMP2 could provide a novel and efficient therapeutic target for clinical use.

Figure 1.1 Differentiation of primitive mesenchymal stem cells into osteoblasts.



Adapted from Rodda *et al*. 2006

Figure 1.2 Signaling transduction of BMP2 pathway.

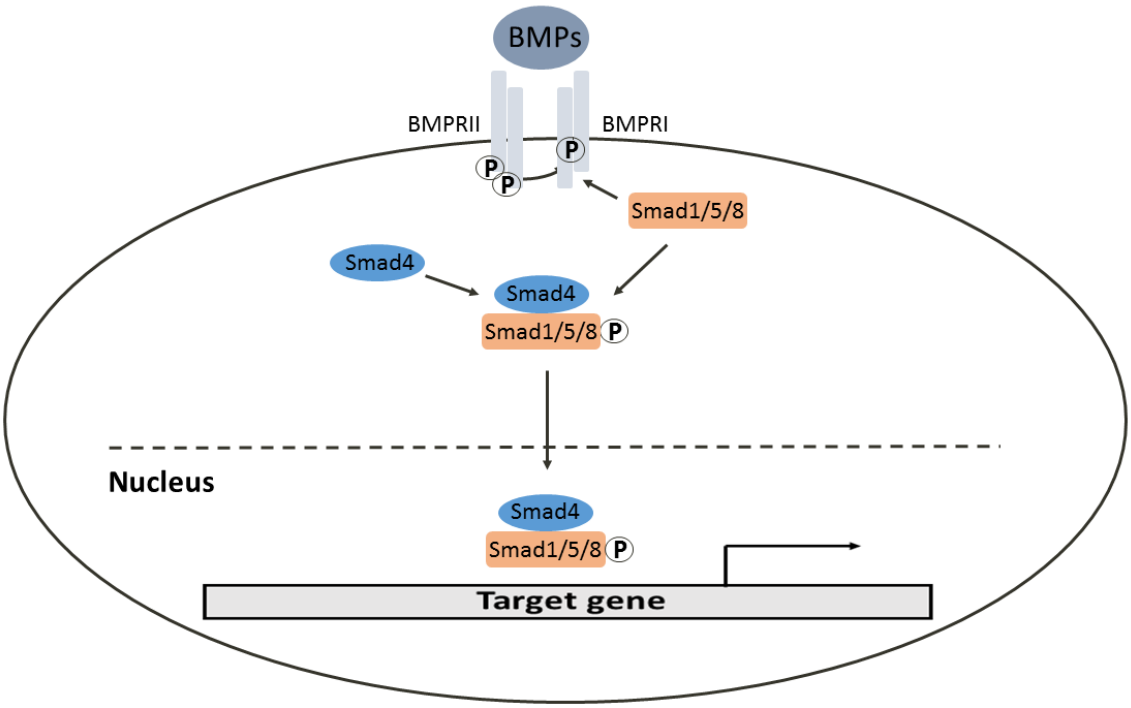
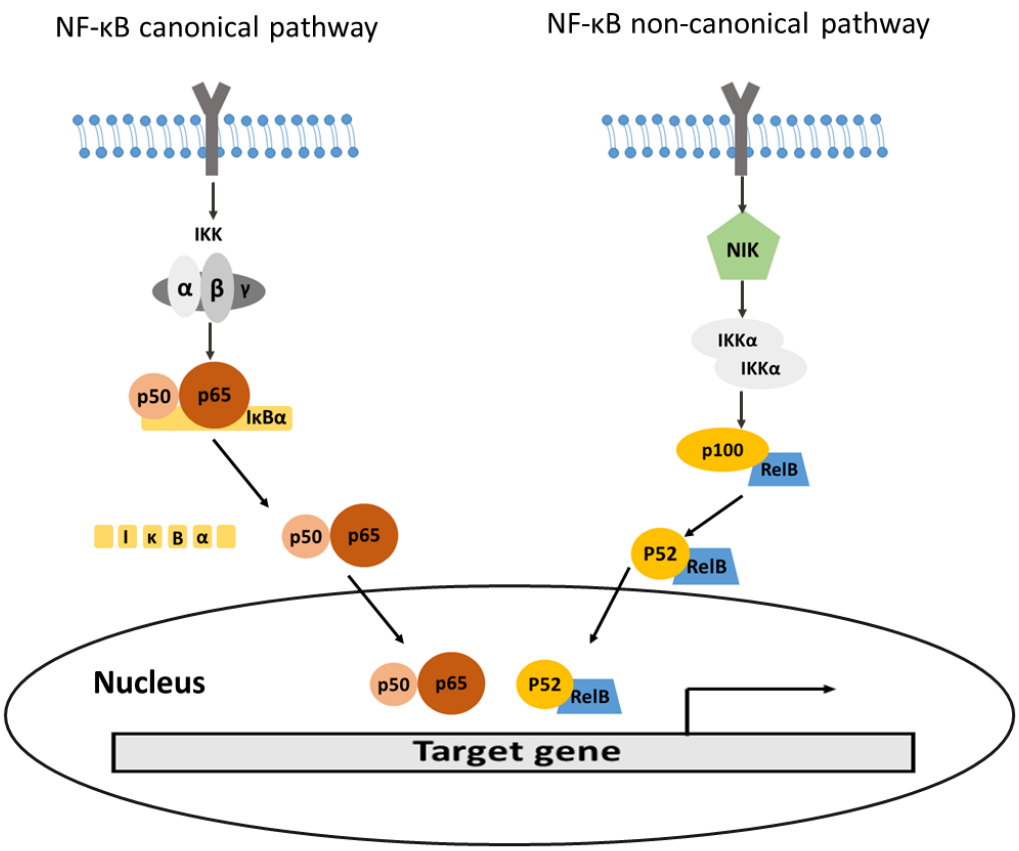


Figure 1.3 Canonical and non-canonical NF-κB signaling pathways in bone cells.



REFERNCES

Agarwal, R., Williams, K., Umscheid, C.A., and Welch, W.C. (2009). Osteoinductive bone graft substitutes for lumbar fusion: a systematic review. Journal of neurosurgery Spine *11*, 729-740.

Aghaloo, T., Cowan, C.M., Zhang, X., Freymiller, E., Soo, C., Wu, B., Ting, K., and Zhang, Z. (2010). The effect of NELL1 and bone morphogenetic protein-2 on calvarial bone regeneration. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons *68*, 300-308.

Ai-Aql, Z.S., Alagl, A.S., Graves, D.T., Gerstenfeld, L.C., and Einhorn, T.A. (2008). Molecular mechanisms controlling bone formation during fracture healing and distraction osteogenesis. Journal of dental research *87*, 107-118.

Akeel, S., El-Awady, A., Hussein, K., El-Refaey, M., Elsalanty, M., Sharawy, M., and Al-Shabrawey, M. (2012). Recombinant bone morphogenetic protein-2 induces up-regulation of vascular endothelial growth factor and interleukin 6 in human pre-osteoblasts: role of reactive oxygen species. Archives of oral biology *57*, 445-452.

Allori, A.C., Sailon, A.M., and Warren, S.M. (2008a). Biological basis of bone formation, remodeling, and repair-part I: biochemical signaling molecules. Tissue engineering Part B, Reviews *14*, 259-273.

Allori, A.C., Sailon, A.M., and Warren, S.M. (2008b). Biological basis of bone formation, remodeling, and repair-part II: extracellular matrix. Tissue engineering Part B, Reviews *14*, 275-283.

Amini, A.R., Laurencin, C.T., and Nukavarapu, S.P. (2012). Bone tissue engineering: recent advances and challenges. Critical reviews in biomedical engineering *40*, 363-408.

Attisano, L., and Wrana, J.L. (2002). Signal transduction by the TGF-beta superfamily. Science *296*, 1646-1647.

Bais, M., McLean, J., Sebastiani, P., Young, M., Wigner, N., Smith, T., Kotton, D.N., Einhorn, T.A., and Gerstenfeld, L.C. (2009). Transcriptional analysis of fracture healing and the induction of embryonic stem cell-related genes. PloS one *4*, e5393.

Baroli, B. (2009). From natural bone grafts to tissue engineering therapeutics: Brainstorming on pharmaceutical formulative requirements and challenges. Journal of pharmaceutical sciences *98*, 1317-1375.

Beg, A.A., Sha, W.C., Bronson, R.T., Ghosh, S., and Baltimore, D. (1995). Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. Nature *376*, 167-170.

Bellows, C.G., Reimers, S.M., and Heersche, J.N. (1999). Expression of mRNAs for type-I collagen, bone sialoprotein, osteocalcin, and osteopontin at different stages of osteoblastic differentiation and their regulation by 1,25 dihydroxyvitamin D3. Cell and tissue research *297*, 249-259.

Bhattacharyya, T., Levin, R., Vrahas, M.S., and Solomon, D.H. (2005). Nonsteroidal antiinflammatory drugs and nonunion of humeral shaft fractures. Arthritis and rheumatism *53*, 364-367.

Blokhuis, T.J., Calori, G.M., and Schmidmaier, G. (2013). Autograft versus BMPs for the treatment of non-unions: what is the evidence? Injury *44 Suppl 1*, S40-42.

Bonizzi, G., and Karin, M. (2004). The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends in immunology *25*, 280-288.

Boskey, A.L., and Posner, A.S. (1984). Bone structure, composition, and mineralization. The Orthopedic clinics of North America *15*, 597-612.

Bouleftour, W., Boudiffa, M., Wade-Gueye, N.M., Bouet, G., Cardelli, M., Laroche, N., Vanden-Bossche, A., Thomas, M., Bonnelye, E., Aubin, J.E.*, et al.* (2014). Skeletal development of mice lacking bone sialoprotein (BSP)--impairment of long bone growth and progressive establishment of high trabecular bone mass. PloS one *9*, e95144.

Caron, M.M., Emans, P.J., Surtel, D.A., Cremers, A., Voncken, J.W., Welting, T.J., and van Rhijn, L.W. (2012). Activation of NF-kappaB/p65 facilitates early chondrogenic differentiation during endochondral ossification. PloS one *7*, e33467.

Carreira, A.C., Lojudice, F.H., Halcsik, E., Navarro, R.D., Sogayar, M.C., and Granjeiro, J.M. (2014). Bone morphogenetic proteins: facts, challenges, and future perspectives. Journal of dental research *93*, 335-345.

Chambers, C.A. (1994). TKO'ed: lox, stock and barrel. BioEssays : news and reviews in molecular, cellular and developmental biology *16*, 865-868.

Chang, J., Wang, Z., Tang, E., Fan, Z., McCauley, L., Franceschi, R., Guan, K., Krebsbach, P.H., and Wang, C.Y. (2009). Inhibition of osteoblastic bone formation by nuclear factor-kappaB. Nature medicine *15*, 682-689.

Chen, G., Deng, C., and Li, Y.P. (2012). TGF-beta and BMP signaling in osteoblast differentiation and bone formation. International journal of biological sciences *8*, 272-288.

Chenard, K.E., Teven, C.M., He, T.C., and Reid, R.R. (2012). Bone morphogenetic proteins in craniofacial surgery: current techniques, clinical experiences, and the future of personalized stem cell therapy. Journal of biomedicine & biotechnology *2012*, 601549.

Cho, H.H., Shin, K.K., Kim, Y.J., Song, J.S., Kim, J.M., Bae, Y.C., Kim, C.D., and Jung, J.S. (2010). NF-kappaB activation stimulates osteogenic differentiation of mesenchymal stem cells derived from human adipose tissue by increasing TAZ expression. Journal of cellular physiology *223*, 168-177.

Clohisy, J.C., Hirayama, T., Frazier, E., Han, S.K., and Abu-Amer, Y. (2004). NF-kB signaling blockade abolishes implant particle-induced osteoclastogenesis. Journal of orthopaedic research : official publication of the Orthopaedic Research Society *22*, 13-20.

Cunningham, N.S., Paralkar, V., and Reddi, A.H. (1992). Osteogenin and recombinant bone morphogenetic protein 2B are chemotactic for human monocytes and stimulate transforming growth factor beta 1 mRNA expression. Proceedings of the National Academy of Sciences of the United States of America *89*, 11740-11744.

Dimitriou, R., Jones, E., McGonagle, D., and Giannoudis, P.V. (2011). Bone regeneration: current concepts and future directions. BMC medicine *9*, 66.

Dimitriou, R., Tsiridis, E., and Giannoudis, P.V. (2005). Current concepts of molecular aspects of bone healing. Injury *36*, 1392-1404.

Edgar, C.M., Chakravarthy, V., Barnes, G., Kakar, S., Gerstenfeld, L.C., and Einhorn, T.A. (2007). Autogenous regulation of a network of bone morphogenetic proteins (BMPs) mediates the osteogenic differentiation in murine marrow stromal cells. Bone *40*, 1389-1398.

Einhorn, T.A. (1998). The cell and molecular biology of fracture healing. Clinical orthopaedics and related research, S7-21.

Farhadieh, R.D., Gianoutsos, M.P., Yu, Y., and Walsh, W.R. (2004). The role of bone morphogenetic proteins BMP-2 and BMP-4 and their related postreceptor signaling system (Smads) in distraction osteogenesis of the mandible. The Journal of craniofacial surgery *15*, 714-718.

Feng, J.Q., Xing, L., Zhang, J.H., Zhao, M., Horn, D., Chan, J., Boyce, B.F., Harris, S.E., Mundy, G.R., and Chen, D. (2003). NF-kappaB specifically activates BMP-2 gene expression in growth plate chondrocytes in vivo and in a chondrocyte cell line in vitro. The Journal of biological chemistry *278*, 29130-29135.

Feng, X., Feng, G., Xing, J., Shen, B., Li, L., Tan, W., Xu, Y., Liu, S., Liu, H., Jiang, J.*, et al.* (2013). TNF-alpha triggers osteogenic differentiation of human dental pulp stem cells via the NF-kappaB signalling pathway. Cell biology international *37*, 1267-1275.

Franz-Odendaal, T.A. (2011). Induction and patterning of intramembranous bone. Frontiers in bioscience *16*, 2734-2746.

Fukui, N., Zhu, Y., Maloney, W.J., Clohisy, J., and Sandell, L.J. (2003). Stimulation of BMP-2 expression by pro-inflammatory cytokines IL-1 and TNF-alpha in normal and osteoarthritic chondrocytes. The Journal of bone and joint surgery American volume *85-A Suppl 3*, 59-66.

Geiger, M., Li, R.H., and Friess, W. (2003). Collagen sponges for bone regeneration with rhBMP-2. Advanced drug delivery reviews *55*, 1613-1629.

Gerstenfeld, L.C., Cho, T.J., Kon, T., Aizawa, T., Tsay, A., Fitch, J., Barnes, G.L., Graves, D.T., and Einhorn, T.A. (2003a). Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research *18*, 1584-1592.

Gerstenfeld, L.C., Cullinane, D.M., Barnes, G.L., Graves, D.T., and Einhorn, T.A. (2003b). Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. Journal of cellular biochemistry *88*, 873-884.

Giannoudis, P.V., Dinopoulos, H., and Tsiridis, E. (2005). Bone substitutes: an update. Injury *36 Suppl 3*, S20-27.

Gordon, J.A., Tye, C.E., Sampaio, A.V., Underhill, T.M., Hunter, G.K., and Goldberg, H.A. (2007). Bone sialoprotein expression enhances osteoblast differentiation and matrix mineralization in vitro. Bone *41*, 462-473.

Harada, S., and Rodan, G.A. (2003). Control of osteoblast function and regulation of bone mass. Nature *423*, 349-355.

Hayden, M.S., and Ghosh, S. (2008). Shared principles in NF-kappaB signaling. Cell *132*, 344-362.

Helbing, T., Rothweiler, R., Ketterer, E., Goetz, L., Heinke, J., Grundmann, S., Duerschmied, D., Patterson, C., Bode, C., and Moser, M. (2011). BMP activity controlled by BMPER regulates the proinflammatory phenotype of endothelium. Blood *118*, 5040-5049.

Hess, K., Ushmorov, A., Fiedler, J., Brenner, R.E., and Wirth, T. (2009). TNFalpha promotes osteogenic differentiation of human mesenchymal stem cells by triggering the NF-kappaB signaling pathway. Bone *45*, 367-376.

Hsu, H., Lacey, D.L., Dunstan, C.R., Solovyev, I., Colombero, A., Timms, E., Tan, H.L., Elliott, G., Kelley, M.J., Sarosi, I.*, et al.* (1999). Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. Proceedings of the National Academy of Sciences of the United States of America *96*, 3540-3545.

Huang, R.L., Yuan, Y., Zou, G.M., Liu, G., Tu, J., and Li, Q. (2014). LPS-stimulated inflammatory environment inhibits BMP-2-induced osteoblastic differentiation through crosstalk between TLR4/MyD88/NF-kappaB and BMP/Smad signaling. Stem cells and development *23*, 277-289.

Iotsova, V., Caamano, J., Loy, J., Yang, Y., Lewin, A., and Bravo, R. (1997). Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2. Nature medicine *3*, 1285-1289.

Javed, A., Chen, H., and Ghori, F.Y. (2010). Genetic and transcriptional control of bone formation. Oral and maxillofacial surgery clinics of North America *22*, 283-293, v.

Jeffcoach, D.R., Sams, V.G., Lawson, C.M., Enderson, B.L., Smith, S.T., Kline, H., Barlow, P.B., Wylie, D.R., Krumenacker, L.A., McMillen, J.C.*, et al.* (2014). Nonsteroidal anti-inflammatory drugs' impact on nonunion and infection rates in long-bone fractures. The journal of trauma and acute care surgery *76*, 779-783.

Katavic, V., Grcevic, D., Lukic, I.K., Vucenik, V., Kovacic, N., Kalajzic, I., and Marusic, A. (2003). Non-functional Fas ligand increases the formation of cartilage early in the endochondral bone induction by rhBMP-2. Life sciences *74*, 13-28.

Kawabata, M., Imamura, T., Inoue, H., Hanai, J., Nishihara, A., Hanyu, A., Takase, M., Ishidou, Y., Udagawa, Y., Oeda, E.*, et al.* (1999). Intracellular signaling of the TGF-beta superfamily by Smad proteins. Annals of the New York Academy of Sciences *886*, 73-82.

Khan, S.N., Solaris, J., Ramsey, K.E., Yang, X., Bostrom, M.P., Stephan, D., and Daluiski, A. (2008). Identification of novel gene expression in healing fracture callus tissue by DNA microarray. HSS journal : the musculoskeletal journal of Hospital for Special Surgery *4*, 149-160.

Khanal, A., Yoshioka, I., Tominaga, K., Furuta, N., Habu, M., and Fukuda, J. (2008). The BMP signaling and its Smads in mandibular distraction osteogenesis. Oral diseases *14*, 347-355.

Kim, C.S., Kim, J.I., Kim, J., Choi, S.H., Chai, J.K., Kim, C.K., and Cho, K.S. (2005). Ectopic bone formation associated with recombinant human bone morphogenetic proteins-2 using absorbable collagen sponge and beta tricalcium phosphate as carriers. Biomaterials *26*, 2501-2507.

Komori, T. (2005). Regulation of skeletal development by the Runx family of transcription factors. Journal of cellular biochemistry *95*, 445-453.

Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R.T., Gao, Y.H., Inada, M.*, et al.* (1997). Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell *89*, 755-764.

Kon, T., Cho, T.J., Aizawa, T., Yamazaki, M., Nooh, N., Graves, D., Gerstenfeld, L.C., and Einhorn, T.A. (2001). Expression of osteoprotegerin, receptor activator of NF-kappaB ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research *16*, 1004-1014.

Kugimiya, F., Kawaguchi, H., Kamekura, S., Chikuda, H., Ohba, S., Yano, F., Ogata, N., Katagiri, T., Harada, Y., Azuma, Y.*, et al.* (2005). Involvement of endogenous bone morphogenetic protein (BMP) 2 and BMP6 in bone formation. The Journal of biological chemistry *280*, 35704-35712.

Lee, K.B., Murray, S.S., Taghavi, C.E., Song, K.J., Brochmann, E.J., Johnson, J.S., Keorochana, G., Liao, J.C., and Wang, J.C. (2011). Bone morphogenetic protein-binding peptide reduces the inflammatory response to recombinant human bone morphogenetic protein-2 and recombinant human bone morphogenetic protein-7 in a rodent model of soft-tissue inflammation. The spine journal : official journal of the North American Spine Society *11*, 568-576.

Lee, M.H., Kim, Y.J., Kim, H.J., Park, H.D., Kang, A.R., Kyung, H.M., Sung, J.H., Wozney, J.M., Kim, H.J., and Ryoo, H.M. (2003). BMP-2-induced Runx2 expression is mediated by Dlx5, and TGF-beta 1 opposes the BMP-2-induced osteoblast differentiation by suppression of Dlx5 expression. The Journal of biological chemistry *278*, 34387-34394.

Li, J., Yan, M., Wang, Z., Jing, S., Li, Y., Liu, G., Yu, J., and Fan, Z. (2014). Effects of canonical NF-kappaB signaling pathway on the proliferation and odonto/osteogenic differentiation of human stem cells from apical papilla. BioMed research international *2014*, 319651.

Lin, C.Y., Chang, Y.H., Li, K.C., Lu, C.H., Sung, L.Y., Yeh, C.L., Lin, K.J., Huang, S.F., Yen, T.C., and Hu, Y.C. (2013). The use of ASCs engineered to express BMP2 or TGF-beta3 within scaffold constructs to promote calvarial bone repair. Biomaterials *34*, 9401-9412.

Lu, Z., Wang, G., Dunstan, C.R., Chen, Y., Lu, W.Y., Davies, B., and Zreiqat, H. (2013). Activation and promotion of adipose stem cells by tumour necrosis factor-alpha preconditioning for bone regeneration. Journal of cellular physiology *228*, 1737-1744.

Luu, H.H., Song, W.X., Luo, X., Manning, D., Luo, J., Deng, Z.L., Sharff, K.A., Montag, A.G., Haydon, R.C., and He, T.C. (2007). Distinct roles of bone morphogenetic proteins in osteogenic differentiation of mesenchymal stem cells. Journal of orthopaedic research : official publication of the Orthopaedic Research Society *25*, 665-677.

Malaval, L., Monfoulet, L., Fabre, T., Pothuaud, L., Bareille, R., Miraux, S., Thiaudiere, E., Raffard, G., Franconi, J.M., Lafage-Proust, M.H.*, et al.* (2009). Absence of bone sialoprotein (BSP) impairs cortical defect repair in mouse long bone. Bone *45*, 853-861.

Miyazono, K. (2000a). Positive and negative regulation of TGF-beta signaling. Journal of cell science *113 (Pt 7)*, 1101-1109.

Miyazono, K. (2000b). TGF-beta signaling by Smad proteins. Cytokine & growth factor reviews *11*, 15-22.

Monfoulet, L., Malaval, L., Aubin, J.E., Rittling, S.R., Gadeau, A.P., Fricain, J.C., and Chassande, O. (2010). Bone sialoprotein, but not osteopontin, deficiency impairs the mineralization of regenerating bone during cortical defect healing. Bone *46*, 447-452.

Mori-Akiyama, Y., Akiyama, H., Rowitch, D.H., and de Crombrugghe, B. (2003). Sox9 is required for determination of the chondrogenic cell lineage in the cranial neural crest. Proceedings of the National Academy of Sciences of the United States of America *100*, 9360-9365.

Mountziaris, P.M., and Mikos, A.G. (2008). Modulation of the inflammatory response for enhanced bone tissue regeneration. Tissue engineering Part B, Reviews *14*, 179-186.

Mountziaris, P.M., Spicer, P.P., Kasper, F.K., and Mikos, A.G. (2011). Harnessing and modulating inflammation in strategies for bone regeneration. Tissue engineering Part B, Reviews *17*, 393-402.

Nakahama, K. (2010). Cellular communications in bone homeostasis and repair. Cellular and molecular life sciences : CMLS *67*, 4001-4009.

Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J.M., Behringer, R.R., and de Crombrugghe, B. (2002). The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell *108*, 17-29.

Novack, D.V. (2011). Role of NF-kappaB in the skeleton. Cell research *21*, 169-182.

Oeckinghaus, A., and Ghosh, S. (2009). The NF-kappaB family of transcription factors and its regulation. Cold Spring Harbor perspectives in biology *1*, a000034.

Otto, F., Thornell, A.P., Crompton, T., Denzel, A., Gilmour, K.C., Rosewell, I.R., Stamp, G.W., Beddington, R.S., Mundlos, S., Olsen, B.R.*, et al.* (1997). Cbfa1, a candidate gene for

cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell *89*, 765-771.

Owens, J.M., Gallagher, A.C., and Chambers, T.J. (1996). Bone cells required for osteoclastic resorption but not for osteoclastic differentiation. Biochemical and biophysical research communications *222*, 225-229.

Peng, Y., Kang, Q., Cheng, H., Li, X., Sun, M.H., Jiang, W., Luu, H.H., Park, J.Y., Haydon, R.C., and He, T.C. (2003). Transcriptional characterization of bone morphogenetic proteins (BMPs)-mediated osteogenic signaling. Journal of cellular biochemistry *90*, 1149-1165.

Rahman, C.V., Ben-David, D., Dhillon, A., Kuhn, G., Gould, T.W., Muller, R., Rose, F.R., Shakesheff, K.M., and Livne, E. (2014). Controlled release of BMP-2 from a sintered polymer scaffold enhances bone repair in a mouse calvarial defect model. Journal of tissue engineering and regenerative medicine *8*, 59-66.

Rawadi, G., Vayssiere, B., Dunn, F., Baron, R., and Roman-Roman, S. (2003). BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research *18*, 1842-1853.

Rifas, L. (2006). T-cell cytokine induction of BMP-2 regulates human mesenchymal stromal cell differentiation and mineralization. Journal of cellular biochemistry *98*, 706-714.

Rodan, G.A., and Noda, M. (1991). Gene expression in osteoblastic cells. Critical reviews in eukaryotic gene expression *1*, 85-98.

Rundle, C.H., Wang, H., Yu, H., Chadwick, R.B., Davis, E.I., Wergedal, J.E., Lau, K.H., Mohan, S., Ryaby, J.T., and Baylink, D.J. (2006). Microarray analysis of gene expression during the inflammation and endochondral bone formation stages of rat femur fracture repair. Bone *38*, 521-529.

Schmidt-Bleek, K., Schell, H., Schulz, N., Hoff, P., Perka, C., Buttgereit, F., Volk, H.D., Lienau, J., and Duda, G.N. (2012). Inflammatory phase of bone healing initiates the regenerative healing cascade. Cell and tissue research *347*, 567-573.

Simpson, A.H., Mills, L., and Noble, B. (2006). The role of growth factors and related agents in accelerating fracture healing. The Journal of bone and joint surgery British volume *88*, 701-705.

Soysa, N.S., and Alles, N. (2009). NF-kappaB functions in osteoclasts. Biochemical and biophysical research communications *378*, 1-5.

Stein, G.S., Lian, J.B., and Owen, T.A. (1990). Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *4*, 3111-3123.

Suda, T., Nakamura, I., Jimi, E., and Takahashi, N. (1997). Regulation of osteoclast function. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research *12*, 869-879.

Suda, T., Takahashi, N., Udagawa, N., Jimi, E., Gillespie, M.T., and Martin, T.J. (1999). Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocrine reviews *20*, 345-357.

Swarnkar, G., Zhang, K., Mbalaviele, G., Long, F., and Abu-Amer, Y. (2014). Constitutive activation of IKK2/NF-kappaB impairs osteogenesis and skeletal development. PloS one *9*, e91421.

Szpalski, C., Barr, J., Wetterau, M., Saadeh, P.B., and Warren, S.M. (2010). Cranial bone defects: current and future strategies. Neurosurgical focus *29*, E8.

ten Berge, D., Brouwer, A., Korving, J., Martin, J.F., and Meijlink, F. (1998). Prx1 and Prx2 in skeletogenesis: roles in the craniofacial region, inner ear and limbs. Development *125*, 3831-3842.

Tsuji, K., Bandyopadhyay, A., Harfe, B.D., Cox, K., Kakar, S., Gerstenfeld, L., Einhorn, T., Tabin, C.J., and Rosen, V. (2006). BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. Nature genetics *38*, 1424-1429.

Tuan, R.S., Boland, G., and Tuli, R. (2003). Adult mesenchymal stem cells and cell-based tissue engineering. Arthritis research & therapy *5*, 32-45.

Urist, M.R. (1965). Bone: formation by autoinduction. Science *150*, 893-899.

Urist, M.R., and Strates, B.S. (1971). Bone morphogenetic protein. Journal of dental research *50*, 1392-1406.

Vallabhapurapu, S., and Karin, M. (2009). Regulation and function of NF-kappaB transcription factors in the immune system. Annual review of immunology *27*, 693-733.

Wang, E.A., Rosen, V., Cordes, P., Hewick, R.M., Kriz, M.J., Luxenberg, D.P., Sibley, B.S., and Wozney, J.M. (1988). Purification and characterization of other distinct bone-inducing factors. Proceedings of the National Academy of Sciences of the United States of America *85*, 9484-9488.

Yu, K., Xu, J., Liu, Z., Sosic, D., Shao, J., Olson, E.N., Towler, D.A., and Ornitz, D.M. (2003). Conditional inactivation of FGF receptor 2 reveals an essential role for FGF signaling in the regulation of osteoblast function and bone growth. Development *130*, 3063-3074.

Zhang, X., Schwarz, E.M., Young, D.A., Puzas, J.E., Rosier, R.N., and O'Keefe, R.J. (2002). Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair. The Journal of clinical investigation *109*, 1405-1415.

CHAPTER 2

EFFECT OF NF-κB HAPLOINSUFFICIENCY ON CALVARIAL BONE HEALING

INTRODUCTION

Critical sized bony defect caused by congenital disorders, trauma, tumors or infection remains a challenge for dental and orthopedic surgeons and represents a great economic burden to health resources (Amini et al., 2012; Baroli, 2009). The treatment for such clinical conditions requires enhancement and acceleration of bone regeneration with the application of bone grafts (Pneumaticos et al., 2010; Tevlin et al., 2014). To avoid the intrinsic shortcoming of natural bone grafts (i.e donor site morbidity, immunogenic property, pain, etc.), synthetic bone graft substitutes using biocompatible scaffold carrier, with osteoinductive agents including BMPs and other growth factors, have been developed (Amini et al., 2012; Chenard et al., 2012).

Bone morphogenic protein 2 (BMP2) is a growth factor that belongs to the transforming growth factor-β superfamily. At the cellular level, BMP2 triggers the phosphorylation of downstream Smad1, 5 and 8, leading to the transcriptional regulation of major osteogenic markers such as *Runx2*, *Sp7*, *Alp*, *Bsp* and *Bglap* (Chen et al., 1997; Luu et al., 2007; Nakashima et al., 2002). As a potent and promising osteogenic/osteoinducing factor, the Food and Drug Administration (FDA) approved BMP2 for the treatment of non-union fractures and spinal fusion in 2002. Both early clinical trial, as well as basic science

research experiments using animal models, showed improved bone formation and healing following local delivery of BMP2 by an absorbable collagen carrier (Kim et al., 2005; Lin et al., 2013; Simpson et al., 2006). BMP2-mediated bone regeneration involves in an initial inflammatory response associated with the production of cytokines such as tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6) (Gerstenfeld et al., 2003). *In vitro* studies revealed that BMP2 induces expression of inflammatory mediators in various cell types including preosteoblasts (Akeel et al., 2012; Helbing et al., 2011). Inflammatory signals are crucial for the recruitment and subsequent differentiation of stem and progenitor cells, reflecting a signaling mechanism through which BMP2 promotes bone regeneration. Furthermore, the osteoinductive capability of BMP2 is impaired in the absence of proper inflammatory signals (Katavic et al., 2003; Zhang et al., 2002).

Nuclear factor kappa B (NF-κB) is recognized as a master regulator for the immune system and inflammatory response. The NF-κB family is comprised of transcription factors that mediate many biological activities including cell proliferation, survival and apoptosis (Li and Verma, 2002). The NF-κB family consists of 5 proteins in mammalian cells: RelA (p65), RelB, c-Rel, p50 and p52. These members all share an N-terminal Rel homology domain which is responsible for dimerization and DNA binding. The p65-p50 heterodimer is the most common form of NF-κB in mammals (Oeckinghaus and Ghosh, 2009; Vallabhapurapu and Karin, 2009). In unstimulated cells, inactive NF-κB complexes remain in the cytoplasm in association with IκB proteins (IκBα, IκBβ and IκBε). Upon activation, IκB proteins are phosphorylated by the IκB kinase complex (IKKα, IKKβ and IKKγ), resulting in the release of the NF-κB dimers into the nucleus. Many pro-inflammatory factors such as receptor

activator of NF-κB ligand (RANKL) and TNFα are able to induce NF-κB activation through the classical pathway, which requires the p65-p50 and cRel-p50 heterodimers. (Hayden and Ghosh, 2008; Oeckinghaus and Ghosh, 2009).

NF-κB signaling is required for bone development. Its interaction with bone resorbing cells (osteoclasts) and role in relevant inflammatory bone disorders has been extensively investigated (Abu-Amer, 2013). Growing evidence suggests that NF-κB, as a vital inflammatory mediator, is vital for osteoblastic regulation and osteogenesis (Caron et al., 2012; Cho et al., 2010; Hess et al., 2009). NF-κB/p65 signaling activation facilitates BMP2-mediated chondrogenic differentiation during endochondral ossification (Caron et al., 2012). Moreover, TNFα treatment and p65 activation positively influences osteogenic differentiation of human mesenchymal stromal cells (hMSCs) *via* the regulation of *BMP2* expression (Hess et al., 2009). These findings indicate an interaction between NF-κB mediated inflammatory signals and BMP2-induced bone formation and repair. The precise role of NF-κB in the context of bone forming cells (osteoblasts), however, remains unclear. Moreover, little is known regarding the role of the NF-κB signaling pathway in the osteoinductivity of BMP2 during bone healing.

In the present study, we sought to investigate the interplay between NF-κB signaling and BMP2 mediated bone repair in a critical size calvarial defect model using a genetically modified mouse model. Given the fact that the global deletion of p65, a major NF-κB component, is embryonic lethal, we utilized a Cre/Loxp strategy to generate transgenic mice with a NF-κB deficiency restricted to bone tissue. By crossing the p65 floxed mice with Osx-

Cre mice, we obtained mice with a p65 haploinsufficiency in osteoblastic cell lineages. p65 expression was regulated by the *Sp7* promoter.

Uncovering the function of NF-κB during BMP2-mediated bone repair will enhance the understanding of bone regeneration mechanisms, thereby providing new therapeutic targets for improved treatment that promotes integrated skeletal regeneration.

Materials and Methods

Mice

p65-floxed mice were crossed with Osterix-Cre mice (Osx-Cre) in order to generate mice with an osteoblast-restricted p65 ablation ($p65^{fl/+}$Osx-Cre) (see Figure 2.1). p65 floxed mice ($p65^{fl/fl}$) in which exons 5-8 of p65 were flanked with loxp constructs were provided by Dr. Albert S Baldwin, University of North Carolina at Chapel Hill (Steinbrecher et al., 2008). Osterix-Cre mice (Osx-Cre) which express a GFP fused Cre recombinase gene downstream of the *Sp7* promoter were purchased from the Jackson Laboratory (Bar Harbor, ME). The phenotype of Osx-Cre mice has been described previously (Rodda and McMahon, 2006). All animals were on the C57BL/6 background.

Genotyping of mice was performed using PCR of genomic DNA, which was isolated from tail samples taken from mice. Primer sequences used for genotyping are listed in Table 2.1. The animals were housed in a pathogen-free environment with controlled temperature and humidity. Mice were maintained on a 12-hour light/dark cycle. Routine husbandry procedures included cage cleaning, feeding and watering and were conducted every other day. All procedures were performed in accordance with the animal protocol approved by the Institutional Animal Care and Use Committees of the University of North Carolina (IACUC).

Calvarial Defect Model and Surgery procedures

Mice at 9-11 weeks old (n=5-7) were anesthetized by intraperitoneal injection of ketamine/xylazine (124/8.8 mg/kg) (PSS, West Columbia, SC). After the incision region on

the head was shaved, skin was sterilized using 70% ethanol and betadine. A midline incision parallel to the sagittal suture was made using a scalpel in order to expose the parietal bones of the animal. A trephine bur attached to a dental handpiece was then used to create a defect with a diameter of 3.5 mm (Figure 2.2). Great caution was taken to minimize injury to the dura mater. An absorbable collagen sponge (diameter ≈ 4 mm, thickness ≈ 1 mm) (DuraGen, Integra, Plainsboro, NJ) containing saline or 500 ng rhBMP2 (Medtronic, Minneapolis, MN) was placed over the defect. The collagen sponge was molded into a disk shape (diameter ≈4 mm, thickness ≈1 mm) using a tissue biopsy punch. The skin was sutured using absorbable 4 or 5-0 chromic gut sutures (Ethicon, Somerville, NJ). Animals were allowed to recover on a heating pad before being transferred to clean recovery cages with soft bedding. Pain was managed using ketoprofen (5mg/kg, s.c) following surgery. Full time veterinarians were available at all times in the facility to ensure the health and care of the animals. All surgical procedures were approved by the Institutional Animal Care and Use Committees of the University of North Carolina (IACUC).

Micro-CT imaging and analysis

Separate groups of mice were euthanized 4 and 8 weeks following surgery, using CO_2 asphyxiation followed by cervical dislocation. The heads were harvested and immediately soaked in 4% paraformaldehyde for 3 days at room temperature to achieve fixation. Calvarial defects of studied animals were scanned following fixation, using a µCT imaging system (Skyscan 1074HR, Aartselaar, Belgium) at a resolution of 20.5 µm/pixel. The following settings were used for the scan: X-ray voltage of 40 kVp and anode current of 1000 µA for the 360 rotational steps. Scanned images were reconstructed using NRecon

software (Skyscan, Aartselaar, Belgium). Representative 3-Dementional images were created using CTAn and CTVol software provided by Skyscan.

In order to compare the level of the healing between $p65^{fl/+}$ and $p65^{fl/+}$Osx-Cre mice, a parameter "healing ratio" was introduced into the analysis. This was defined as follows: Healing ratio= (1-[Current Volume of defect – Original Volume of defect]) X 100 percent. The volume of the calvarial defect was determined using ITK-SNAP (www.itksnap.org).

Histology analysis

For separate groups of animals, heads were harvested 4 and 8 weeks following surgeries. The tissue was immediately immersed into 4% paraformaldehyde for 3 days and then decalcified in 10% Ethylenediamine-tetraacetic acid (EDTA, Sigma, St. Louis, MO, USA) at pH 7.4 at 4 ^{o}C for 2 weeks. The samples were then dehydrated through a serial dilution of ethanol before paraffin embedding. The embedded samples were cut at the center of defect into 5 μm thick sections. Paraffin sections were then stained for Hematoxylin and eosin as well as Masson's trichrome staining according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). The stained sections were examined and photographed using a light microscope (Nikon Eclipse50i, Nikon, Melville, NY).

Immunohistochemical analysis

To evaluate the osteoblast differentiation between groups, immunohistochemical analysis was performed using a rabbit anti-BSP antibody (LF-84), kindly provided by Dr. Larry Fisher (NIDCR, Bethesda, MD). Briefly, paraffin sections of samples were deparaffinized in Hemo-D (Fisher Scientific, Waltham, MA) and then rehydrated through graded alcohol

(50%-100%). Antigen was retrieved by incubating specimens in 10 mM sodium citrate buffer (PH=6.0) for 20 minutes at 70 ^{o}C. Endogenous peroxidase was quenched using 3% hydrogen peroxide in methanol. The slides were then incubated with primary antibody overnight at 4 ^{o}C after blocking with 2.5% normal goat serum in 0.1% bovine serum albumin (BSA) for 30 minutes at room temperature. The Avidin/Biotin Blocking Kit (SP-2001, Vector Laboratories, Burlingame, CA) was used to block all endogenous biotin and avidin binding sites present in tissues. A biotinylated secondary antibody was applied to the slides for 45 minutes at room temperature. The immunoreaction was amplified using RTU ABC Elite reagents (Vector Laboratories, Burlingame, CA). Finally, the slides were detected using DAB substrate kit (Vector Laboratories, Burlingame, CA) and then counterstained with hematoxylin (Sigma-Aldrich, St. Louis, MO) and mounted. Non-specific rabbit IgG was used in place of a primary antibody for the negative control slides. The primary antibody was used at a 1:400 dilution and the secondary antibody was performed at 1:800 dilution in 2.5% goat serum in 0.1% BSA. Photographs of stained sections were taken using a Nikon Eclipse 50i microscope (Nikon, Melville, NY).

CFU-F and CFU-OB assay

Animals were euthanized at 13-14 weeks old. Following euthanisa, and the femur was separated from the tibia on both hind limbs. Skin and muscle were then carefully removed. A scalpel was used to cut the epiphysis of the long bones to expose the bone marrow cavity. Bone marrow stromal cells were isolated from femur and tibia by flushing the bone marrow with a 27G syringe (BD, Franklin Lakes, NJ). The cells were then seeded into 6-well plates (2×10^6 cells/well) in MesenCult™ MSC Basal Medium (STEMCELL

Technologies, Vancouver, British Columbia, Canada) supplemented with MesenCult™ Mesenchymal Stem Cell Stimulatory Supplements (STEMCELL Technologies), 2 mM L-Glutamine and 100 units/ml penicillin/streptomycin. The medium was changed after 24 hours to allow adequate adhesion of cells. For the CFU-OB assay, rhBMP2 (50 ng/ml, Medtronic) and 0.2 mM ascorbic acid were added into the culture as an osteogenic supplement (osteogenic medium). The medium was changed every 2 days for a period of 14 days.

After 14 days of culture, the cells were washed with PBS twice and then fixed with citrate-acetone-formaldehyde fixation solution for 1 minute. For the CFU-F assay, the cells were stained with 2.5% crystal violet for 20 minutes at room temperature. Formation of osteoblastic colonies was detected using an alkaline phosphatase assay (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Colonies containing more than 50 cells were enumerated under a light microscope.

Primary osteoprogenitors isolation

The osteoprogenitor cells were isolated based on an established protocol (STEMCELL Technologies, Vancouver, British Columbia, Canada). Briefly, tibia and femur bones from adult mice (13-14 week old) were separated and trimmed to remove skin and muscle. Bone marrow was flushed away using a 27G syringe with HG-DMEM medium. A scalpel was used to slice the compact bone into fine pieces. The bone pieces were incubated in 0.25% Collagenase Type I in PBS containing 20% FBS (STEMCELL Technologies) in a shaking 37 ^{o}C water bath for 45 minutes. Supernatant was collected and filtered through a 70 µm cell

strainer (Falcon, Corning Life Sciences). Collected supernatant was centrifuged at 300 x g for 10 minutes at room temperature. The cell pellet was resuspended and maintained in HG-DMEM regular growth medium (Lonza) until reaching 70% confluence. The medium was changed every other day.

Total RNA isolation from tissue

$p65^{fl/+}$, $p65^{fl/+}$Osx-Cre and Osx- Cre mice (N = 4-5 per group) were subjected to calvarial surgery. The rhBMP2 (500 ng) with the Absorbable Collagen Sponge (ACS) carrier was placed over the defect as described previously. After 4 weeks of surgery, mice were euthanized using CO_2 asphyxiation followed by cervical dislocation. A trephine bur attached to a dental handpiece was used to remove the bony tissue in the defect area. Tissue samples were immediately flash-frozen in liquid nitrogen. The samples were kept at -80 ^{o}C until homogenization procedures. Specimens were homogenized using a mortar and pestle in liquid nitrogen. QIAzol lysis reagent (Qiagen, Valencia, CA, USA) was added into the powdered tissue for further homogenization and total RNA was then extracted using the Qiagen RNeasy Micro kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. RNA concentrations were measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA) and total RNA was then submitted for quantitative real-time PCR analysis.

Quantitative real-time PCR

Primary osteoprogenitors (P1-P3) isolated from animals were seeded into 12-well plates for overnight incubation. rhBMP2 (50 ng/ml, Medtronic), 0.2 mM ascorbic acid and

10 mM β- glycerophosphate were then added to cell cultures to induce osteogenic differentiation. The medium, with or without rhBMP2, was changed every other day. For separate groups of animals, total RNA was extracted using TriZol reagent (Invitrogen, Carlsbad, CA) after 4 and 7 days. Reverse transcription was completed using the SuperScript VILO™ cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. Analysis by quantitative real-time PCR was conducted using Taqman Universal PCR Master Mix (Applied Biosystems, Foster city, CA) with the ABI Prism 7500 system. Standard, commercially available primers and probes for osteogenic markers were used: *Sp7*, *Alp*, *Bsp* and *Satb2* (ABI assay No. Mm00504574_ml, Mm00475831_ml, Mm00492555_ml, Mm00507331_m1). The expression levels of target genes were standardized by housekeeping gene GAPDH (ABI assay No. 4308313) using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Luciferase reporter assay

To test the effect of TNFα on NF-κB activity, C2C12 cells were co-transfected with a NF-κB luciferase reporter (3xkB.luc), Renilla luciferase (pRL-TK, Promega, Madison, WI) and the Attractene transfection reagent (Qiagen, Valencia, CA, USA) in a 48-well plate. A pGL3 plasmid was used as an empty vector control for 3xkB.luc. After 24 hours of transfection, TNFα (Sigma-Aldrich) was added to the transfected cells at various concentration levels (0.1 ng/ml, 1 ng/ml, 10 ng/ml and 100 ng/ml). Luciferase activity was determined using a Dual Luciferase assay (Promega) 12 hours following stimulation with TNFα according to established protocol. The transfection efficiency was normalized using renilla luciferase activity. The results were displayed as fold change.

Immunoblotting analysis

A murine myoblast cell line, C2C12 cells (P11-P14) and primary osteoprogenitors (P1-P3) isolated from animals were seeded on 6- well plates in HG-DMEM (Lonza) supplemented with 10% FBS, 100 units/ml penicillin/streptomycin medium (regular growth medium). The cells were cultured overnight to allow cell adherence. A combination of rhBMP2 (50 ng/ml, Medtronic) and TNFα (10 ng/ml and 100 ng/ml, Sigma-Aldrich) was then added to the C2C12 culture. The protein was isolated using RIPA lysis buffer (Invitrogen) with a mixture of protease and phosphatase inhibitors at 1 and 2 hours following stimulation. For the primary osteoprogenitors, rhBMP2 was added to the culture and total protein was extracted after 15-, 30- and 60-minute intervals.

Protein concentration was determined using a Bradford Protein Assay Kit (Bio-Rad, Hercules, CA). An equal amount of protein (15-20 ug) was then electrophoresed through 10% SDS-polyacrylamide gel (SDS-PAGE) and transferred electrophoretically onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). The membrane was probed for targeted protein expression using primary antibodies after blocking with 5% non-fat milk for 1 hour at room temperature. The following primary antibodies were used: phosphorylated Smad 1/5/8, Smad 1, GAPDH and beta-actin (all from Cell signaling Technology). The membrane was then incubated with a horseradish peroxidase conjugated goat anti-rabbit antibody (Amersham Biosciences, Pittsburgh, PA) for 1 hour at room temperature. Signals were visualized using a chemiluminescent ECL prime kit (Amersham Biosciences). ImageJ software (http://imagej.nih.gov/ij/) was used to perform densitometric analysis.

Statistical analysis

The experiments were performed in duplicate or triplicate for each time point and treatment. Data were expressed as mean ± SD. Difference among groups was determined using student t tests with SPSS software. Statistical significance was defined as $p \leq 0.05$.

Results:

Effects of NF-κB haploinsufficiency on bone healing in a critical size calvarial defect model

Micro-CT assessment of bone regeneration and image analysis

In an attempt to investigate the role of NF-κB signaling in BMP2 mediated bone repair in the perspective of bone forming cells, we utilized transgenic mice with a deficiency in NF-κB in specific to the osteoblastic cell lineage. The $p65^{fl/+}$Osx-Cre mice harbor both an *Ostrix* driven Cre transgene and a floxed p65 gene on one allele, leading to the ablation of p65 on osteoprogenitors. Calvarial defects were generated on $p65^{fl/+}$Osx-Cre (n≥5) and p65 fl/+ (n≥7) mice. An absorbable collagen sponge (ACS) containing rhBMP2 (500 ng), or saline as control, was implanted at the bone defect. The dosage of rhBMP2 (500 ng) was selected to effectively induce bone regneration based on previous ecotopic bone formation experiments performed by our lab (manuscript in preparation) as well as studies by other groups (Behr et al., 2012). To assess the level of new bone formation, micro-CT was performed at 4 and 8 weeks post-surgery. The level of calvarial healing in $p65^{fl/+}$Osx-Cre mice was compared to that of $p65^{fl/+}$ mice, which was used as littermate controls. For optimal interpretation of results, Osx-Cre mice at 4 weeks following surgery were also observed as a control.

As shown in 3D resonstruction images, there was minimal or limited bone formation in ACS+saline at 4 (Figure 2.3) and 8 weeks (Figure 2.4) in the control group. At 4 weeks, no newly formed tissue was observed in $p65^{fl/+}$Osx-Cre mice. The treatment with rhBMP2 effectively enhanced bone healing in both controls and $p65^{fl/+}$Osx-Cre mice. The Osx-Cre mice displayed a similar healing pattern as $p65^{fl/+}$ mice at 4 weeks when treated with saline

or rhBMP2. At 8 weeks, extensively new bone formation was observed in the control group when treated with rhBMP2. However, $p65^{fl/+}$Osx-Cre mice demonstrated decreased bone formation as compared to $p65^{fl/+}$ mice in the rhBMP2 treated group at both time points.

In order to quantitatively evaluate the level of bone healing, we utilized the percentage of the volume of newly regenerated bone tissue to the total defect volume as a parameter (healing ratio). The bone healing ratio of the $p65^{fl/+}$Osx-Cre mice was significantly lower than that of the $p65^{fl/+}$ mice when treated with saline (0 vs 0.21 at 4 week, $p<0.01$; 0.11 vs 0.35 at 8 week, $p<0.01$) or BMP2 (0.42 vs 0.72 at 4 week, $p<0.01$; 0.45 vs 0.91 at 8 week, $p<0.01$) (Figure 2.5). At 4 weeks, the haploinsufficient animals showed significant reduction of bone healing (0 vs 0.17, $p<0.01$, saline group; 0.42 vs 0.77, $p<0.01$, rhBMP2 group) as compared to Osx-Cre mice (Figure 2.5). The healing level in Osx-Cre mice at 8 weeks following implantation was not tested in this study. Moreover, the healing level of Osx-Cre mice showed no difference ($p>0.05$) as compared to $p65^{fl/+}$ mice at 4 weeks in both saline and rhBMP2 groups.

It is important to note that there was no statistical difference in the bone repair level of $p65^{fl/+}$Osx-Cre mice at 4 and 8 weeks in the rhBMP2 group, implicating a defective bone regeneration process in the absence of sufficient NF-κB signaling. The micro-CT analysis indicates that p65 haploinfficiency negatively affects rhBMP2 mediated bone formation during bone healing.

Histological analysis of bone formation

Histologically, the defect area was bridged mainly by large amount of fibrous tissue with an abundant infiltration of inflammatory cells in both animal groups without rhBMP2.

In some defect regions of $p65^{fl/+}$Osx-Cre mice receiving saline, the residual collagen scaffold was clearly observed (Figure 2.6A, "S" indicates the residual collagen sponge). In the rhBMP2 group, moderate new bone formation was observed at 4 weeks, and extensive bony tissue was observed at 8 weeks. Consistent with the micro-CT findings, $p65^{fl/+}$Osx-Cre mice revealed significantly less new bone formation in comparison with control animals. The defect area was markedly filled with connective tissue and few bone-like tissues, both at the periphery of native bones and in the center of the graft. Preosteoblastic like cells were found lining the newly formed bone tissue with osteocytes embedded within lacuna from H&E staining. Bone marrow-like structures with blood vessels as well as osteobclast-like cells were identified in ACS with rhBMP at 8 weeks, indicating the presence of the bone remodelling process.

Masson's thrichrome staining was used to distinguish the unmineralized (osteoid) and mineralized bone matrix. Woven bone formation at the peripheral of the defect and also in the center of graft, was observed in the rhBMP2 group (Figure 2.6B).

At 8 weeks, control animals treated with rhBMP2 demonstrated a high abundance of bone sialoprotein (BSP) (as shown by the dark brown immunohistochemical staining) in the newly formed bone at the peripheral defect (Figure 2.7B). In contrast, $p65^{fl/+}$Osx-Cre mice treated with rhBMP2 demonstrated a weak/light stain, similar to the staining pattern observed in the control group treated ACS+ saline at 8 weeks (Figure 2.7A). BSP is a marker for osteoblastic differentiation and a nucleator for bone mineralization (Gordon et al., 2007).

CFU-F and CFU-OB analysis

The onset of bone injury stimulates the release and homing of multipotential stem cells from the bone marrow niche to the damaged sites for regeneration. Therefore, we intended to assess the number and osteogenic property of early and late osteoprogenitors within the bone marrow MSCs population (Chou et al., 2009).

The CFU-F assay demonstrated that bone marrow stromal cells isolated from $p65^{fl/+}$Osx-Cre mice showed a ~25% reduction of CFU-Fs as compared to the control animals ($p65^{fl/+}$Osx-Cre vs $p65^{fl/+}$, 44 vs 61 colonies/well, $p<0.01$). It is noteworthy that the number of CFU-OB colonies was decreased threefold in $p65^{fl/+}$Osx-Cre mice as compared to the control group ($p65^{fl/+}$Osx-Cre vs $p65^{fl/+}$, 22 vs 56 colonies/well; $p<0.01$) (Figure 2.8B). When bone marrow stromal cells from Osx-Cre mice were used as another control, a statistically significant reduction of CFU-F colonies ($p65^{fl/+}$Osx-Cre vs Osx-Cre, 44 vs 59 colonies/well, $p<0.01$) and CFU-OB colonies ($p65^{fl/+}$Osx-Cre vs Osx-Cre , 22 vs 51 colonies/well, $p<0.01$) were observed in $p65^{fl/+}$Osx-Cre mice. There was no difference observed in colony numbers between Osx-Cre and $p65^{fl/+}$ mice. These obseravations implicate that fewer osteoprogenitors may be available to contribute to bone in mice with partially ablated p65 expression.

Effects of NF-κB haploinsufficiency on BMP2 mediated osteogensis *in vitro*

One of the mechanisms by which BMP2 contributes to bone formation is *via* the regulation of osteoblastic differentiation. To understand whether the impaired bone regeneration directed by BMP2 in NF-κB haploinsufficient animal results from defective osteoblast differentiation, we evaluated the gene expression levels of osteoblastic markers using quantitative real-time PCR in both control and p65 haploinsufficient animals.

Osterix/Sp7 is a transcription factor that promotes the osteogenic lineage commitment as well as osteoprogenitor differentiation (Nakashima et al., 2002). It is regarded as an early marker for osteogenic differentiation. Alkaline phosphatase (ALP) and bone sialoprotein (BSP) are osteogenic markers corresponding to synthesis and maturation of extracellular matrix (Cowles et al., 1998; Harada and Rodan, 2003).

In the absence of osteogenic supplements, osteogenic gene expression was comparable in both groups in all time points. With the addition of rhBMP2, there was significant mRNA upregulation of all osteoblast markers, in both animal groups, at day 4 (Figure 2.9A) However, the expression levels of the *Sp7*, *Alp* and *Bsp* genes were markedly less in the cells of $p65^{fl/+}$Osx-Cre mice at day 4 compared with those of $p65^{fl/+}$ mice ($p<0.05$). Consistent with day 4 results, *Alp* and *Bsp* mRNA levels remained downregulated at day 7 ($p<0.05$) (Figure 2.9B). As a result, the haploinsufficient mice displayed reduced osteoblastic differentiation. This appears to be particularly evident at the early stages of osteoblastic differentiation (Figure 2.9A).

To further investigate the cellular and molecullar events that occur during the bone healing process, we collected the RNA samples from the defect area after 4 weeks of implatation of ACS loaded with rhBMP2 (500 ng). The expression of osteogenic markers were evaluated using real-time PCR. At 4 weeks post-surgery when there was active bone regeneration present in the defect (Figure 2.6), the mRNA level of *Sp7*, *Alp* and *Bsp* were detectable in both animal groups. The $p65^{fl/+}$Osx-Cre mice exhibited a statistically significant decrease in *Sp7*, *Alp* and *Bsp* expression as compared to the $p65^{fl/+}$ mice (Figure 2.10). There was a 55% reduction of *Sp7* expression in $p65^{fl/+}$Osx-Cre mice as compared to $p65^{fl/+}$ mice

($p<0.05$) and over a 70% reduction as compared to Osx-Cre mice ($p<0.01$). Similarly, another early osteoblastic gene, *Satb2*, downstream of *Runx2* was down-regulated by almost 60% in p65 insufficient mice as compared to $p65^{fl/+}$ mice ($p<0.01$) and by 80% as compared to the Osx-Cre group ($p<0.01$) (Conner and Hornick, 2013; Dobreva et al., 2006).

We also oberved an over 50% reduction in *Alp* mRNA expression in $p65^{fl/+}$Osx-Cre mice as compared to both $p65^{fl/+}$ ($p<0.01$) and Osx-Cre mice ($p<0.05$). The expression of *Bsp* was also reduced in $p65^{fl/+}$Osx-Cre mice as compared to $p65^{fl/+}$ mice ($p<0.05$), but not Osx-Cre mice ($p>0.05$). These data implicate a trend of increased expression of *Sp7*, *Alp* and *Satb2* in Osx-Cre mice, as compared to $p65^{fl/+}$ mice. However, there is no statistical significance detected between Osx-Cre and $p65^{fl/+}$ mice. These results suggest that one possible mechanism by which bone healing is impaired in p65 haploinsufficient mice is the reduced preosteoblast population in the regenerated tissue. This may be caused by impaired osteoblastic differentiation of osteoprogenitors within the bone marrow reservoir of the $p65^{fl/+}$Osx-Cre mice(Figure 2.9). Another possibility could be that the migration of progenitors in the $p65^{fl/+}$Osx-Cre mice to injured sites is impaired or delayed.

Effects of NF-κB haploinsufficiency on BMP2 mediated Smads phosphorylation

Upon binding to the BMP2 receptor (BMPR) type I and II complex, BMP2 induces the phosphorylation of downstream Smad 1/5/8, leading to the transcriptional regulation of osteogenic related gene expression. It remains unknown whether or not the downregulation of osteoblastic markers expression in $p65^{fl/+}$Osx-Cre mice was caused by the disruption of BMP2/Smad signaling axis. Osteoprogenitors isolated from control and $p65^{fl/+}$Osx-Cre mice were treated with rhBMP2 (50 ng/ml) for various time courses (15-, 30-

and 60-minutes). Cell lysates were electrophoresed and then probed for anti-phosphorylated Smad 1/5/8 and anti-Smad 1 antibodies. To evaluate the level of p65 expression in cells, anti-p65 antibody was applied for detection. Immediately following 15 minutes stimulation of rhBMP2, the osteoprogenitors isolated from p65 haploinsufficient mice displayed a 40-50% reduction of p65 production (Figure 2.11A-B).

Cell lysates were electrophoresed and then probed for anti-phosphorylated Smad 1/5/8 and anti-Smad 1 antibodies. As shown in Figure 2.11C-D , rhBMP2 at 50 ng/ml successfully induced Smad 1/5/8 phosphorylation following 15 minutes of treatment. Cells from $p65^{fl/+}$Osx-Cre mice displayed decreased phosphorylation of Smad 1/5/8 levels at all time points following BMP2 stimulation (Figure 2.11C-D). The densotometric analysis further confirm that pSmad 1/5/8 levels were significantly reduced in osteoprogenitors from $p65^{fl/+}$Osx-Cre mice (Figure 2.11D). These findings suggest that sufficient NF-κB signaling is crucial for the maintenance of a normal BMP2/Smad signaling axis.

The effects of TNFα on BMP2- mediated Smads signaling in C2C12

To further confirm our previous findings that there is an interaction between NF-κB and BMP2/Smads signaling, we treated C2C12 cells with TNFα and/or BMP2 and evaluated the phosphorylated Smads by immunoblot. TNFα is a pro-inflammatory cytokine and is well-known as a potent activator for the NF-κB pathway. As shown in Figure 2.12A , TNFα was able to induce the transcriptional activity of NF-κB in C2C12 cells by the NF-κB luciferase reporter assay.

In the absence of rhBMP2, TNFα (10 ng/ml and 100 ng/ml) alone did not affect the phosphorylation level of Smad 1/5/8. Upon the stimulation of rhBMP2 (50 ng/ml), there

was an increase in p-Smad expression. Of note, the addition of TNFα further enhanced Smads phosphorylation by rhBMP2 (Figure 2.12B). One consequence of this acute TNFα treatment and NF-κB activation is the enhancement of p-Smad abundance following BMP2 signaling (Figure 2.12C). This is possibly caused by inceased Smad 1 expression induced by TNFα, followed by greater p-Smads abundance. At 1 hour following stimulation, the level of p-Smads expression was further elevated as a result of the higher concentration of TNFα (100 ng/ml), whereas the Smad 1 level did not change. This may indicate that TNFα treatment may not only increase p-Smads expression via Smad 1 stimulation, but it may also directly enhance Smads phosphorylation. This will be further investigated in future studies.

Taken together, the immunoblotting data (Figure 2.11-2.12) implicate that the relative absences of NF-κB in haploinsufficient animals results in reduced signaling along the Smad pathway.

Discussion

The successful regeneration of BMP2 induced bone grafts requires a complex of signaling cascades which ensure sufficicient osteoconductivity and osteogenesis of osteoprgenitor cells (Carreira et al., 2014; Chenard et al., 2012). The importance of inflammatory signals in the regulation of bone regeneration processes has been addressed previously (Schmidt-Bleek et al., 2012). NF-κB is a central mediater in inflammation, making it an attractive target for researchers. Previous work in our lab has found that mice with insufficient p65 (a major NF-κB component) signaling in osteoblastic cells display retarded bone growth and development (manuscript in preparation) in a physiological context. In the current study, we further examine the effect of p65 haploinsufficiency on osteoblasts during bone healing in a calvarial defect mouse model. Critical-sized defects are defined as defects that can not be healed naturally during the life-time of an animal. Critical-sized animal models are a well-established tool for researchers to investigate bone healing processes (Szpalski et al., 2010). To the extent of our knowledge, this is the first study to show the effects of direct p65 ablation in osteogenic cell lines and its influence in bone regeneration *in vivo*.

In the present study, we showed that rhBMP2-mediated healing processes in mice with a p65 haploinsufficiency are compromised, as demonstrated by a significant reduction in bony tissue regeneration as indicated by micro-CT analysis (Figure 2.3-5). Bone regeneration in control animals displayed a physiological bone repair pattern inflammation, fibrous tissue infiltration and bone matrix deposition (Cameron et al., 2013). Histological

evaluation further confirmed that p65 haploinsufficient animals illustrated a delayed and impaired healing process.

CFU-F and CFU-OB assays are commonly used to assess the relative concentrations and osteogenic potential of mesenchymal stem cell (MSC) lineages within the bone marrow niche. MSCs are multipotential cells that possess the capacity to differentiate into several cell lineages such as osteoblastic, chondrogenic, adipogenic and myogenic lineages. The specification of stem cells into a cell lineage is regulated by many factors within the microenvironment. The expression of *Runx2* and *Sp7* genes drives stem cell commitment into osteoblasts while *Sox9* drives a chondrogenic specification (Komori, 2006). Peroxisome proliferator-activated receptor gamma (*Pparγ*) and *MyoD* are the master genes that drive apiogenesis and myogenesis, respectively (Gang et al., 2004; Takada et al., 2010).

In the current study, $p65^{fl/+}$Osx-Cre mice demonstrated a comparable number of CFU-Fs, but significantly less CFU-OBs as compared to the control group (Figure 2.8). In response to rhBMP2, nearly 90-100% of the mesenchymal stem cells were shown to be osteogenic in $p65^{fl/+}$ and Osx-Cre mice (Figure 2.8B). Only 50% of MSCs in $p65^{fl/+}$Osx-Cre mice demonstrated osteogenic property. It is possible that the MSCs of $p65^{fl/+}$Osx-Cre mice enter other lineages such as chondrogenic or adipogenic cell lineages. Fewer osteogenic progenitors within MSC populations in p65 haploinsufficient mice may lead to the impaired bone regeneration observed in the micro-CT (Figure 2.3-5) and histology analyses (Figure 2.6).

Furthermore, $p65^{fl/+}$Osx-Cre mice displayed significantly reduced expression of several osteogenic genes (bone markers) during rhBMP2 mediated repair at 4 weeks as

compared to both control groups (Figure 2.10). In accordance with the decreased mRNA expression levels for various bone markers, immunohistochemial analysis on bone defects demonstrated less BSP protein production after 8 weeks for both natural and rhBMP2 mediated bone healing (Figure 2.7). These findings further suggest that the defective bone repair in $p65^{fl/+}$Osx-Cre mice may be caused by the reduction in osteoprogenitors at regions corresponding to bone defects. The reduced osteoprogenitor populations may be caused by compromised osteogenic commitment of MSCs in $p65^{fl/+}$Osx-Cre mice. Another possibility is defective migration of progenitors to regions of injury, as a result of insufficient p65.

We next showed that the osteoblastic differentiation of osteoprogenitors in the p65 haploinsufficient animals was impaired. Early osteogenic marker genes (*Sp7*, *Alp* and *Bsp*) were significantly downregulated in cells taken from $p65^{fl/+}$Osx-Cre mice at 4 days following rhBMP2 treatment (Figure 2.9).

rhBMP2 as an osteoinductive factor has been extensively used in bone regenrative therapy. Interestingly, we identified a mechanism by which NF-κB haploinsufficiency affects bone repair in the BMP2 signaling pathway. The BMP2 signaling pathway stimulates osteoblastic differention and mineralization by enhancing the expression of bone matrix proteins such as type I collagen, osterix, alkaline phosphotase, osteopontin and bone sialoprotein (Lai and Cheng, 2005; Matsubara et al., 2008; Rawadi et al., 2003). Our data demonstrated a downregulation of rhBMP2 mediated osteogenic differentiation in cells with insufficient NF-κB signals, suggesting a positive role for NF-κB in osteogenesis. Consistent with our findings, it has been previously reported that NF-κB activation and TNFα treatment induces osteogenic differentitaion and matrix mineralization in stem cells (Caron

et al., 2012; Cho et al., 2010; Feng et al., 2013; Hess et al., 2009; Li et al., 2014). Moreover, it was shown that the osteoblastic differentiation of mouse osteoblastic MC3T3-E1 cells (subclone 4) is regulated *via* NF-κB signaling (Kim et al., 2008).

The BMP2 signaling pathway is regulated at multipal molecular levels. Signal stimulation is induced by binding of BMP2 ligands to the type I and type II receptor complexes, triggering intracelluar cascades (Carreira et al., 2014; Chenard et al., 2012). R-Smad proteins (Smad 1/5/8) are generally recognized as the primary components downstream of BMP2 signaling (Heldin et al., 1997; Miyazono et al., 2001). Phosphorylation and nuclear translocation of R-Smads induces the transcriptional modulation of genes responsible for osteogenesis (Chen et al., 2012; Retting et al., 2009). Two putative NF-κB response elements were found located in the promoter region of the BMP2 gene, implicating a direct regulatory mechanism of NF-κB in the BMP2 pathway (Feng et al., 2003). Previous work in our lab also demonstrated that NF-κB induces BMP2 mRNA expression (manuscript in preparation). In the current study, we further demonstrated that the negative effect of NF-κB haploinsufficiency on osteogenic differentiation results from disruption of BMP2 induced Smad phosphorylation. To further comfirm the role of NF-κB in rhBMP2/Smad signaling, we tested Smad phosphorylation following stimulation of TNFα and rhBMP2 in a mouse myogenic stem cell line. Accordingly, we found that activation of NF-κB signaling by TNFα significantly enhanced rhBMP2 induced Smad phosphorylation.

It is worth noting that previous studies suggest a negative role for NF-κB in osteogenesis, as demonstrated by *bglap2*-IKK-DN mice with decreased NF-κB function in osteoblasts and *Col2*-IKK2a mice with constitutively activated NF-κB signaling in early

progentors (Chang et al., 2009; Swarnkar et al., 2014). The discrepancy between our findings with previous work lies in the differentiation stage at which NF-κB function was manipulated in the animal model. In our study, the differentiation of primitive stem cells into osteoblasts was regulated by osteogenic genes in a definable temporal manner. Type 2 collagen is expressed in early bipotential skeletal progenitors with a capability to differentiate into either a chondrogenic (with coexpression of *Sox9*) or an osteoblastic lineage (with further expression of *Runx2* and *Sp7* genes) (Rodda and McMahon, 2006). Osteocalcin is generally accepted as a marker for mature osteoblasts, representing a final stage of osteoblastogenesis (Javed et al., 2010). The distinct outcome of bone formation in these animal models may indicate that the effect of NF-κB signaling on osteobalstic differentiation is stage-specific.

In addition, previous work by our team indicates an obvious reduction of p65 in the $p65^{fl/+}$Osx-Cre mice, while other subunits in the NF-κB pathway remain unaffected (manuscript in preparation). These results exclude the possibility of a compensation mechanism directed by other molecules. Furthermore, the previous studies were established in a physiological context of bone development/homeostasis, while our current studies hereby tested the role of NF-κB in bone graft induced regeneration. The inflammatory phase following bone injury was suggested to be critical for progenitor recruitment, proliferation and further differention (Mountziaris and Mikos, 2008; Mountziaris et al., 2011; Schmidt-Bleek et al., 2012). Accordingly, we proposed NF-κB mediated inflammatory signaling as an indispensible factor in a pathological bone healing context.

In this study, we created a transgenic animal model with partial p65 ablation in osteoblastic cells without affecting other cell lineages that could contribute to bone repair. This strategy provided a way to elucidate the direct role of NF-κB signaling in cell signaling as it relates to bone formation. Using MSCs graft with an IKK small molecule inhibitor (IKKIV), Chang el al. (Chang et al., 2013) showed improved calvarial repair in rats, suggesting a negative role of NF-κB on bone repair. Bone defect sites are commonly infiltrated with an abundance of various cell types (e.g. osteobalstic cells, hematopoietic lineages, fibroblasts.) that may either have previously resided at the site pre-injury, or been recruited to the site post injury. It is possible that the application of inhibitors could influence some, or all, of the resident cells. It is therefore possible that distinct populations of cells at different injury sites or in different models could influence the study results. This is important to note for future studies and clinical applications.

Successful bone repair is achieved by balanced cooperation of osteoblast-mediated bone formation and osteoclast-regulated bone resorption. Mechanistically the p65 ablation in $p65^{fl/+}$Osx-Cre mice is restricted to osteoblastic cell lineages, and thus there was no direct effect on other cell types. However, other cells may have been indirectly affected. For example, osteoblasts are known to affect osteoclast expression and subsequent bone resoprtion (Sims and Martin, 2014). Osteoblasts directly regulate osteoclast maturation and activity by secreting RANKL and OPG. RANKL binds to its receptor RANK on osteoclasts and promotes osteocalstogenesis. In contrast, OPG counteracts RANKL-RANK mediated osteoclastogenic activity. This RANKL-OPG-RANK axis represents an essential regulatory mechanism by which osteoblastic cells affect osteoclast function (Boyce and Xing, 2008).

There is possibility that the defective osteoblastogenesis in $p65^{fl/+}$Osx-Cre mice may influence osteoclast activity, thereby contributing to impaired bone healing. Previous studies in our lab, however, have shown that the number and activity of osteoclasts are not affected in $p65^{fl/+}$Osx-Cre mice as compared to $p65^{fl/+}$ mice (manuscript in preparation). Still, in future studies should address the role of osteoclastogenesis in bony defect and bone repair.

Conclusion

In summary, we proposed that NF-κB in osteobastic cells plays an important role in bone repair. As hypothesized, we found that intact NF-κB signaling is imperative for BMP2 induced osteogenic differentiation of osteoprogenitors through canonical Smad pathways during bone healing. The current results demonstrate that in addition to playing a key role in osteoclastogenesis, NF-κB is necessary for for osteoblastogenesis.

Figure 2.1 Schematic pictures describing the generation of transgenic mouse model.

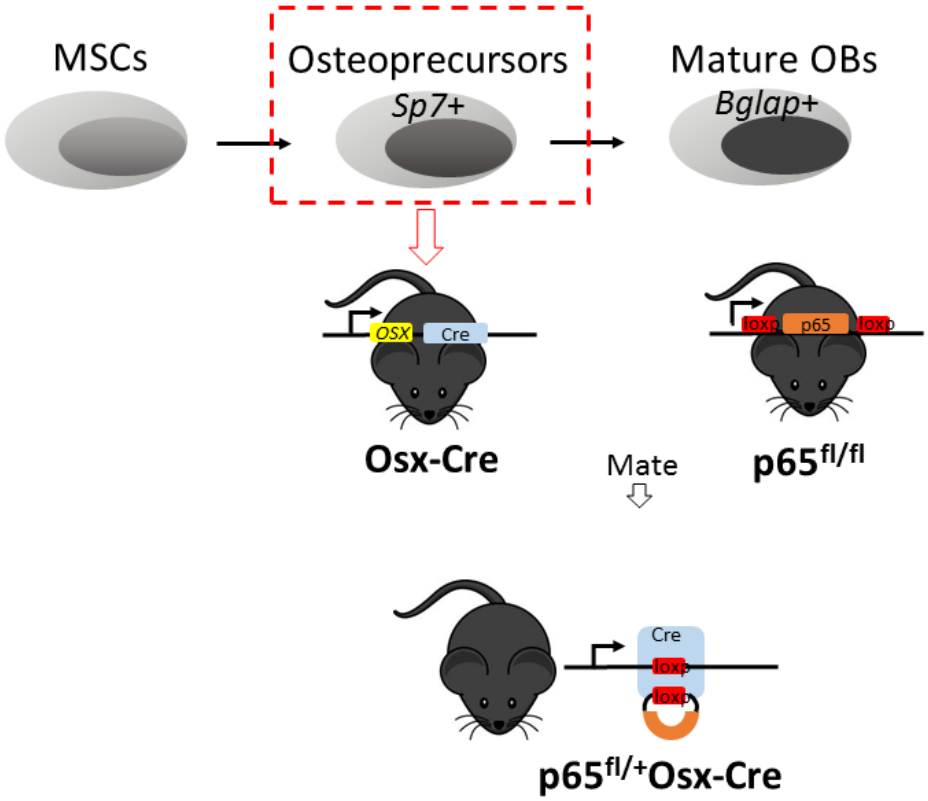
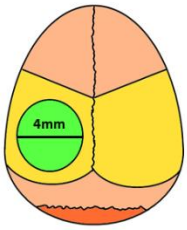


Figure 2.2 Illustration of the critical-sized defect model and procedures in mouse.

(A) Schematic drawing of the calvarial defect mouse model. (B) A 4 mm diameter defect was made on the parietal bone of mice.



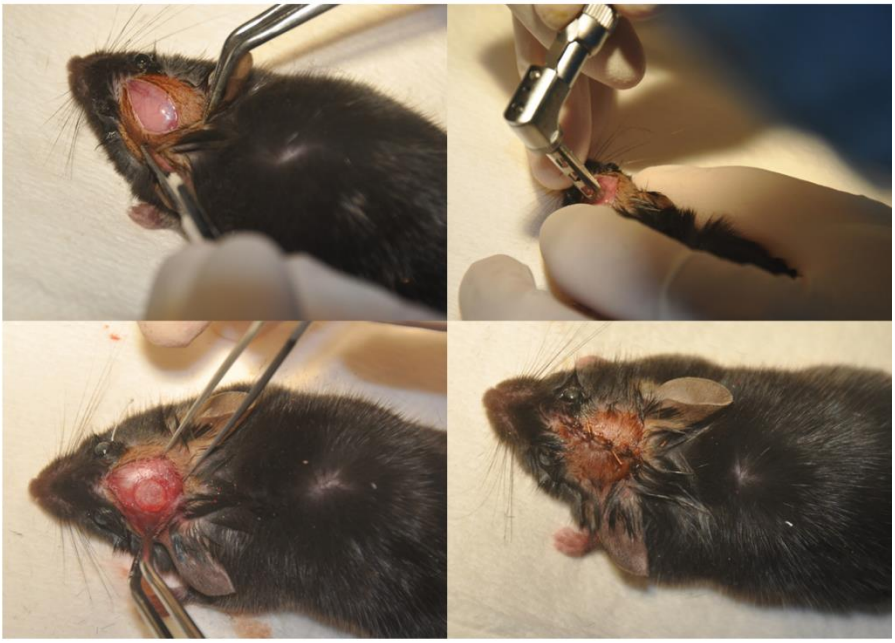


Figure 2.3 Micro-CT analysis of new bone formation in p65$^{fl/+}$Osx-Cre, p65$^{fl/+}$ and Osx-Cre mice at 4 weeks following surgery.

Representative 3-D reconstruction images of parietal bones of each group at 4 weeks. Each group received either ACS (Absorbable collagen sponge) with saline or ACS loaded with rhBMP2 (500 ng).

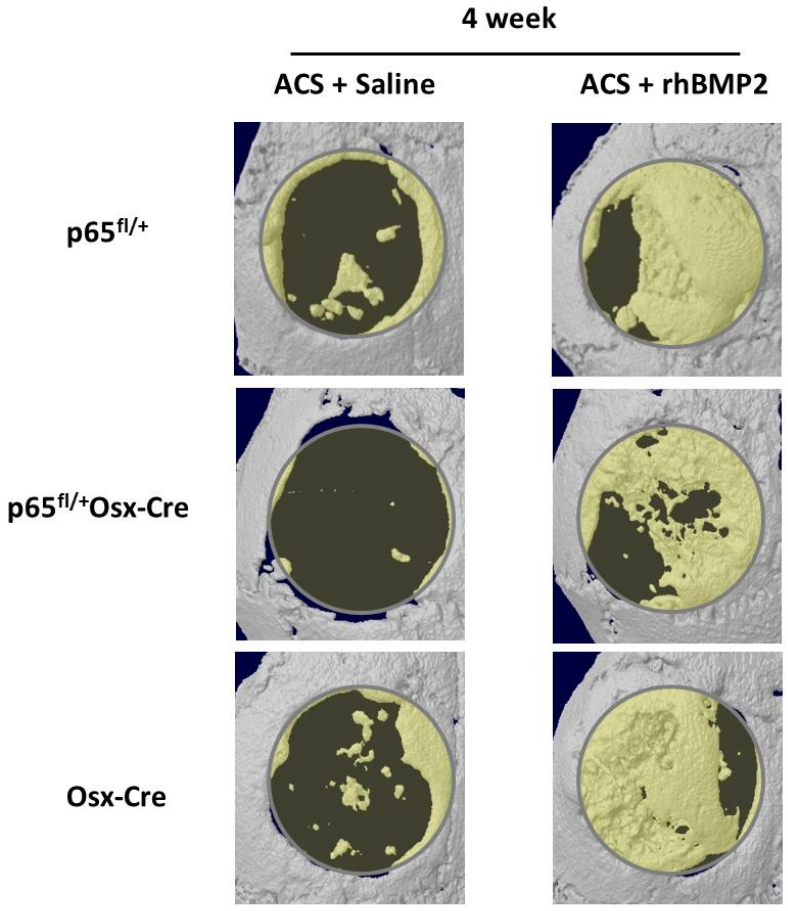


Figure 2.4 Micro-CT analysis of new bone formation in p65$^{fl/+}$Osx-Cre and p65$^{fl/+}$ mice at 8 weeks following surgery.

Representative 3-D reconstruction images of parietal bones of each group at 8 weeks. Each group received either ACS (Absorbable collagen sponge) with saline or ACS loaded with rhBMP2 (500 ng).

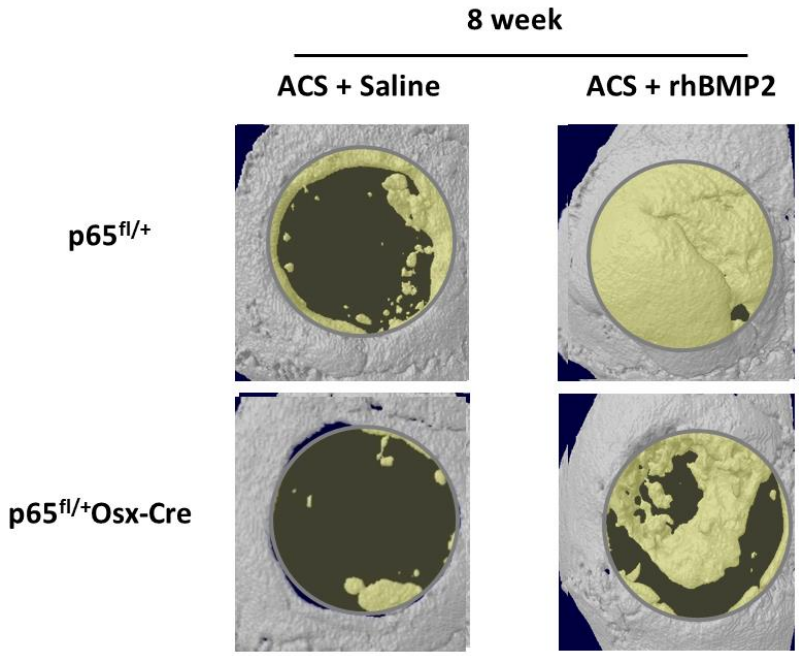


Figure 2.5 Micro-CT analysis of new bone formation in p65$^{fl/+}$Osx-Cre and p65$^{fl/+}$ mice and Osx-Cre mice at 4 and 8 weeks following surgery.

Quantitative assessment of regenerated bone volume of each group at different time points. Healing ratio= (1-[Current Volume of defect – Original Volume of defect]) X 100 percent. (n= 5-8 animal/group; *P<0.05, **P<0.01)

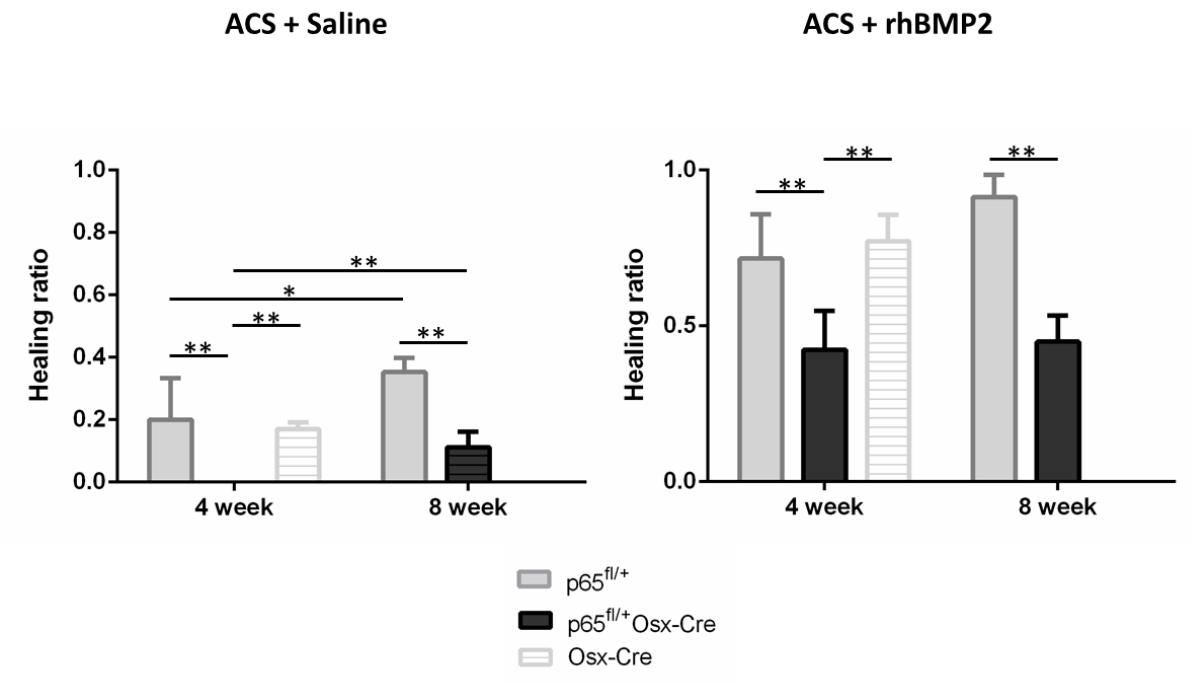


Figure 2.6 Histological evaluation of healing process in calvarial defects in $p65^{fl/+}$Osx-Cre and $p65^{fl/+}$ mice.

(A) Hematoxylin and eosin staining of newly formed bone. The samples were collected at 4 and 8 weeks following implantation of absorbable collagen sponge (ACS) loaded with saline or rhBMP2 (500 ng). There is a lack of healing at 4 weeks in the saline-treated group for both animals. Specifically, no detectable bone tissue was found in the $p65^{fl/+}$Osx-Cre mice. Increased mature bone tissue was demonstrated when treated with rhBMP2. $p65^{fl/+}$Osx-Cre mice exhibited less new bone regeneration versus $p65^{fl/+}$ mice. The upper panel of each group displays the sagittal sections of bone defects (2.5X magnification). The scale bar indicates 1000 um. The lower panels show enlarged views of the red rectangles in the upper panels (40X magnification, scale bar: 50 um). "S" indicates the residual collagen scaffold. (B) Masson's trichrome staining of bone defect in both animal groups at 4 and 8 weeks post-operation. (Upper panel: 2.5X, bar: 1000 um; Lower panel: 40X, bar: 50 um)

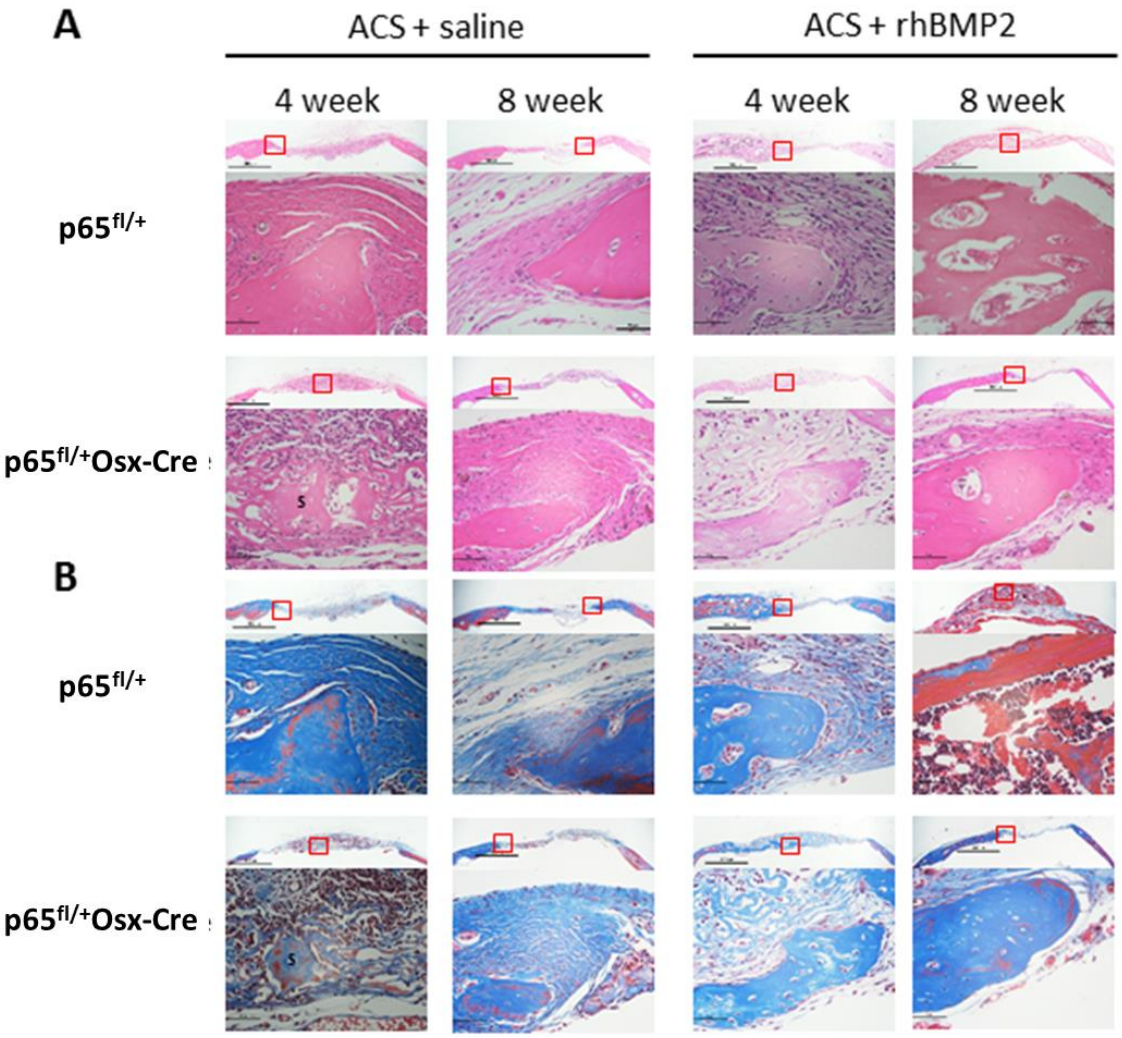

A
ACS + saline
ACS + rhBMP2
4 week
8 week
4 week
8 week
p65fl/+
p65fl/+Osx-Cre
S
B
p65fl/+
p65fl/+Osx-Cre
S

Figure 2.7 Immunohistochemical assessment of bone sialoprotein (BSP) expression in regenerated tissue of $p65^{fl/+}$Osx-Cre and $p65^{fl/+}$ mice.

(A) Immunohistochemical staining of BSP in calvarial defect implanted with ACS+saline after 8 weeks. (B) Immunohistochemical staining of BSP in calvarial defect implanted with ACS+rhBMP2 after 8 weeks. The right panel shows the negative IgG control. Arrow indicates the stained protein within the bone defect area. (Magnification: 40X, Scale bar: 50 um)

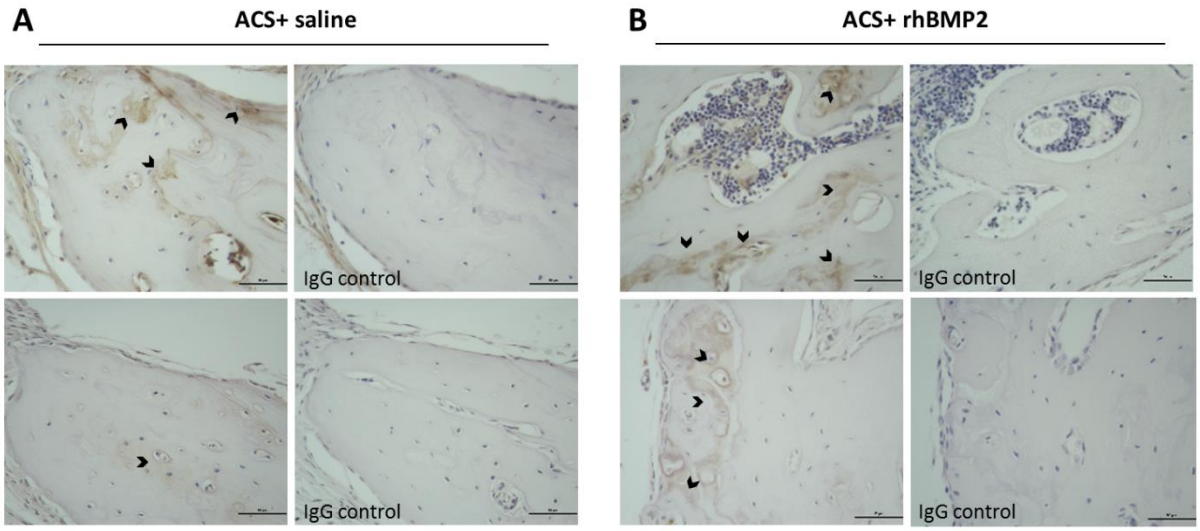
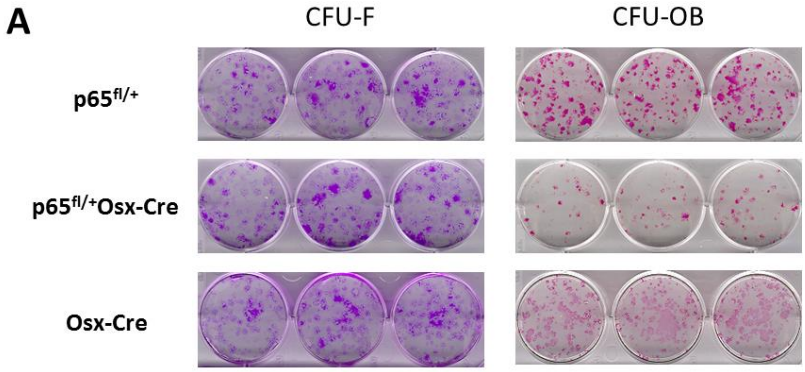


Figure 2.8 Colony-forming unit-fibroblasts (CFU-F) and colony-forming unit-osteoblasts (CFU-OB) were isolated and stained for $p65^{fl/+}$Osx-Cre and $p65^{fl/+}$ mice.

Whole bone marrow stromal cells (2 X 10^6 cells/well) isolated from long bones of studied mice were plated onto 6-well plates. rhBMP2 (50 ng/ml) was used to induce osteogenic differentiation for the CFU-OB assay. (A) Representative culture plates of stained CFU-F and CFU-OB colonies. (B) Quantification of CFU-F and CFU-OB colony numbers. (N ≥ 5 animal per group; **P<0.01)



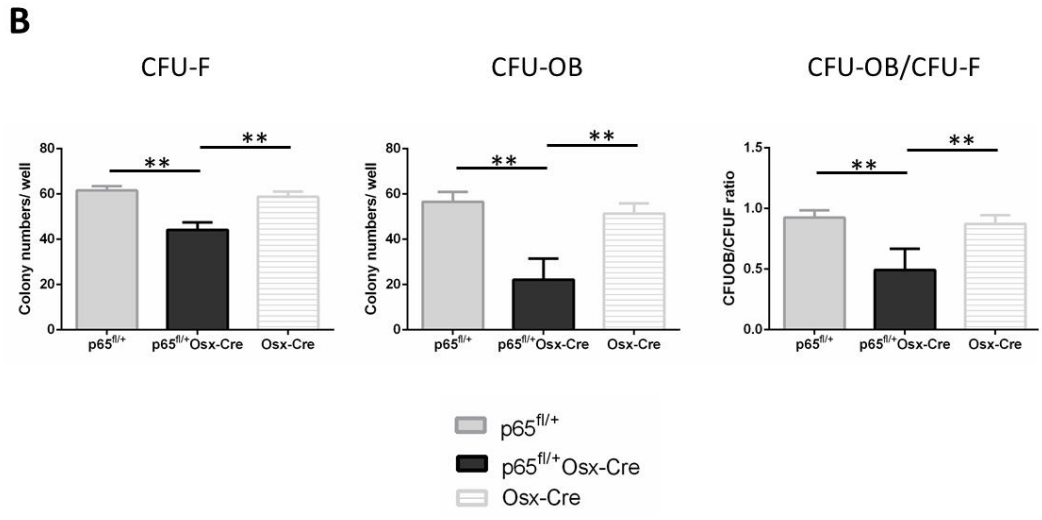


Figure 2.9 Relative mRNA expression levels of osteogenic markers (*Sp7*, *Alp*, *Bsp*) of osteoprogenitors obtained from p65$^{fl/+}$Osx-Cre and p65$^{fl/+}$ mice.

Osteoprogenitor cells (10^5 cells/well) isolated from animals were seeded onto 12-well plates. rhBMP2 (50 ng/ml), 0.2 mM ascorbic acid and 10mM β- glycerophosphate was then added to cell cultures to induce osteogenic differentiation. (A) Expression levels at 4 days of culture (B) 7 days of culture with and without rhBMP2. Data are shown as mean ± SD. (N ≥ 5 animal per group; *P<0.05, **P<0.01)

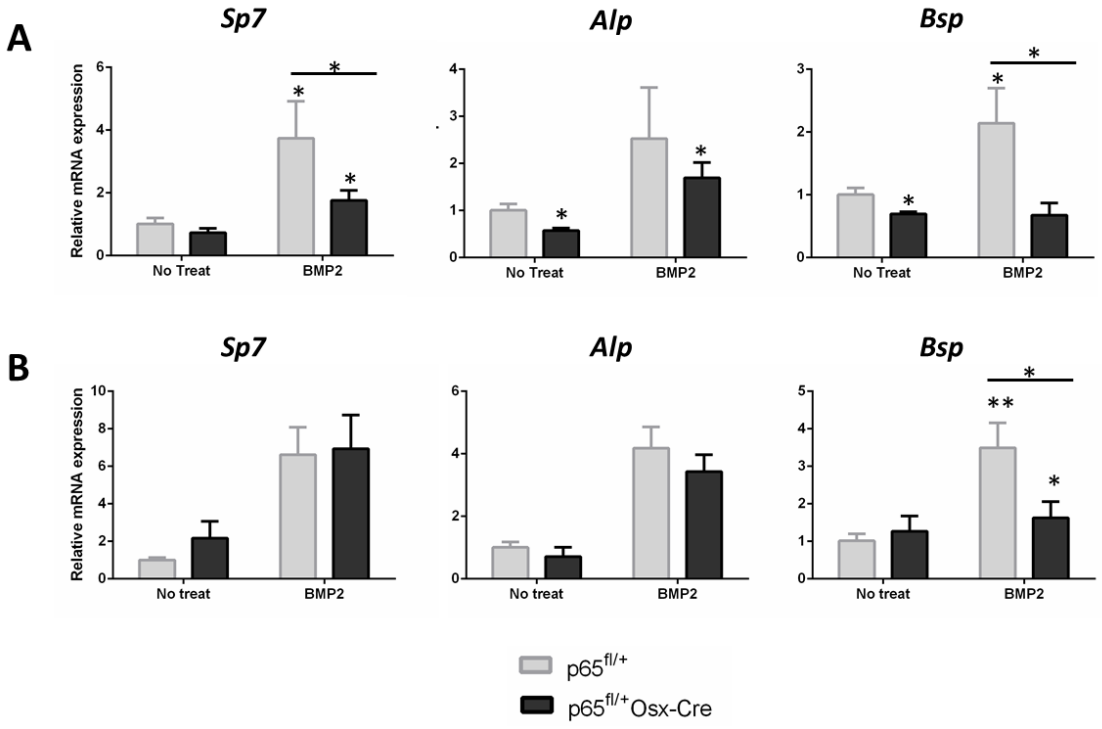


Figure 2.10 Relative mRNA expression levels of several osteogenic genes (*Sp7*, *Alp*, *Bsp*, *Satb2*) during the calvarial healing process in p65$^{fl/+}$Osx-Cre and p65$^{fl/+}$ mice.

Quantification of mRNA expression of *Sp7*, *Alp*, *Bsp* and *Satb2* in regenerated bone tissue after 4 weeks of implantation with ACS+rhBMP2 (500 ng) in mice. Data are shown as mean ± SD. (n= 4-5 animal/group; *P<0.05, **P<0.01)

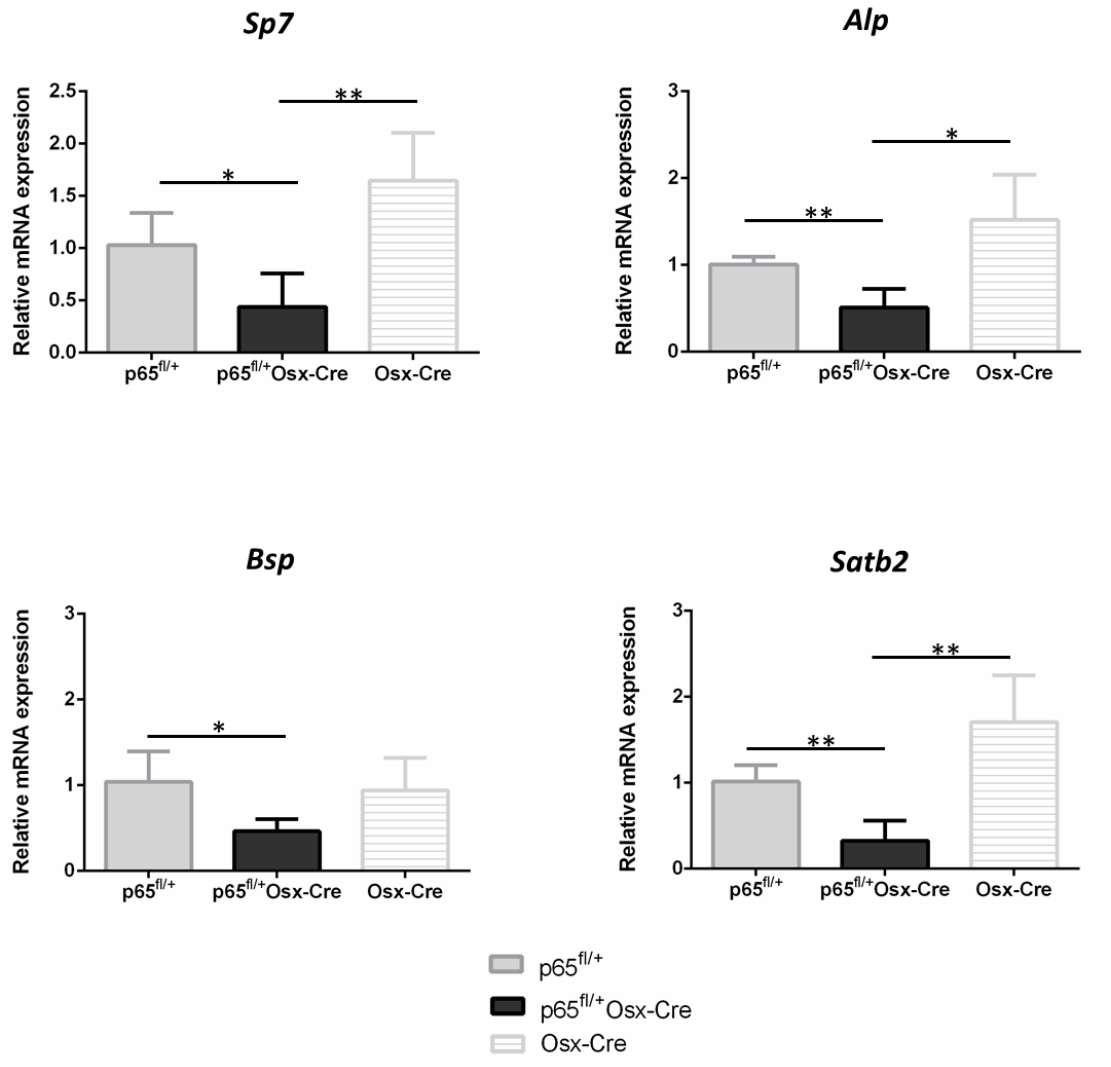


Figure 2.11 The BMP2 mediated Smad signaling pathway is affected by NF-κB signaling.

Osteoprogenitors from $p65^{fl/+}$ and $p65^{fl/+}$Osx-Cre mice were treated with/without rhBMP2 (50 ng/ml) for 15 minutes, 30 minutes and 1 hour. (A) The expression of p65 in both animal groups after 15 minutes when treated with rhBMP2. (B) Relative expression of p65 was quantified using densitometry. (C) The expression of phosphorylated Smads (p-Smad 1/5/8), Smad 1 and GAPDH was examined by western blot. (D) Relative protein expression level of p-Smads to Smad 1. Generated by densitometry analysis using Image J. (*P<0.05, **P<0.01)

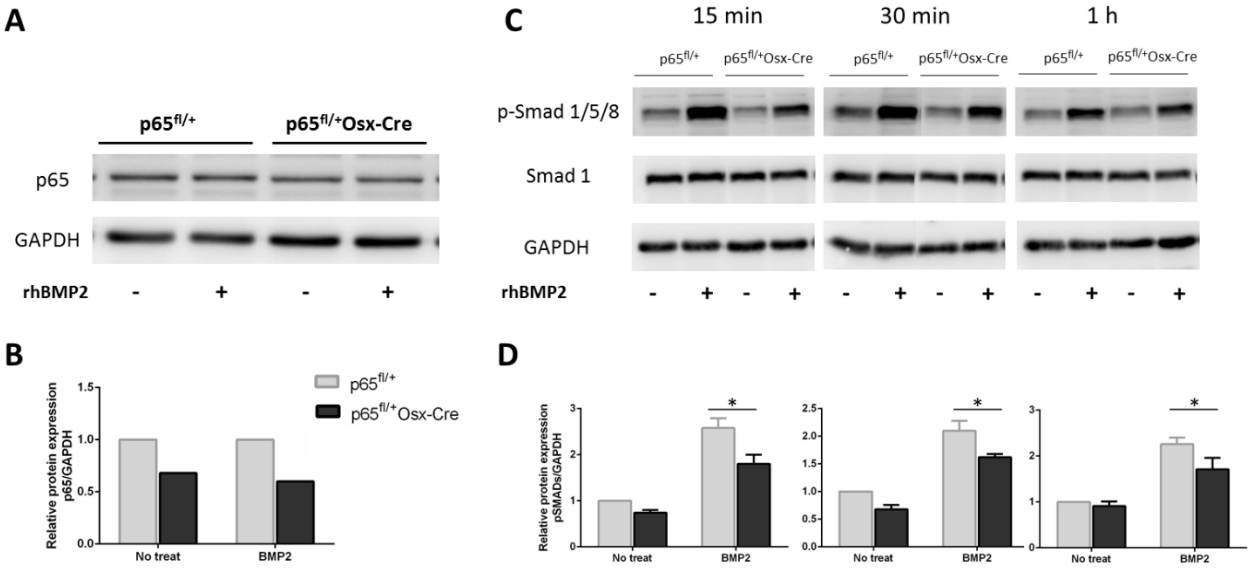


Figure 2.12 The BMP2 mediated Smad signaling pathway is affected by NF-κB signaling.

(A) TNFα was able to enhance the transcriptional activity of NF-κB in C2C12 cells. (B) The expression of p-Smad 1/5/8, Smad 1 and β-actin in C2C12 cells upon treated with rhBMP2 (50ng/ml) and/or TNFα for 1h or 2h. (+: TNFα 10ng/ml, ++: TNFα 100ng/ml) (C) Relative protein expression level of p-Smads and Smad 1 to β-actin at 1 hour and 2 hours. Generated by densitometry analysis using Image J.

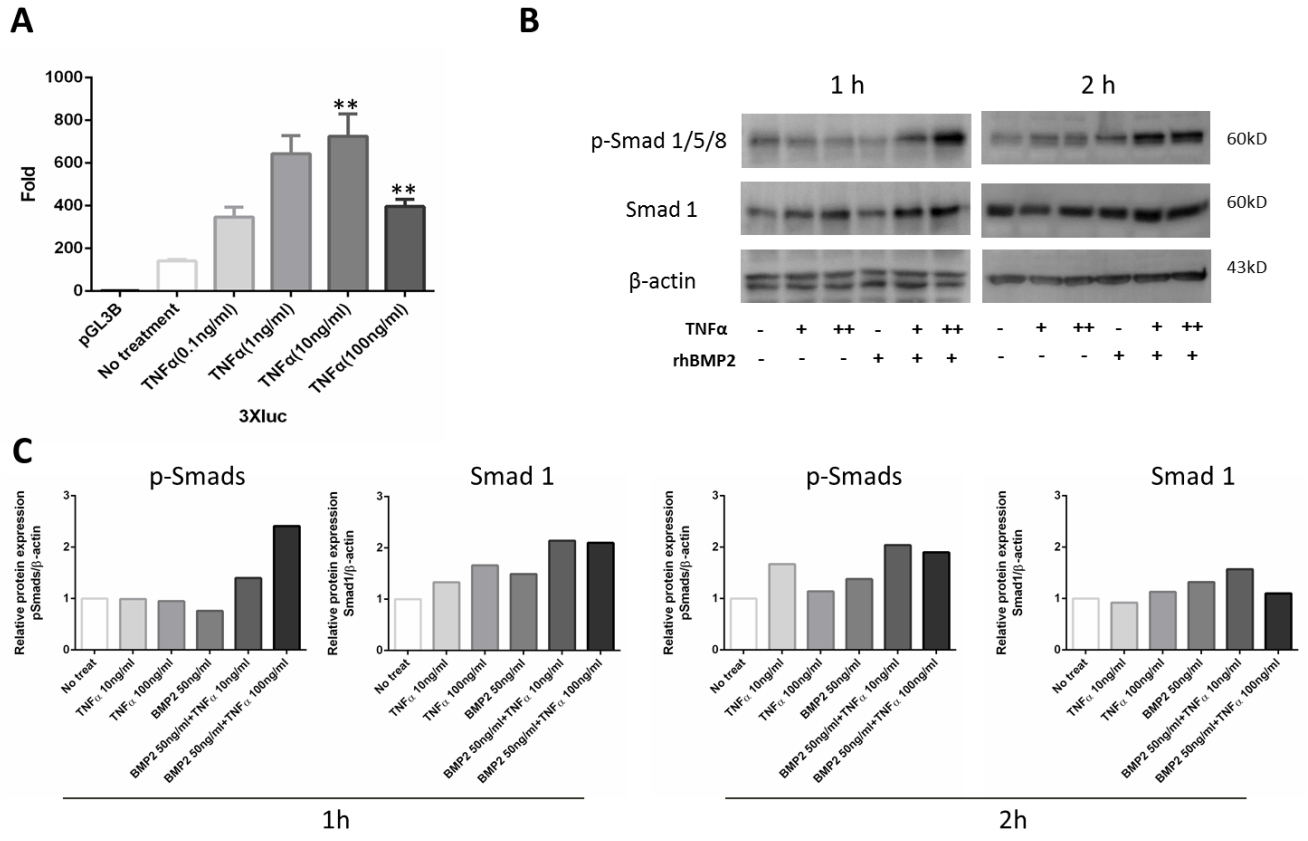


Table 2.1 Primers used in PCR for genotyping.

Gene	Primer Sequence
Cre	
Cre Forward	5‘-GCG GTC TGG CAG TAA AAA CTA TC-3’
Cre Reverse	5‘-GTG AAA CAG CAT TGC TGT CAC TT-3’
Internal Control	
Forward	5‘-CTA GGC CAC AGA ATT GAA AGA TCT-3’
Reverse	5‘-GTA GGT GGA AAT TCT AGC ATC ATC C-3’
p65loxP	
Forward	5‘-CGA CTT TGG GTT GGA GGG TTA CAG AAG GC-3’
Reverse	5‘-TGG TCT GGA TTC GCT GGC TAA TGG C-3’

REFERNCES

Abu-Amer, Y. (2013). NF-kappaB signaling and bone resorption. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA *24*, 2377-2386.

Akeel, S., El-Awady, A., Hussein, K., El-Refaey, M., Elsalanty, M., Sharawy, M., and Al-Shabrawey, M. (2012). Recombinant bone morphogenetic protein-2 induces up-regulation of vascular endothelial growth factor and interleukin 6 in human pre-osteoblasts: role of reactive oxygen species. Archives of oral biology *57*, 445-452.

Amini, A.R., Laurencin, C.T., and Nukavarapu, S.P. (2012). Bone tissue engineering: recent advances and challenges. Critical reviews in biomedical engineering *40*, 363-408.

Baroli, B. (2009). From natural bone grafts to tissue engineering therapeutics: Brainstorming on pharmaceutical formulative requirements and challenges. Journal of pharmaceutical sciences *98*, 1317-1375.

Behr, B., Sorkin, M., Lehnhardt, M., Renda, A., Longaker, M.T., and Quarto, N. (2012). A comparative analysis of the osteogenic effects of BMP-2, FGF-2, and VEGFA in a calvarial defect model. Tissue engineering Part A *18*, 1079-1086.

Boyce, B.F., and Xing, L. (2008). Functions of RANKL/RANK/OPG in bone modeling and remodeling. Archives of biochemistry and biophysics *473*, 139-146.

Cameron, J.A., Milner, D.J., Lee, J.S., Cheng, J., Fang, N.X., and Jasiuk, I.M. (2013). Employing the biology of successful fracture repair to heal critical size bone defects. Current topics in microbiology and immunology *367*, 113-132.

Caron, M.M., Emans, P.J., Surtel, D.A., Cremers, A., Voncken, J.W., Welting, T.J., and van Rhijn, L.W. (2012). Activation of NF-kappaB/p65 facilitates early chondrogenic differentiation during endochondral ossification. PloS one *7*, e33467.

Carreira, A.C., Lojudice, F.H., Halcsik, E., Navarro, R.D., Sogayar, M.C., and Granjeiro, J.M. (2014). Bone morphogenetic proteins: facts, challenges, and future perspectives. Journal of dental research *93*, 335-345.

Chang, J., Liu, F., Lee, M., Wu, B., Ting, K., Zara, J.N., Soo, C., Al Hezaimi, K., Zou, W., Chen, X.*, et al.* (2013). NF-kappaB inhibits osteogenic differentiation of mesenchymal stem cells by promoting beta-catenin degradation. Proceedings of the National Academy of Sciences of the United States of America *110*, 9469-9474.

Chang, J., Wang, Z., Tang, E., Fan, Z., McCauley, L., Franceschi, R., Guan, K., Krebsbach, P.H., and Wang, C.Y. (2009). Inhibition of osteoblastic bone formation by nuclear factor-kappaB. Nature medicine *15*, 682-689.

Chen, D., Harris, M.A., Rossini, G., Dunstan, C.R., Dallas, S.L., Feng, J.Q., Mundy, G.R., and Harris, S.E. (1997). Bone morphogenetic protein 2 (BMP-2) enhances BMP-3, BMP-4, and bone cell differentiation marker gene expression during the induction of mineralized bone matrix formation in cultures of fetal rat calvarial osteoblasts. Calcified tissue international *60*, 283-290.

Chen, G., Deng, C., and Li, Y.P. (2012). TGF-beta and BMP signaling in osteoblast differentiation and bone formation. International journal of biological sciences *8*, 272-288.

Chenard, K.E., Teven, C.M., He, T.C., and Reid, R.R. (2012). Bone morphogenetic proteins in craniofacial surgery: current techniques, clinical experiences, and the future of personalized stem cell therapy. Journal of biomedicine & biotechnology *2012*, 601549.

Cho, H.H., Shin, K.K., Kim, Y.J., Song, J.S., Kim, J.M., Bae, Y.C., Kim, C.D., and Jung, J.S. (2010). NF-kappaB activation stimulates osteogenic differentiation of mesenchymal stem cells derived from human adipose tissue by increasing TAZ expression. Journal of cellular physiology *223*, 168-177.

Chou, M.Y., Yan, D., Jafarov, T., and Everett, E.T. (2009). Modulation of murine bone marrow-derived CFU-F and CFU-OB by in vivo bisphosphonate and fluoride treatments. Orthodontics & craniofacial research *12*, 141-147.

Conner, J.R., and Hornick, J.L. (2013). SATB2 is a novel marker of osteoblastic differentiation in bone and soft tissue tumours. Histopathology *63*, 36-49.

Cowles, E.A., DeRome, M.E., Pastizzo, G., Brailey, L.L., and Gronowicz, G.A. (1998). Mineralization and the expression of matrix proteins during in vivo bone development. Calcified tissue international *62*, 74-82.

Dobreva, G., Chahrour, M., Dautzenberg, M., Chirivella, L., Kanzler, B., Farinas, I., Karsenty, G., and Grosschedl, R. (2006). SATB2 is a multifunctional determinant of craniofacial patterning and osteoblast differentiation. Cell *125*, 971-986.

Feng, J.Q., Xing, L., Zhang, J.H., Zhao, M., Horn, D., Chan, J., Boyce, B.F., Harris, S.E., Mundy, G.R., and Chen, D. (2003). NF-kappaB specifically activates BMP-2 gene expression in growth plate chondrocytes in vivo and in a chondrocyte cell line in vitro. The Journal of biological chemistry *278*, 29130-29135.

Feng, X., Feng, G., Xing, J., Shen, B., Li, L., Tan, W., Xu, Y., Liu, S., Liu, H., Jiang, J.*, et al.* (2013). TNF-alpha triggers osteogenic differentiation of human dental pulp stem cells via the NF-kappaB signalling pathway. Cell biology international *37*, 1267-1275.

Gang, E.J., Jeong, J.A., Hong, S.H., Hwang, S.H., Kim, S.W., Yang, I.H., Ahn, C., Han, H., and Kim, H. (2004). Skeletal myogenic differentiation of mesenchymal stem cells isolated from human umbilical cord blood. Stem cells *22*, 617-624.

Gerstenfeld, L.C., Cho, T.J., Kon, T., Aizawa, T., Tsay, A., Fitch, J., Barnes, G.L., Graves, D.T., and Einhorn, T.A. (2003). Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research *18*, 1584-1592.

Gordon, J.A., Tye, C.E., Sampaio, A.V., Underhill, T.M., Hunter, G.K., and Goldberg, H.A. (2007). Bone sialoprotein expression enhances osteoblast differentiation and matrix mineralization in vitro. Bone *41*, 462-473.

Harada, S., and Rodan, G.A. (2003). Control of osteoblast function and regulation of bone mass. Nature *423*, 349-355.

Hayden, M.S., and Ghosh, S. (2008). Shared principles in NF-kappaB signaling. Cell *132*, 344-362.

Helbing, T., Rothweiler, R., Ketterer, E., Goetz, L., Heinke, J., Grundmann, S., Duerschmied, D., Patterson, C., Bode, C., and Moser, M. (2011). BMP activity controlled by BMPER regulates the proinflammatory phenotype of endothelium. Blood *118*, 5040-5049.

Heldin, C.H., Miyazono, K., and ten Dijke, P. (1997). TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature *390*, 465-471.

Hess, K., Ushmorov, A., Fiedler, J., Brenner, R.E., and Wirth, T. (2009). TNFalpha promotes osteogenic differentiation of human mesenchymal stem cells by triggering the NF-kappaB signaling pathway. Bone *45*, 367-376.

Javed, A., Chen, H., and Ghori, F.Y. (2010). Genetic and transcriptional control of bone formation. Oral and maxillofacial surgery clinics of North America *22*, 283-293, v.

Katavic, V., Grcevic, D., Lukic, I.K., Vucenik, V., Kovacic, N., Kalajzic, I., and Marusic, A. (2003). Non-functional Fas ligand increases the formation of cartilage early in the endochondral bone induction by rhBMP-2. Life sciences *74*, 13-28.

Kim, C.S., Kim, J.I., Kim, J., Choi, S.H., Chai, J.K., Kim, C.K., and Cho, K.S. (2005). Ectopic bone formation associated with recombinant human bone morphogenetic proteins-2 using absorbable collagen sponge and beta tricalcium phosphate as carriers. Biomaterials *26*, 2501-2507.

Kim, J.M., Lee, S.U., Kim, Y.S., Min, Y.K., and Kim, S.H. (2008). Baicalein stimulates osteoblast differentiation via coordinating activation of MAP kinases and transcription factors. Journal of cellular biochemistry *104*, 1906-1917.

Komori, T. (2006). Regulation of osteoblast differentiation by transcription factors. Journal of cellular biochemistry *99*, 1233-1239.

Lai, C.F., and Cheng, S.L. (2005). Alphavbeta integrins play an essential role in BMP-2 induction of osteoblast differentiation. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research *20*, 330-340.

Li, J., Yan, M., Wang, Z., Jing, S., Li, Y., Liu, G., Yu, J., and Fan, Z. (2014). Effects of canonical NF-kappaB signaling pathway on the proliferation and odonto/osteogenic differentiation of human stem cells from apical papilla. BioMed research international *2014*, 319651.

Li, Q., and Verma, I.M. (2002). NF-kappaB regulation in the immune system. Nature reviews Immunology *2*, 725-734.

Lin, C.Y., Chang, Y.H., Li, K.C., Lu, C.H., Sung, L.Y., Yeh, C.L., Lin, K.J., Huang, S.F., Yen, T.C., and Hu, Y.C. (2013). The use of ASCs engineered to express BMP2 or TGF-beta3 within scaffold constructs to promote calvarial bone repair. Biomaterials *34*, 9401-9412.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402-408.

Luu, H.H., Song, W.X., Luo, X., Manning, D., Luo, J., Deng, Z.L., Sharff, K.A., Montag, A.G., Haydon, R.C., and He, T.C. (2007). Distinct roles of bone morphogenetic proteins in osteogenic differentiation of mesenchymal stem cells. Journal of orthopaedic research : official publication of the Orthopaedic Research Society *25*, 665-677.

Matsubara, T., Kida, K., Yamaguchi, A., Hata, K., Ichida, F., Meguro, H., Aburatani, H., Nishimura, R., and Yoneda, T. (2008). BMP2 regulates Osterix through Msx2 and Runx2 during osteoblast differentiation. The Journal of biological chemistry *283*, 29119-29125.

Miyazono, K., Kusanagi, K., and Inoue, H. (2001). Divergence and convergence of TGF-beta/BMP signaling. Journal of cellular physiology *187*, 265-276.

Mountziaris, P.M., and Mikos, A.G. (2008). Modulation of the inflammatory response for enhanced bone tissue regeneration. Tissue engineering Part B, Reviews *14*, 179-186.

Mountziaris, P.M., Spicer, P.P., Kasper, F.K., and Mikos, A.G. (2011). Harnessing and modulating inflammation in strategies for bone regeneration. Tissue engineering Part B, Reviews *17*, 393-402.

Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J.M., Behringer, R.R., and de Crombrugghe, B. (2002). The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell *108*, 17-29.

Oeckinghaus, A., and Ghosh, S. (2009). The NF-kappaB family of transcription factors and its regulation. Cold Spring Harbor perspectives in biology *1*, a000034.

Pneumaticos, S.G., Triantafyllopoulos, G.K., Basdra, E.K., and Papavassiliou, A.G. (2010). Segmental bone defects: from cellular and molecular pathways to the development of novel biological treatments. Journal of cellular and molecular medicine *14*, 2561-2569.

Rawadi, G., Vayssiere, B., Dunn, F., Baron, R., and Roman-Roman, S. (2003). BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research *18*, 1842-1853.

Retting, K.N., Song, B., Yoon, B.S., and Lyons, K.M. (2009). BMP canonical Smad signaling through Smad1 and Smad5 is required for endochondral bone formation. Development *136*, 1093-1104.

Rodda, S.J., and McMahon, A.P. (2006). Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. Development *133*, 3231-3244.

Schmidt-Bleek, K., Schell, H., Schulz, N., Hoff, P., Perka, C., Buttgereit, F., Volk, H.D., Lienau, J., and Duda, G.N. (2012). Inflammatory phase of bone healing initiates the regenerative healing cascade. Cell and tissue research *347*, 567-573.

Simpson, A.H., Mills, L., and Noble, B. (2006). The role of growth factors and related agents in accelerating fracture healing. The Journal of bone and joint surgery British volume *88*, 701-705.

Sims, N.A., and Martin, T.J. (2014). Coupling the activities of bone formation and resorption: a multitude of signals within the basic multicellular unit. BoneKEy reports *3*, 481.

Steinbrecher, K.A., Harmel-Laws, E., Sitcheran, R., and Baldwin, A.S. (2008). Loss of epithelial RelA results in deregulated intestinal proliferative/apoptotic homeostasis and susceptibility to inflammation. In Journal of immunology, pp. 2588-2599.

Swarnkar, G., Zhang, K., Mbalaviele, G., Long, F., and Abu-Amer, Y. (2014). Constitutive activation of IKK2/NF-kappaB impairs osteogenesis and skeletal development. PloS one *9*, e91421.

Szpalski, C., Barr, J., Wetterau, M., Saadeh, P.B., and Warren, S.M. (2010). Cranial bone defects: current and future strategies. Neurosurgical focus *29*, E8.

Takada, I., Kouzmenko, A.P., and Kato, S. (2010). PPAR-gamma Signaling Crosstalk in Mesenchymal Stem Cells. PPAR research *2010*.

Tevlin, R., McArdle, A., Atashroo, D., Walmsley, G.G., Senarath-Yapa, K., Zielins, E.R., Paik, K.J., Longaker, M.T., and Wan, D.C. (2014). Biomaterials for craniofacial bone engineering. Journal of dental research *93*, 1187-1195.

Vallabhapurapu, S., and Karin, M. (2009). Regulation and function of NF-kappaB transcription factors in the immune system. Annual review of immunology *27*, 693-733.

Zhang, X., Schwarz, E.M., Young, D.A., Puzas, J.E., Rosier, R.N., and O'Keefe, R.J. (2002). Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair. The Journal of clinical investigation *109*, 1405-1415.

CHAPTER 3

GENE EXPRESSION ANALYSIS IN A CRANIAL BONE HEALING MODEL USING MICE WITH A NF-κB HAPLOINSUFFICIENCY

INTRODUCTION

Bony defects in the craniofacial skeleton have a large negative impact on society both economically and functionally (Amini et al., 2012; Baroli, 2009). Due to the complexity of bone repair, bone defects remain a challenging problem for surgeons and researchers. In order to enhance bone regeneration, autografts have been utilized as a golden standard for treating bony defects in clinical settings. Despite the advantages and non-immunogenic nature of autografts, the high cost and post-surgery patient morbidity have limited their application (Amini et al., 2012; Blokhuis et al., 2013). Recently, scientists have focused on the development of graft substitutes using synthetic scaffolds in a combination with growth factors or cells (Agarwal et al., 2009; Rahman et al., 2014). As a result of their strong osteoinductive capability, bone morphogenetic proteins hold great promise for tissue engineering application. BMPs belong to the transforming growth factor β (TGF-β) superfamily, which is involved in many biological processes including organogenesis, embryogenesis and wound healing. Elevated BMP2 expression has been observed in native craniofacial fractures (Farhadieh et al., 2004; Khanal et al., 2008). Moreover, previous work suggests a crucial role for BMP2 in the initiation and progression of bone fracture healing

(Kugimiya et al., 2005). Local delivery of BMP2 by an absorbable collagen carrier has been shown to enhance bone healing and regeneration in both experimental and clinical applications (Kim et al., 2005; Lin et al., 2013; Simpson et al., 2006).

Accumulating evidence demonstrates the significant impact of the initial inflammatory response on bone regeneration following bone injury (Gerstenfeld et al., 2003; Lu et al., 2013). Onset of injury induces enhanced acute expression of proinflammatory cytokines such as TNFα, IL-1β and IL-6 (Kon et al., 2001; Mountziaris and Mikos, 2008). The inflammatory signals then stimulate healing cascades by recruiting progenitor cells, thereby priming the environment for stem cell differentiation and angiogenesis (Mountziaris et al., 2011). Proinflammatory factors stimulate osteogenic differentiation of stem cells by increasing the productions of BMP2 (Fukui et al., 2003; Hess et al., 2009; Rifas, 2006). Accordingly, the maximal expression of BMP2 can be observed within 24 hours following injury. Further, the osteoinductive capability of BMP2 is impaired in the absence of proper inflammatory signals (Katavic et al., 2003; Zhang et al., 2002). These findings, taken together, suggest crosstalk between BMP2 mediated bone regeneration and the inflammatory signaling pathway.

In an attempt to understand the relationship between inflammation and bone repair, our lab has been focused on NF-κB, a central mediator in the immune system and inflammatory process. Previously, by utilizing a genetically modified mouse model with a p65 (a major component of NF-κB signaling) haploinsufficiency, we demonstrated that p65 partial ablation leads to delayed and defective calvarial repair. Further studies suggest that the mechanism by which NF-κB insufficiency negatively affects bone repair is through

disruption of osteoblastic differentiation *via* the BMP2/Smad pathway (Refer to Chapter 2). Our findings suggest that the integrity of the NF-κB mediated inflammatory signaling pathway in bone forming cells is vital for BMP2 induced bone regeneration. Consistently, other studies have demonstrated that NF-κB activation and TNFα treatment induces osteogenic differentiation and matrix mineralization in stem cells (Caron et al., 2012; Cho et al., 2010; Feng et al., 2013; Hess et al., 2009). Despite these large advances, the precise role of NF-κB in bone healing, along with the associated cellular and molecular pathways, remains unknown. This is a result of the complexity and intricacy of the bone repair process.

Whole genome microarray analysis serves as a powerful tool that enables researchers to study gene expression and identify signaling pathways of interest on a large, genome-wide scale. Using microarray analysis, previous studies have investigated the gene expression patterns associated with fracture repair (Rundle et al., 2006). In this study, we sought to identify the differentially expressed genes during rhBMP2 mediated calvarial bone repair in $p65^{fl/+}$Osx-Cre mice as compared to that of the $p65^{fl/+}$ (control) group.

Materials and Methods

Mice

p65-floxed mice were crossed with Osterix-Cre mice to create mice with osteoblast-restricted p65 ablation ($p65^{fl/+}$Osx-Cre). p65 floxed mice ($p65^{fl/fl}$) in which exons 5-8 were flanked with loxp constructs were provided by Dr. Albert S Baldwin of University of North Carolina at Chapel Hill (Steinbrecher et al., 2008). Osx-Cre mice, which express a GFP fused Cre recombinase gene downstream of the Osterix promoter, were purchased from Jackson Laboratory (Bar Harbor, ME). The Osx-Cre mice have been described in previous literature (Rodda and McMahon, 2006). All animals were on the C57BL/6 background.

Cranial bone healing model

Mice at 9-11s week old (n=5) were anesthetized using ketamine/xylazine (124/8.8 mg/kg) by intraperitoneal injection. After heads were shaved, the skin was sterilized using 70% ethanol and betadine. Next, a midline incision was made, parallel to the sagittal suture, with a scalpel, thereby exposing the parietal bone. A 4 mm diameter defect was then created using a trephine bur attached to a dental handpiece. Great caution was taken to minimize injury to the dura mater. An absorbable collagen sponge (diameter ≈4 mm, thickness ≈1 mm) (DuraGen, Integra， Plainsboro， NJ) containing 500 ng rhBMP2 (Medtronic, Minneapolis， MN) was placed over the defect. The skin was sutured using absorbable 4 or 5-0 chromic gut sutures (Ethicon, Somerville, NJ). Animals recovered on a heating pad before being transferred to clean recovery cages with soft bedding. Pain was managed using ketoprofen (5 mg/kg, s.c) following surgeries. All procedures were

performed in accordance with protocols approved by the Institutional Animal Care and Use Committees of the University of North Carolina (IACUC).

Total RNA isolation

4 weeks following surgeries, mice were euthanized using CO_2 asphyxiation followed by cervical dislocation. A trephine attached to a dental handpiece was used to remove bony tissue in the defect area. The tissue samples were immediately flash-frozen in liquid nitrogen. The samples were kept at -80 ^{o}C. Specimens were homogenized using a mortar and pestle in liquid nitrogen. QIAzol lysis reagent (Qiagen, Valencia, CA, USA) was added into the powdered tissue for further homogenization. Total RNA was then extracted using the Qiagen RNeasy Micro kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. RNA concentrations were measured using the Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA). RNA integrity was assessed using a Bioanalyzer (Agilent, Santa Clara, CA, USA). RNA samples with an RNA integrity number (RIN) score of 7.5 or higher were submitted for microarray analysis.

Microarray analysis

RNA samples were processed and hybridized onto Affymetrx arrays at the UNC genomic core facility (University of North Carolina at Chapel Hill). The GeneChip® WT PLUS Reagent Kit (Affymetrix, Santa Clara, CA) was used for cDNA synthesis from the total RNA. After amplification, synthesized cDNA were fragmented and labeled using the Affymetrix GeneChip Terminal Labeling Kit. Labeled cDNA fragments were then hybridized to the Affymetrix Mouse Gene 2.0 ST array (Affymetrix). The Affymetrix Mouse Gene 2.0 ST array

system has a comprehensive transcript coverage of 35240 total RefSeq transcripts and 26191 well-established gene annotations, and utilizes an autoloader (http://www.affymetrix.com/catalog/131476/AFFY/Mouse+Gene+ST+Arrays#1_1). The arrays were washed and stained with R-phycoerythrin streptavidin in the GeneChip Fluidics Station 450 (Affymetrix). Arrays were scanned using the GeneChip Scanner 3000 7G Plus.

The resultant image files (.CEL) were processed and analyzed using GeneSpring software v.12.6 (Agilent Technologies, Santa Clara, CA). Gene expression values were compared between the $p65^{fl/+}$Osx-Cre and $p65^{fl/+}$ (control) mice. To identify differentially expressed genes between $p65^{fl/+}$Osx-Cre and control ($p65^{fl/+}$) mice, unpaired t-tests were used, with Benjamini and Hochberg false discovery rate corrections. Statistical significance was defined as $p<0.05$.

Results:

Microarray results

Differential gene expression was not observed between $p65^{fl/+}$Osx-Cre and control ($p65^{fl/+}$) mice. Possible explanations for this observation can be found in the Discussion section.

Gene oncology analysis

Genes that were either upregulated or downregulated with a fold change of 3 or greater were submitted for Gene Ontology (GO) analysis, which was performed using the GO Ontology Browser function provided by GeneSpring software. 55 GO terms satified the p-value cut-off of 0.05. The top 30 GO terms can be found in Table 3.3. 83 GO terms satisfied the corrected p-value cut-off of 0.05.

Next, genes with a fold change of 1.2 or greater were examined. Among the over-represented GO terms, two skeleton/bone related categories were identified: Regulation of Ossification (p=0.0001) and Regulation of Biomineral Tissue Tevelopment (p=0.0238). There were 54 genes identified in the Regulation of Ossification category (Tables 3.4-3.5) and 9 genes identified in the Regulation of Biomineral Tissue Tevelopment category (Table 3.6). A complete list of identified genes in each category can be found in Tables 3.4-6. The p-value listed for each GO term represents a relative enrichment in frequency for that GO category.

$p65^{fl/+}$Osx-Cre mice demonstrated decreased BMP (BMP4, BMP6, BMP7) expression as compared to the $p65^{fl/+}$ group (Table 3.4). The BMP family has over 17 other members, including BMP4, 6 and 7. Due to their osteogenic capability of inducing ecotopic bone formation, these members are commonly recognized as osteogenic BMPs (Bragdon et al.,

2011). A reduction in levels of osteogenic markers osterix (*Sp7*), alkaline phosphotase (*Alp*) was also obeserved in $p65^{fl/+}$Osx-Cre mice.

Additionally, other osteogenic genes were downregulated in mice with a p65 haploinssuficiency (Tables 3.4-6). Wnt element(low-density lipoprotein receptor-related protein 5 (*Lrp5*) is important for maintaining bone mass. The glycoprotein m6b (*Gpm6bA*) gene encodes a membrane glycoprotein that serves as regulator of osteoblast function and bone formation (Drabek et al., 2011). Dlx5 is a key regulator for bone formation (Levi and Gitton, 2014). $p65^{fl/+}$Osx-Cre elicited less *Dlx5* and *Gpm6bA* expression, suggesting impaired bone mineralization. Special AT-rich sequence-binding protein (SATB2) is a potent transcription factor that is critical for skeletal development and osteoblastogenesis (Dobreva et al., 2006). It interacts and synergizes with other osteogenic transcription factors such as runx2 and osterix, robustly enhancing their transcriptional activity to promote osoteogenesis (Zhang et al., 2011). *Satb2* overexpresion has been shown to induce osteoblastic differentiation *in vitro* and also promote bone regeneration *in vivo* (Gong et al., 2014a; Gong et al., 2014b).

In addition, several genes related to bone formation and remodelling were increased in $p65^{fl/+}$Osx-Cre including BMP3 and Extracellular matrix protein 1 (Ecm1) (Table 3.5). Ecm1 is a secreted glycoprotein that negatively regulates endochondral bone formation through inhibition of alkaline phosphotase activity and mineralization (Deckers et al., 2001). BMP3 is a negative regulator for bone morphogenesis and development (Daluiski et al., 2001). BMP3 lacks a cystein residue, which is normally responsible for BMP dimerization. BMP3 serves as

BMP antagonist and competes with other osteogenic BMPs for receptor binding (Rosen, 2006).

Discussion:

In the present study, RNA samples were taken from bone tissue that has been regenerated at calvarial defects in both $p65^{fl/+}$Osx-Cre and $p65^{fl/+}$ mice. A genome wide microarray analysis was utilized for samples in an attempt to identify differentially expressed genes, along with downstream signaling pathways that are associated with bone repair. No genes were differentially expressed between groups when using a p-value cut-off of 0.05. There are, however, several explanations for this outcome.

The bone repair process involves in a series of overlapping events that initiate from an acute inflammatory phase, which lasts for 1-3 days. A combination of intramembranous ossification and endochondral ossification occur subsequently, beginning at 7-14 days following injury. Bone remodeling takes place beginning approximately 10 days and lasting at least 28 days following injury (Ai-Aql et al., 2008; Khan et al., 2008; Kon et al., 2001). The vigorous renewal phase in bone regeneration is associated with great amount of signaling cascades, as compared to the much quieter remodeling phase, which involves significantly less pathways (Khan et al., 2008). A previously reported, relevant study evaluated the expression pattern of transcription factors during bone repair in rat, using microarray analysis based on 1254 probe sets. The number of significant transcription factors peaked at Day 5, and gradually decreases to minimal basal levels at Day 56, following injury (Wise et al., 2010). Taken together, these previous findings suggest that the dramatic bone regeneration process occurs in the first 2 weeks following bone injury (Hadjiargyrou et al., 2002).

In a previous investigation, we observed decreased bone repair (reflected by the healing ratio using micro-CT) in p65-ablated mice implanted with ACS+rhBMP2 (0.72 vs 0.42 at 4 week, $p<0.01$), as compared to controls. The healing level in the control group slightly improved to 0.91 at 8 weeks. In accordance with the temporal bone healing phases described previously, the control group demonstrated a greater regeneration within the first 4 weeks, with mild enhancement in the following 4 weeks. Interestingly, the p65 haploinsufficient mice did not improve significantly (0.42 vs 0.45, $p>0.05$) from 4 weeks to 8 weeks. These findings suggest a very likely possibility that the selective time point (4 weeks post surgery) exceeds the time frame when the most active bone regeneration occurs. At 4 weeks, the biological and molecular events associated with bone repair remain at a lower level than typical for the bone defect model. Fewer participating signaling pathways could be one explanation for the resulting outcome. Furthermore, the precise mechanism by which NF-κB signaling affects osteoblast expression is yet to be elucidated. It is possible that NF-κB signaling pathway is essential for osteoblastic cells in an early phase (e.g. before 4 weeks) during repair in our bone defect model.

In this study, we studied gene expression in five experimental $p65^{fl/+}$Osx-Cre mice and four control $p65^{fl/+}$ mice. The sample sizes may be too small to achieve adequate statistical power. Additionally, we observed a high variation within sample replicates for each group during the microarray analysis. As shown in Figure 3.1, the replicates varied and scattered for both the control and experimental groups. Each animal in its respective group (either control or p65 haploinsufficient) was regarded as a replicate. Each replicate shared the same genetic background and underwent the same experimental treatment. Since the

replicates of one group represent similar experimental conditions, they are assumed to be similar to values within the same group, and different than values in the other group. High biological variation among sample replicates could be another reason for the observed failure to gain statistically significant results.

In summary, using a global survey of gene expression using microarray analysis, no differential gene expression was observed between $p65^{fl/+}$Osx-Cre and $p65^{fl/+}$ mice. Three possible explanations for this outcome include that 1) the selected time point may be beyond the timeframe for occurrence of dramatic biological bone repair events, 2) the number of samples may not be sufficient to achieve statistical significance and 3) large variations among sample replicates within groups may have affected the results. Still, the current results implicate an obvious trend, demonstrating that mice with a p65 haploinsufficiency express lower levels of osteogenic genes, including osteogenic differentiation markers as well as key mediators involved various osteogenic signaling pathways (BMPs, Wnts, etc.).

Table 3.1 Gene downregulation observed in p65$^{fl/+}$Osx-Cre mice at 4 weeks.

Transcripts Cluster Id	Fold Change	Gene symbol	Gene description
17305520	-8.17	Ear1	eosinophil-associated, ribonuclease A family, member1
17284568	-6.55	LOC636067	Ig heavy chain Mem5-like
17284379	-5.89	Igh-VJ558	immunoglobulin heavy chain (J558family)
17502789	-5.75	Gypa	glycophorinA
17531482	-4.97	Camp	cathelicidin antimicrobial peptide
17522555	-4.96	Ltf	lactotransferrin
17467453	-4.95	Igkv4-59	immunoglobulin kappa variable 4-59
17522369	-4.87	Ngp	neutrophilic granuleprotein
17372662	-4.86	Prg2	proteoglycan2, bone marrow
17284500	-4.34	Ighg	Immunoglobulin heavychain (gamma polypeptide)
17284660	-4.28	Ighm	immunoglobulin heavy constant mu
17362922	-4.26	Ms4a1	membrane-spanning4-domains, subfamilyA, member1
17217035	-4.20	Ctse	cathepsinE
17337706	-4.15	Rhag	Rhesusblood group-associated A glycoprotein
17404180	-4.06	Car1	carbonic anhydrase 1
17475127	-4.02	Cd79a	CD79A antigen (immunoglobulin-associated alpha)
17235011	-4.00	Elane	elastase,neutrophil expressed
17267520	-3.91	Epx	eosinophil peroxidase
17284507	-3.87	Ighg|Ighm	Immunoglobulin heavy chain (gamma polypeptide)|immunoglobulin heavy constant mu
17467540	-3.82	Igk-V28	immunoglobulin kappa chain variable 28(V28)
17254877	-3.82	Mpo	myeloperoxidase
17270162	-3.71	Slc4a1	solute carrier family 4 (anion exchanger), member1
17514553	-3.70	Mmp8	matrix metallopeptidase 8
17396162	-3.62	Car2	carbonic anhydrase2
17312219	-3.51	Ly6g	lymphocyte antigen 6 complex, locusG
17383892	-3.48	Lcn2	lipocalin2
17329023	-3.46	Iglv1	immunoglobulin lambda variable1
17462373	-3.46	Cecr2	cat eye syndrome chromosome region, candidate 2
17239664	-3.46	Myb	myeloblastosis oncogene
17229178	-3.42	Atp1b1	ATPase, Na+/K+ transporting, beta1 polypeptide

Table 3.2 Gene upregulation observed in p65$^{fl/+}$Osx-Cre mice at 4 weeks.

Transcripts Cluster Id	Fold Change	Gene symbol	Gene description
17239845	3.94	Arg1	arginase, liver
17294934	3.75	Thbs4	thrombospondin4
17326069	3.37	Retnla	resistin like alpha
17218927	3.31	Dpt	dermatopontin
17238558	3.21	Mmp19	matrix metallopeptidase19
17254053	2.90	Ccl11	chemokine (C-C motif) ligand11
17302475	2.75	Irg1	immunoresponsive gene1
17523659	2.73	Ccr5	chemokine (C-C motif) receptor5
17254047	2.65	Ccl7	chemokine (C-C motif) ligand7
17232215	2.64	Moxd1	monooxygenase,DBH-like1
17245231	2.61	Lyz1	lysozyme1
17230111	2.61	Ifi205	interferon activated gene 205
17310339	2.60	C1qtnf3	C1q and tumor necrosis factor related protein3
17460891	2.56	Fbln2	fibulin2
17493949	2.56	Folr2	folate receptor2(fetal)
17353747	2.52	Cd14	CD14 antigen
17254065	2.52	Ccl8	chemokine (C-C motif) ligand8
17499212	2.52	F7	coagulation factor VII
17403268	2.51	Gbp2|Gbp1	guanylate binding protein2|guanylate binding protein1
17453819	2.51	Serpine1	serine(or cysteine)peptidase inhibitor, cladeE, member1
17278328	2.49	Serpina3n	serine(or cysteine) peptidase inhibitor, cladeA, member3N
17459196	2.45	Tnip3	TNFAIP 3interacting protein3
17514515	2.45	Mmp3	matrix metallopeptidase3
17449710	2.45	Cxcl9	chemokine (C-X-C motif) ligand9
17462788	2.41	Clec4n	C-type lectin domain family4, member n
17350925	2.36	Iigp1	interferon inducible GTPase1
17229931	2.35	Slamf8	SLAM family member8
17254041	2.29	Ccl2	chemokine (C-C motif) ligand2
17512611	2.25	Dpep2	dipeptidase2
17306983	2.25	Gzme|Gzmd	granzymeE|granzymeD

Table 3.3 Gene Ontology Results for in $p65^{fl/+}$Osx-Cre mice (Terms with Fold Change > 3).

GO ACCESSION	GO Term	corrected p-value	Count in Selection
GO:0005488	binding	0.01	39
GO:0043170|GO:0043283	macromolecule metabolic process	0.05	24
GO:0006807	nitrogen compound metabolic process	0.05	20
GO:0034641	cellular nitrogen compound metabolic process	0.05	19
GO:0016043|GO:0044235|GO:0071842	cellular component organization	0.02	18
GO:0071840|GO:0071841	cellular component organization or biogenesis	0.03	18
GO:0090304	nucleic acid metabolic process	0.02	17
GO:0005576	extracellular region	4.49E-04	16
GO:0043933|GO:0034600|GO:0034621	macromolecular complex subunit organization	1.92E-07	16
GO:0071822	protein complex subunit organization	2.20E-07	15
GO:0003677	DNA binding	0.01	14
GO:0065003	macromolecular complex assembly	6.89E-07	14
GO:0006461	protein complex assembly	1.21E-07	14
GO:0022607|GO:0071844	cellular component assembly	5.88E-05	14
GO:0044085|GO:0071843	cellular component biogenesis	2.52E-04	14
GO:0070271	protein complex biogenesis	1.21E-07	14
GO:0006996	organelle organization	0.02	13
GO:0006259|GO:0055132	DNA metabolic process	6.46E-07	13
GO:0051276|GO:0007001|GO:0051277	chromosome organization	2.57E-06	12
GO:0000785|GO:0005717	chromatin	1.03E-09	12
GO:0044427	chromosomal part	5.15E-07	12
GO:0006325	chromatin organization	1.71E-07	12
GO:0044421	extracellular region part	6.99E-04	12
GO:0005694	chromosome	2.75E-06	12
GO:0006323	DNA packaging	9.78E-13	11
GO:0006333	chromatin assembly or disassembly	1.35E-13	11
GO:0006334	nucleosome assembly	3.68E-15	11
GO:0034622	cellular macromolecular complex assembly	1.60E-07	11
GO:0034728	nucleosome organization	2.80E-14	11
GO:0031497	chromatin assembly	2.80E-14	11

Table 3.4 Gene Downregulation of the GO Regulation of Ossification Category in $p65^{fl/+}$Osx-Cre mice (Fold Change > 1.2).

Transcript Cluster ID	Gene symbol	Gene description	Fold change
17223283	Satb2	special AT-rich sequence binding protein2	-1.59
17255458	Phospho1|Zfp652	phosphatase,orphan1|zinc finger protein652	-1.29
17260633	Fignl1	fidgetin-like1	-1.90
17280327	Rsad2	radical S-adenosyl methionine domain containing 2	-1.90
17286340	Foxc1	fork head boxC1	-1.58
17286682	Bmp6	bone morphogenetic protein6	-1.82
17288917	Mef2c	myocyte enhancer factor2C	-1.47
17305709	Bmp4	bone morphogenetic protein44	-1.79
17316793	Rspo2	R-spondin2 homolog (Xenopuslaevis)	-1.26
17322219	Sp7		-1.40
17336338	Col11a2	collagen,typeXI,alpha2	-1.24
17337513	Gabbr1	gamma-aminobutyric acid(GABA)B Receptor,1	-1.39
17354730	Slc26a2	solute carrier family 26 (sulfate transporter), member 2	-1.35
17359816	Kazald1	Kazal-type serine peptidase inhibitor domain 1	-1.46
17360977	Lrp5	low density lipoprotein receptor-related protein 5	-1.23
17395041	Bmp7	bone morphogenetic protein7	-1.37
17396878	Sox2	SRY-box containing gene2	-1.23
17398970	Mef2d	myocyte enhancer factor 2D	-1.22
17401673	Sort1	sortilin1	-1.45
17402662	Lef1	lymphoid enhancer binding factor1	-1.42
17404011	Hey1	hairy/enhancer-of-split related with YRPW motif1	-1.28
17431720	Alpl	alkaline phosphatase,liver/bone/kidney	-1.28
17464706	Dlx5	distal-less homeobox5	-1.40
17465942	Atp6v0a4	ATPase,H+ transporting, lysosomal V0 subunit A4	-1.33
17498142	Igf2	insulin-like growth factor2	-1.35
17506296	Foxc2	forkhead boxC2	-1.45
17522167	Plxnb1	plexinB1	-1.36
17539611	Gpm6b	glycoprotein m6b	-1.25
17545087	Chrdl1	chordin-like1	-1.31

Table 3.5 Gene Upregulation of the GO Ossification Category in $p65^{fl/+}$Osx-Cre mice (Fold Change > 1.2).

Transcript Cluster ID	Gene symbol	Gene description	Fold change
17218060	Ptgs2	prostaglandin-endoperoxide synthase2	1.37
17232235	Ctgf	connective tissue growth factor	1.53
17232529	Col10a1	collagen,typeX,alpha1	1.74
17244726	Myf5	myogenic factor5	1.25
17261542	Fgf18	fibroblast growth factor18	1.28
17263388	Wnt3a	wingless-related MMTV integration site3A	1.22
17265229	Alox15	arachidonate15-lipoxygenase	1.66
17287160	Aspn	asporin	1.82
17308817	Tnfsf11	tumor necrosis factor (ligand) superfamily,member11	1.24
17399347	Thbs3	thrombospondin3	1.32
17405489	Igsf10|Med12l	immunoglobulin superfamily, member10|mediator of RNA polymeraseII transcription, subunit12 homolog (yeast)-like	1.33
17407850	Ecm1	extracellular matrix protein1	1.92
17409075	Csf1	colony stimulating factor1 (macrophage)	1.28
17410974	Cyr61	cysteine rich protein61	1.63
17420171	Hspg2	perlecan(heparan sulfate proteoglycan2)	1.28
17435107	Lrrc17	leucine rich repeat containing 17	1.95
17439535	Bmp3	bone morphogenetic protein3	1.95
17445325	Fzd1	frizzled homolog1 (Drosophila)	1.44
17502001	Jund	Junproto-oncogene related gene d	1.28
17503825	Mmp2	matrix metallopeptidase2	1.29
17509758	Csgalnact1	chondroitin sulfate N-acetyl-galactosaminyltransferase1	1.53
17511259	Junb	Jun-Boncogene	1.45
17523566	Clec3b	C-type lectin domain family3, memberb	1.58
17541681	Gpc3	glypican3	1.35
17544517	Gla	galactosidase,alpha	1.61

Table 3.6 Gene Dysregulation of the GO Regulation of Biomineral Tissue Development Category in $p65^{fl/+}$Osx-Cre mice (Fold Change > 1.2).

Transcript Cluster ID	Gene symbol	Gene description	Fold change
17286682	Bmp6	bone morphogenetic protein6	-1.82
17305709	Bmp4	bone morphogenetic protein44	-1.79
17439825	Mepe	matrix extracellular phosphoglycoprotein with ASARM motif (bone)	-1.59
17497709	Ifitm5	interferon induced transmembrane protein5	-1.65
17539966	Gata1	GATAbindingprotein1	-1.56
17287160	Aspn	asporin	1.82
17310982	Osr2	odd-skipped related2	1.61
17322337	Nfe2	nuclearfactor, erythroid derived2	1.29
17407850	Ecm1	extracellular matrix protein1	1.92

Figure 3.1 Sample Quality Control

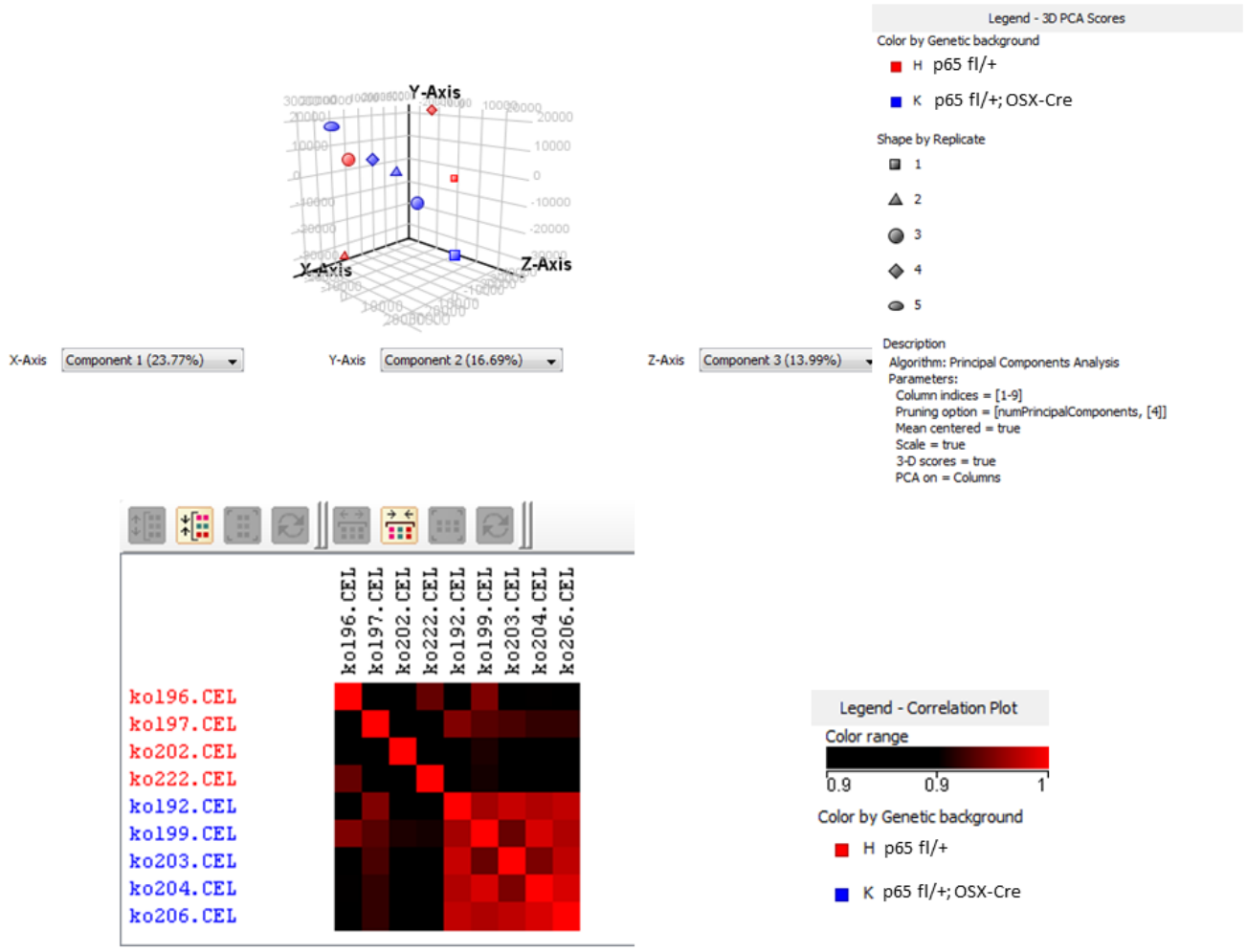
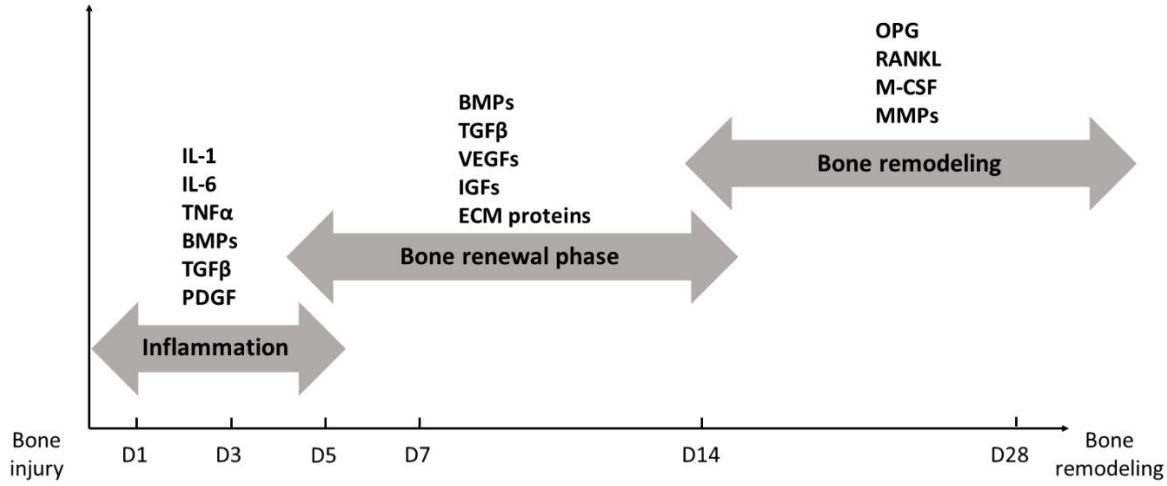


Figure 3.2 A schematic of temporal gene expression profiling during the bone healing process.



Adapted from Ai-Aql et al., 2008; Hadjiargyrou et al., 2002; Khan et al., 2008; Kon et al., 2001

REFERENCES

Agarwal, R., Williams, K., Umscheid, C.A., and Welch, W.C. (2009). Osteoinductive bone graft substitutes for lumbar fusion: a systematic review. Journal of neurosurgery Spine *11*, 729-740.

Ai-Aql, Z.S., Alagl, A.S., Graves, D.T., Gerstenfeld, L.C., and Einhorn, T.A. (2008). Molecular mechanisms controlling bone formation during fracture healing and distraction osteogenesis. Journal of dental research *87*, 107-118.

Amini, A.R., Laurencin, C.T., and Nukavarapu, S.P. (2012). Bone tissue engineering: recent advances and challenges. Critical reviews in biomedical engineering *40*, 363-408.

Baroli, B. (2009). From natural bone grafts to tissue engineering therapeutics: Brainstorming on pharmaceutical formulative requirements and challenges. Journal of pharmaceutical sciences *98*, 1317-1375.

Blokhuis, T.J., Calori, G.M., and Schmidmaier, G. (2013). Autograft versus BMPs for the treatment of non-unions: what is the evidence? Injury *44 Suppl 1*, S40-42.

Bragdon, B., Moseychuk, O., Saldanha, S., King, D., Julian, J., and Nohe, A. (2011). Bone morphogenetic proteins: a critical review. Cellular signalling *23*, 609-620.

Caron, M.M., Emans, P.J., Surtel, D.A., Cremers, A., Voncken, J.W., Welting, T.J., and van Rhijn, L.W. (2012). Activation of NF-kappaB/p65 facilitates early chondrogenic differentiation during endochondral ossification. PloS one *7*, e33467.

Cho, H.H., Shin, K.K., Kim, Y.J., Song, J.S., Kim, J.M., Bae, Y.C., Kim, C.D., and Jung, J.S. (2010). NF-kappaB activation stimulates osteogenic differentiation of mesenchymal stem cells derived from human adipose tissue by increasing TAZ expression. Journal of cellular physiology *223*, 168-177.

Daluiski, A., Engstrand, T., Bahamonde, M.E., Gamer, L.W., Agius, E., Stevenson, S.L., Cox, K., Rosen, V., and Lyons, K.M. (2001). Bone morphogenetic protein-3 is a negative regulator of bone density. Nature genetics *27*, 84-88.

Deckers, M.M., Smits, P., Karperien, M., Ni, J., Tylzanowski, P., Feng, P., Parmelee, D., Zhang, J., Bouffard, E., Gentz, R*., et al.* (2001). Recombinant human extracellular matrix protein 1

inhibits alkaline phosphatase activity and mineralization of mouse embryonic metatarsals in vitro. Bone *28*, 14-20.

Dobreva, G., Chahrour, M., Dautzenberg, M., Chirivella, L., Kanzler, B., Farinas, I., Karsenty, G., and Grosschedl, R. (2006). SATB2 is a multifunctional determinant of craniofacial patterning and osteoblast differentiation. Cell *125*, 971-986.

Drabek, K., van de Peppel, J., Eijken, M., and van Leeuwen, J.P. (2011). GPM6B regulates osteoblast function and induction of mineralization by controlling cytoskeleton and matrix vesicle release. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research *26*, 2045-2051.

Farhadieh, R.D., Gianoutsos, M.P., Yu, Y., and Walsh, W.R. (2004). The role of bone morphogenetic proteins BMP-2 and BMP-4 and their related postreceptor signaling system (Smads) in distraction osteogenesis of the mandible. The Journal of craniofacial surgery *15*, 714-718.

Feng, X., Feng, G., Xing, J., Shen, B., Li, L., Tan, W., Xu, Y., Liu, S., Liu, H., Jiang, J.*, et al.* (2013). TNF-alpha triggers osteogenic differentiation of human dental pulp stem cells via the NF-kappaB signalling pathway. Cell biology international *37*, 1267-1275.

Fukui, N., Zhu, Y., Maloney, W.J., Clohisy, J., and Sandell, L.J. (2003). Stimulation of BMP-2 expression by pro-inflammatory cytokines IL-1 and TNF-alpha in normal and osteoarthritic chondrocytes. The Journal of bone and joint surgery American volume *85-A Suppl 3*, 59-66.

Gerstenfeld, L.C., Cho, T.J., Kon, T., Aizawa, T., Tsay, A., Fitch, J., Barnes, G.L., Graves, D.T., and Einhorn, T.A. (2003). Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research *18*, 1584-1592.

Gong, Y., Qian, Y., Yang, F., Wang, H., and Yu, Y. (2014a). Lentiviral-mediated expression of SATB2 promotes osteogenic differentiation of bone marrow stromal cells in vitro and in vivo. European journal of oral sciences *122*, 190-197.

Gong, Y., Xu, F., Zhang, L., Qian, Y., Chen, J., Huang, H., and Yu, Y. (2014b). MicroRNA expression signature for Satb2-induced osteogenic differentiation in bone marrow stromal cells. Molecular and cellular biochemistry *387*, 227-239.

Hadjiargyrou, M., Lombardo, F., Zhao, S., Ahrens, W., Joo, J., Ahn, H., Jurman, M., White, D.W., and Rubin, C.T. (2002). Transcriptional profiling of bone regeneration. Insight into the molecular complexity of wound repair. The Journal of biological chemistry *277*, 30177-30182.

Hess, K., Ushmorov, A., Fiedler, J., Brenner, R.E., and Wirth, T. (2009). TNFalpha promotes osteogenic differentiation of human mesenchymal stem cells by triggering the NF-kappaB signaling pathway. Bone *45*, 367-376.

Katavic, V., Grcevic, D., Lukic, I.K., Vucenik, V., Kovacic, N., Kalajzic, I., and Marusic, A. (2003). Non-functional Fas ligand increases the formation of cartilage early in the endochondral bone induction by rhBMP-2. Life sciences *74*, 13-28.

Khan, S.N., Solaris, J., Ramsey, K.E., Yang, X., Bostrom, M.P., Stephan, D., and Daluiski, A. (2008). Identification of novel gene expression in healing fracture callus tissue by DNA microarray. HSS journal : the musculoskeletal journal of Hospital for Special Surgery *4*, 149-160.

Khanal, A., Yoshioka, I., Tominaga, K., Furuta, N., Habu, M., and Fukuda, J. (2008). The BMP signaling and its Smads in mandibular distraction osteogenesis. Oral diseases *14*, 347-355.

Kim, C.S., Kim, J.I., Kim, J., Choi, S.H., Chai, J.K., Kim, C.K., and Cho, K.S. (2005). Ectopic bone formation associated with recombinant human bone morphogenetic proteins-2 using absorbable collagen sponge and beta tricalcium phosphate as carriers. Biomaterials *26*, 2501-2507.

Kon, T., Cho, T.J., Aizawa, T., Yamazaki, M., Nooh, N., Graves, D., Gerstenfeld, L.C., and Einhorn, T.A. (2001). Expression of osteoprotegerin, receptor activator of NF-kappaB ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research *16*, 1004-1014.

Kugimiya, F., Kawaguchi, H., Kamekura, S., Chikuda, H., Ohba, S., Yano, F., Ogata, N., Katagiri, T., Harada, Y., Azuma, Y.*, et al.* (2005). Involvement of endogenous bone morphogenetic protein (BMP) 2 and BMP6 in bone formation. The Journal of biological chemistry *280*, 35704-35712.

Levi, G., and Gitton, Y. (2014). Dlx genes and the maintenance of bone homeostasis and skeletal integrity. Cell death and differentiation *21*, 1345-1346.

Lin, C.Y., Chang, Y.H., Li, K.C., Lu, C.H., Sung, L.Y., Yeh, C.L., Lin, K.J., Huang, S.F., Yen, T.C., and Hu, Y.C. (2013). The use of ASCs engineered to express BMP2 or TGF-beta3 within scaffold constructs to promote calvarial bone repair. Biomaterials *34*, 9401-9412.

Lu, Z., Wang, G., Dunstan, C.R., Chen, Y., Lu, W.Y., Davies, B., and Zreiqat, H. (2013). Activation and promotion of adipose stem cells by tumour necrosis factor-alpha preconditioning for bone regeneration. Journal of cellular physiology *228*, 1737-1744.

Mountziaris, P.M., and Mikos, A.G. (2008). Modulation of the inflammatory response for enhanced bone tissue regeneration. Tissue engineering Part B, Reviews *14*, 179-186.

Mountziaris, P.M., Spicer, P.P., Kasper, F.K., and Mikos, A.G. (2011). Harnessing and modulating inflammation in strategies for bone regeneration. Tissue engineering Part B, Reviews *17*, 393-402.

Rahman, C.V., Ben-David, D., Dhillon, A., Kuhn, G., Gould, T.W., Muller, R., Rose, F.R., Shakesheff, K.M., and Livne, E. (2014). Controlled release of BMP-2 from a sintered polymer scaffold enhances bone repair in a mouse calvarial defect model. Journal of tissue engineering and regenerative medicine *8*, 59-66.

Rifas, L. (2006). T-cell cytokine induction of BMP-2 regulates human mesenchymal stromal cell differentiation and mineralization. Journal of cellular biochemistry *98*, 706-714.

Rodda, S.J., and McMahon, A.P. (2006). Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. Development *133*, 3231-3244.

Rosen, V. (2006). BMP and BMP inhibitors in bone. Annals of the New York Academy of Sciences *1068*, 19-25.

Rundle, C.H., Wang, H., Yu, H., Chadwick, R.B., Davis, E.I., Wergedal, J.E., Lau, K.H., Mohan, S., Ryaby, J.T., and Baylink, D.J. (2006). Microarray analysis of gene expression during the inflammation and endochondral bone formation stages of rat femur fracture repair. Bone *38*, 521-529.

Simpson, A.H., Mills, L., and Noble, B. (2006). The role of growth factors and related agents in accelerating fracture healing. The Journal of bone and joint surgery British volume *88*, 701-705.

Steinbrecher, K.A., Harmel-Laws, E., Sitcheran, R., and Baldwin, A.S. (2008). Loss of epithelial RelA results in deregulated intestinal proliferative/apoptotic homeostasis and susceptibility to inflammation. In Journal of immunology, pp. 2588-2599.

Wise, J.K., Sena, K., Vranizan, K., Pollock, J.F., Healy, K.E., Hughes, W.F., Sumner, D.R., and Virdi, A.S. (2010). Temporal gene expression profiling during rat femoral marrow ablation-induced intramembranous bone regeneration. PloS one *5*.

Zhang, J., Tu, Q., Grosschedl, R., Kim, M.S., Griffin, T., Drissi, H., Yang, P., and Chen, J. (2011). Roles of SATB2 in osteogenic differentiation and bone regeneration. Tissue engineering Part A *17*, 1767-1776.

Zhang, X., Schwarz, E.M., Young, D.A., Puzas, J.E., Rosier, R.N., and O'Keefe, R.J. (2002). Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair. The Journal of clinical investigation *109*, 1405-1415.

CHAPTER 4

GENERAL DISCUSSION

Bone defect is a significant healthcare problem that largely jeopardizes general social welfare. In the United States, over 2.5 billion dollars are used to treat patients with bone defect annually (Amini et al., 2012; Baroli, 2009). There is an urgent need for the development of efficient treatments for bone defect that integrate bone repair and regeneration techniques.

While many investigations demonstrate an inhibitory effect of inflammation on bone formation (Pape et al., 2010; Thomas and Puleo, 2011), short-term exposure of inflammation can actually enhance osteogenesis. Previous studies show that pro-inflammatory cytokines including TNFα, IL-1 and IL-6 increase BMP synthesis and induce osteogenic differentiation of chondrocytes and human mesenchymal stem cells (Fukui et al., 2003; Rifas, 2006). Local administration of TNFα during the early stages of injury accelerates fracture healing *in vivo*, suggesting that pro-inflammatory cytokines play important role in the initial osteogenic differentiation of progenitors involved with bone healing (Glass et al., 2011). Accordingly, the absence of sufficient inflammatory signals results in impaired bone healing in animal models (Gerstenfeld et al., 2003; Katavic et al., 2003; Zhang et al., 2002). Further, application of anti-inflammatory medication to patients with long bone fracture increases the risk of nonunion, malunion and infection

(Bhattacharyya et al., 2005; Jeffcoach et al., 2014). These seemingly contradicting results raise many questions regarding the effects of inflammation on wound repair.

NF-κB is a central mediator of inflammation and the immune response, and is therefore an attractive target for investigating the role of the inflammatory reaction in bone repair. NF-κB signaling pathway has long been known to regulate the function of hematopoietic stem cell (HSC) derived cell lineages (e.g. T cell, B cells, monocytes.) (Stein and Baldwin, 2013). Accumulating research suggests a role for NF-κB in MSC modulation. NF-κB signaling regulates stem cell fate by targeting expression of critical transcription factors. NF-κB also functions as an inhibitor of chondrogenesis (Sitcheran et al., 2003) myogenesis (Langen et al., 2001) and adipogenesis. However, the effect of NF-κB on osteogenesis is not well studied and the current knowledge of this interaction is controversial.

We and several other groups have suggested a positive effect of NF-κB on osteogenic differentiation of stem cells including hMSCs and human stem cells derived from apical papilla (Caron et al., 2012; Cho et al., 2010; Feng et al., 2013; Hess et al., 2009; Li et al., 2014). In contrast, several other studies have indicated a negative role of NF-κB on osteogenesis (Chang et al., 2009; Swarnkar et al., 2014). Given that they employed transgenic mice with manipulated NF-κB function on osteoblasts in a later or earlier stage then the one studied by our group, one possible explanation for the discrepancy is that the role of NF-κB is dependent on the stage of differentiation. Furthermore, the observations made by other groups were based on the results of manipulating NF-κB regulatory elements

such as IκB kinase. The effect of this manipulation could possibly extend to several other NF-κB components or subunits sharing the same modulators as NF-κB/p65.

In this study, we generated a p65 conditional knock down mouse with p65 haploinsufficiency restricted to preosteoblasts, by crossing p65 floxed mice with *Sp7* driven Cre mice. In this model, NF-κB was impaired at an early stage of osteoblastic differentiation in the Sp7 expressing cell lineage. Direct targeting of p65, a major component of the NF-κB signaling pathway, ensured that other subunits of the NF-κB pathway were not affected. This model enabled the study of the direct influence of p65 in early osteoblastogenesis during bone repair.

In the present study, the healing process in $p65^{fl/+}$Osx-Cre mice was impaired or delayed as shown by the micro-CT analysis and histological assessments. We next sought to elucidate the underlying mechanisms by which p65 influences osteogenesis.

Bone is able to self-repair without formation of scar tissue through a process that recapitulates the natural bone development of both intramembranous and endochondral bone (Dimitriou et al., 2011; Einhorn, 1998). Bone repair is a well-defined and strictly orchestrated process where many cellular and molecular events take place in order to regenerate new bone. These complex events occur in overlapping phases including MSC recruitment, proliferation, differentiation and bone remodeling.

The initial step to obtaining successful regeneration is the recruitment of sufficient amounts of MSCs and progenitor cells to the site of injury (Schmidt-Bleek et al., 2012). MSCs are stem cells that can further differentiate into osteoblasts. The presence of MSCs in the

bone healing process is critical for the outcome of bone repair due to its ability to modulate inflammation and its stimulatory effect on tissue regeneration. Various cell types, such as endothelial and immune cells, produce a broad spectrum of inflammatory cytokines and matrix proteins within minutes of bone trauma (Mountziaris and Mikos, 2008; Mountziaris et al., 2011). In response to these chemoattractants, MSCs from local sources (e.g. periosteum, muscle and local bone marrow) and circulating MSCs migrate to injured sites (Maxson et al., 2012).

Bone marrow contains primitive MSCs that can be recruited to bone defects for repair following trauma. In the present work, $p65^{fl/+}$Osx-Cre mice showed less CFU-F colonies, whereas the CFU-OB colonies were dramatically reduced, as compared to both control groups ($p65^{fl/+}$ mouse and Osx-Cre mice). MSCs from $p65^{fl/+}$Osx-Cre mice showed less osteogenic potential in response to rhBMP2. Fewer osteoprogenitors within the bone marrow reservoir in p65 haploinsufficient mice, possibly contributing to the defective bone repair processes. Furthermore, in $p65^{fl/+}$Osx-Cre mice, fewer preosteoblasts displayed expression of early bone repair markers within the regenerated tissue 4 weeks following rhBMP2 application. Previous research in our lab demonstrates that the cell proliferation rate in p65 haploinsufficient mice is not different from that of the control group. MSCs are multipotent stem cells that can develop into a variety of cells, including adipocytes and chondrocytes. Instead of osteogenic specification, MSCs in mice with the p65 haploinsufficiency may be predisposed to an adipogenic or chondrogenic specification.

The migration of MSCs induced by secreted chemoattractants at the site of injury also plays an important role in bone healing. The migration capability of these progenitors

influences the outcome of bone repair. In an attempt to investigate the broader effects of the p65 haploinsufficiency on rhBMP2-induced osteoblastic differentiation, we evaluated the gene expression profile of bone defects 4 weeks following rhBMP2-induced bone repair. Although no statistically significant genes were identified in $p65^{fl/+}$Osx-Cre mice as compared to the control animals ($p65^{fl/+}$ mice), we still observed upregulation of several genes that are responsible for chemotaxis and cytoskeleton modulation (Table 3.2). Furthermore, we also found that several osteogenic genes were downregulated in p65 haploinsufficient mice (Table 3.4).

In order to regenerate new bone, the recruited MSCs undergo osteogenic differentiation into osteoblasts, which then produce bone matrix and promote mineralization. Resembling bone morphogenesis during embryonic development, new bone is formed through intramembranous and endochondral ossification. During intramembranous ossification, primitive MSCs directly differentiate into osteoblasts. In endochondral ossification, MSCs first differentiate into chondrocytes and produce a cartilage template, which osteoblasts later replace with bone tissue (Javed et al., 2010). Differentiation of MSCs into mature osteoblasts requires sequential expression of many transcription factors and matrix proteins.

Owing to their pivotal role in osteoblastogenesis, these factors are commonly referred to as bone markers. Runx2 and osterix are key transcription factors that promote an osteogenic lineage commitment as well as osteoprogenitor differentiation during early osteogenic differentiation (Nakashima et al., 2002). Alkaline Phosphatase (ALP) and Bone Sialoprotein (BSP) are early osteogenic markers important for synthesis and maturation of

extracellular matrix. Osteocalcin (OCN) is a definitive marker of terminally differentiated osteoblasts (Maxson et al., 2012). Biochemical and biomechanical cues within the microenvironment regulate bone formation *via* bone precursors and various intracellular and extracellular molecular pathways. Wnt, TGF-β, BMP, VEGF and MAPK signaling pathways have been implicated in osteogenic differentiation of MSCs (Allori et al., 2008). Although many key players have been identified, the complex interaction and crosstalk between the various signaling cascades remains only partially understood.

We demonstrated that osteoprogenitors isolated from bone marrow of $p65^{fl/+}$Osx-Cre mice displayed compromised osteoblastic differentiation with a corresponding decrease in the expression of several osteogenic genes (*Sp7*, *Alp*, *Bsp*). This appears to be particularly evident during the early stages of differentiation. These data are consistent with microarray results that demonstrated downregulation of *Sp7*, *Alp* and *Stab2* in $p65^{fl/+}$Osx-Cre mice. Together with data from previous studies, this suggests that NF-κB signaling plays a distinct role in different stages during osteoblastogenesis. Our data hereby support that sufficient NF-κB function is required for early osteoblastic differentiation.

The BMP2/Smad signaling pathway is a key regulator of osteoinduction and osteogenesis. Smads, which are immediately downstream of BMP2, mediate many osteogenic cascades. In our study, the immunoblotting demonstrated that rhBMP2-induced phosphorylation of Smads was impaired in $p65^{fl/+}$Osx-Cre mice. Further, C2C12 cells with intact NF-κB function demonstrated increased p-Smads expression following activation by acute TNFα. One consequence of acute TNFα treatment and NF-κB activation is the enhancement of p-Smads abundance following BMP2 signaling. The Increased p-Smads

abundance may also result from enhanced Smad 1 expression following the TNFα application. In summary, the relatively low NF-κB levels observed in the p65 haploinsufficient animals likely causes reduced signaling along the Smad pathway.

Recent studies in this field discussed concerns regarding the unexpected skeletal abnormalities of Osterix-Cre mice, which could possibly confound the results of bone studies utilizing this model (Chen et al., 2014; Huang and Olsen, 2015; Wang et al., 2015). These findings were based on the observations of bone phenotypes during physiological bone development in pups or young mice, without investigation of the bone healing and regeneration mechanisms. Here, we demonstrated that Osx-Cre mice possess comparable bone repair processes to that of the p65-floxed control mice. We did not observe any defective healing in the Osx-Cre mice during natural or rhBMP2 induced bone repair at 4 weeks. The healing level was significantly decreased in p65 haploinsufficient mice as compared to the Osx-Cre mice. Further, Osx-Cre mice displayed a similar relative percentage of MSCs and osteoprogenitors within whole bone marrow stromal cells (CFU-Fs and CFU-OBs) as compared to the p65-floxed control mice. Enhanced osteogenic gene expression was also observed in Osx-Cre mice during bone healing, implying the presence of an active bone regeneration process. Our data therefore suggest that the absence of p65 function primarily contributes to the compromised bone healing in p65 haploinsufficient mouse.

In conclusion, we have demonstrated that mice with ablated p65 function have decreased efficinecy of bone repair. Cells of $p65^{fl/+}$Osx-Cre mice have fewer CFU-OBs as well as decreased *Sp7*, *Alp*, *Bsp* and *Satb2* expression, indicating defective osteoblastogenesis.

The adverse effect of the p65 haploinsuffiency on osteoblastogenesis and bone repair can be attributed to the diminished canonical Smad pathway. These data suggest that intact NF-κB is essential for osteoblastogenesis during the bone healing process.

REFERENCES

Allori, A.C., Sailon, A.M., and Warren, S.M. (2008). Biological basis of bone formation, remodeling, and repair-part I: biochemical signaling molecules. Tissue engineering Part B, Reviews *14*, 259-273.

Amini, A.R., Laurencin, C.T., and Nukavarapu, S.P. (2012). Bone tissue engineering: recent advances and challenges. Critical reviews in biomedical engineering *40*, 363-408.

Baroli, B. (2009). From natural bone grafts to tissue engineering therapeutics: Brainstorming on pharmaceutical formulative requirements and challenges. Journal of pharmaceutical sciences *98*, 1317-1375.

Bhattacharyya, T., Levin, R., Vrahas, M.S., and Solomon, D.H. (2005). Nonsteroidal antiinflammatory drugs and nonunion of humeral shaft fractures. Arthritis and rheumatism *53*, 364-367.

Caron, M.M., Emans, P.J., Surtel, D.A., Cremers, A., Voncken, J.W., Welting, T.J., and van Rhijn, L.W. (2012). Activation of NF-kappaB/p65 facilitates early chondrogenic differentiation during endochondral ossification. PloS one *7*, e33467.

Chang, J., Wang, Z., Tang, E., Fan, Z., McCauley, L., Franceschi, R., Guan, K., Krebsbach, P.H., and Wang, C.Y. (2009). Inhibition of osteoblastic bone formation by nuclear factor-kappaB. Nature medicine *15*, 682-689.

Chen, J., Shi, Y., Regan, J., Karuppaiah, K., Ornitz, D.M., and Long, F. (2014). Osx-Cre targets multiple cell types besides osteoblast lineage in postnatal mice. PloS one *9*, e85161.

Cho, H.H., Shin, K.K., Kim, Y.J., Song, J.S., Kim, J.M., Bae, Y.C., Kim, C.D., and Jung, J.S. (2010). NF-kappaB activation stimulates osteogenic differentiation of mesenchymal stem cells derived from human adipose tissue by increasing TAZ expression. Journal of cellular physiology *223*, 168-177.

Dimitriou, R., Jones, E., McGonagle, D., and Giannoudis, P.V. (2011). Bone regeneration: current concepts and future directions. BMC medicine *9*, 66.

Einhorn, T.A. (1998). The cell and molecular biology of fracture healing. Clinical orthopaedics and related research, S7-21.

Feng, X., Feng, G., Xing, J., Shen, B., Li, L., Tan, W., Xu, Y., Liu, S., Liu, H., Jiang, J.*, et al.* (2013). TNF-alpha triggers osteogenic differentiation of human dental pulp stem cells via the NF-kappaB signalling pathway. Cell biology international *37*, 1267-1275.

Fukui, N., Zhu, Y., Maloney, W.J., Clohisy, J., and Sandell, L.J. (2003). Stimulation of BMP-2 expression by pro-inflammatory cytokines IL-1 and TNF-alpha in normal and osteoarthritic chondrocytes. The Journal of bone and joint surgery American volume *85-A Suppl 3*, 59-66.

Gerstenfeld, L.C., Cho, T.J., Kon, T., Aizawa, T., Tsay, A., Fitch, J., Barnes, G.L., Graves, D.T., and Einhorn, T.A. (2003). Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research *18*, 1584-1592.

Glass, G.E., Chan, J.K., Freidin, A., Feldmann, M., Horwood, N.J., and Nanchahal, J. (2011). TNF-alpha promotes fracture repair by augmenting the recruitment and differentiation of muscle-derived stromal cells. Proceedings of the National Academy of Sciences of the United States of America *108*, 1585-1590.

Hess, K., Ushmorov, A., Fiedler, J., Brenner, R.E., and Wirth, T. (2009). TNFalpha promotes osteogenic differentiation of human mesenchymal stem cells by triggering the NF-kappaB signaling pathway. Bone *45*, 367-376.

Huang, W., and Olsen, B.R. (2015). Skeletal defects in Osterix-Cre transgenic mice. Transgenic research *24*, 167-172.

Javed, A., Chen, H., and Ghori, F.Y. (2010). Genetic and transcriptional control of bone formation. Oral and maxillofacial surgery clinics of North America *22*, 283-293, v.

Jeffcoach, D.R., Sams, V.G., Lawson, C.M., Enderson, B.L., Smith, S.T., Kline, H., Barlow, P.B., Wylie, D.R., Krumenacker, L.A., McMillen, J.C.*, et al.* (2014). Nonsteroidal anti-inflammatory drugs' impact on nonunion and infection rates in long-bone fractures. The journal of trauma and acute care surgery *76*, 779-783.

Katavic, V., Grcevic, D., Lukic, I.K., Vucenik, V., Kovacic, N., Kalajzic, I., and Marusic, A. (2003). Non-functional Fas ligand increases the formation of cartilage early in the endochondral bone induction by rhBMP-2. Life sciences *74*, 13-28.

Langen, R.C., Schols, A.M., Kelders, M.C., Wouters, E.F., and Janssen-Heininger, Y.M. (2001). Inflammatory cytokines inhibit myogenic differentiation through activation of nuclear factor-kappaB. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *15*, 1169-1180.

Li, J., Yan, M., Wang, Z., Jing, S., Li, Y., Liu, G., Yu, J., and Fan, Z. (2014). Effects of canonical NF-kappaB signaling pathway on the proliferation and odonto/osteogenic differentiation of human stem cells from apical papilla. BioMed research international *2014*, 319651.

Maxson, S., Lopez, E.A., Yoo, D., Danilkovitch-Miagkova, A., and Leroux, M.A. (2012). Concise review: role of mesenchymal stem cells in wound repair. Stem cells translational medicine *1*, 142-149.

Mountziaris, P.M., and Mikos, A.G. (2008). Modulation of the inflammatory response for enhanced bone tissue regeneration. Tissue engineering Part B, Reviews *14*, 179-186.

Mountziaris, P.M., Spicer, P.P., Kasper, F.K., and Mikos, A.G. (2011). Harnessing and modulating inflammation in strategies for bone regeneration. Tissue engineering Part B, Reviews *17*, 393-402.

Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J.M., Behringer, R.R., and de Crombrugghe, B. (2002). The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell *108*, 17-29.

Pape, H.C., Marcucio, R., Humphrey, C., Colnot, C., Knobe, M., and Harvey, E.J. (2010). Trauma-induced inflammation and fracture healing. Journal of orthopaedic trauma *24*, 522-525.

Rifas, L. (2006). T-cell cytokine induction of BMP-2 regulates human mesenchymal stromal cell differentiation and mineralization. Journal of cellular biochemistry *98*, 706-714.

Schmidt-Bleek, K., Schell, H., Schulz, N., Hoff, P., Perka, C., Buttgereit, F., Volk, H.D., Lienau, J., and Duda, G.N. (2012). Inflammatory phase of bone healing initiates the regenerative healing cascade. Cell and tissue research *347*, 567-573.

Sitcheran, R., Cogswell, P.C., and Baldwin, A.S., Jr. (2003). NF-kappaB mediates inhibition of mesenchymal cell differentiation through a posttranscriptional gene silencing mechanism. Genes & development *17*, 2368-2373.

Stein, S.J., and Baldwin, A.S. (2013). Deletion of the NF-kappaB subunit p65/RelA in the hematopoietic compartment leads to defects in hematopoietic stem cell function. Blood *121*, 5015-5024.

Swarnkar, G., Zhang, K., Mbalaviele, G., Long, F., and Abu-Amer, Y. (2014). Constitutive activation of IKK2/NF-kappaB impairs osteogenesis and skeletal development. PloS one *9*, e91421.

Thomas, M.V., and Puleo, D.A. (2011). Infection, inflammation, and bone regeneration: a paradoxical relationship. Journal of dental research *90*, 1052-1061.

Wang, L., Mishina, Y., and Liu, F. (2015). Osterix-Cre transgene causes craniofacial bone development defect. Calcified tissue international *96*, 129-137.

Zhang, X., Schwarz, E.M., Young, D.A., Puzas, J.E., Rosier, R.N., and O'Keefe, R.J. (2002). Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair. The Journal of clinical investigation *109*, 1405-1415.