**ROLE OF NFκB P65 IN OSTEOGENESIS THROUGH
OSTEOBLAST REGULATION**

Sodsi Wirojchanasak

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in
partial fulfillment of the requirements for the degree of Doctor of Philosophy
in the Curriculum of Oral Biology (School of Dentistry)

Chapel Hill
2012

Approved by

Professor Lyndon F. Cooper

Professor Albert S. Baldwin

Professor Eric T. Everett

Professor Paul Krebsbach

Assoc. Prof. Patrick Flood

ABSTRACT

SODSI WIROJCHANASAK: Role of NFκB p65 in osteogenesis through
osteoblast regulation
(Under the direction of Professor Lyndon F. Cooper)

Inflammation affects bone repair and strongly impacts bone regeneration
therapy. The transcription factor NFκB mediates inflammatory responses which exert
regulatory effects on bone homeostasis. Concepts regarding the effect of inflammation
on osteogenesis during bone development remain elusive. The current study
investigated the influence of NFκB on osteogenesis through osteoblast regulation *in
vivo* using a Cre-LoxP recombination strategy. Mice with *p65* haploinsufficiency
targeting osteoprogenitors were generated and the phenotypes were characterized. The
mice lacking one *p65* allele in osteogenic cells displayed reduced skeletal dimension,
less bone mineralization and altered bone structure. Cellular assays provided evidence
that reduced NFκB activities due to *p65* haploinsufficiency resulted in decreased
differentiation and elevated apoptosis in the osteogenic cells. Increased *in situ*
apoptosis in bones from the mutant mice confirmed the protective role of NFκB in
osteogenic cell survival. Lack of one *p65* allele attenuated osteogenic potential of
mesenchymal stem cells derived from the mutant mice. The heterotopic bone
formation approach using BMP2 was employed to assess bone formation potential in

the mice with *p65* haploinsufficiency in osteoprogenitors. The *p65* haploinsufficienty mice showed less newly formed bone in the BMP2-induced bone regeneration process in compared with the controls due to reduced osteoblast differentiation. In conclusion, NFκB modulates osteoblastic activities. Diminution of NFκB activities in osteoprogenitors retards bone development which alters skeletal size and bone architecture. Decreased osteoblastic differentiation and increased apoptosis in the p65 haploinsufficient osteogenic cells indicate that there are multiple levels which NFκB acts in control of bone formation.

To my mother,

who has been giving me immeasurable support and courage

to follow my dream.

ACKNOWLEDGEMENTS

This part of the dissertation holds more than my heart-felt appreciation during the culmination of my studying period. It also reflects the invaluable relationships with people whom I have met and influenced my entire life since the beginning of my graduate study.

First and foremost, I would like to show my deep gratitude to my mentor, Dr. Lyndon F Cooper for giving me the privilege to conduct research in his laboratory. His brilliant mind with substantial scientific expertise has inspired me to explore the research world from the very first days of my Ph.D. program. His challenging questions have developed my thoughts in both academic and scientific aspects. Despite the great amount of responsibilities he has, he always gives me meticulous guidance and supports during the period of my investigation. His immeasurable encouragement throughout the years of my studying makes me feel fortunate and grateful to have him as my mentor.

I am deeply grateful to Dr. Eric T Everett, my committee member for his friendship and thoughtful guidance. His enthusiasm in teaching and his constant encouragement have motivated me in the pursuit of my dissertation. My gratefulness goes to Dr. Patrick Flood, the Oral Biology Program director and my committee member for giving me the opportunity to enroll in this program and for being

supportive in every aspect of my study. I also would like to express my gratitude to my committee members Dr. Albert S Baldwin, and Dr. Paul Krebsbach for their continued support. Their valuable and constructive comments and criticism have greatly contributed to the completion of my dissertation.

My sincere appreciation also goes to Naresuan University for the Commission of high education Thailand scholarship, which enables my advanced studies at the University of North Carolina at Chapel Hill accomplished.

I would like to thank previous and current members of Dr. Cooper's lab, especially Dr. Daniela Mendonça and Dr. Gustavo Mendonça. Their presence and companionship have established an excellent research environment with diligence and genuine friendship. The joyful times in the lab are so memorable.

My sincere thanks go to Dr. Marnisa Sricholpech for her help and kindness, my classmates, the people in the Dental Research Center, my friends at UNC and in Thailand for a network of support in various forms that I have received which helped me go through various obstacles in life. The great experiences I have had here are to be with them. My thankful thoughts also go to Cindy Blake, the Oral biology program manager, for her affectionate help.

My deepest thanks are for my beloved mother and my family members for their love and faith in me during the long years of my education. My graduation will bring pride to them.

Lastly, I would like to thank my boyfriend Mohammed, for his love and tremendous support throughout my study life. I cherish all the moments I have shared with you.

TABLE OF CONTENTS

LIST OF TABLES

Table

Page

LIST OF FIGURES

Figures

Page

ABBREVIATIONS

ALP   Alkaline phosphatase

BAFF   B-cell activatin factor

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

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

DMEM   Dulbecco's modified Eagle's medium

EDTA   Ethylenediaminetetraacetic acid

FBS   Fetal bovine serum

GAPDH   Glyceraldehyde-3-pohophate dehydrogenase

GFP	Green fluorescent protein
IκB	Protein inhibitor of NFκB
IKK	IκB kinase
IL	Interleukin
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
α-MEM	Modified Eagle's medium alpha
MOI	Multiplicity of infection
MSC	Mesenchymal stem cell
MSX	Msh homeobox
NFATC1	Nuclear factor of activated T cells, cytoplasmic 1
NFκB	Nuclear factor kappa B
NIK	NFκB inducing kinase
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
RANK	Receptor activator of NFκB
RANKL	Ligand of receptor activator of NFκB
RT-PCR	Reverse transcriptase-polymerase chain reaction
RUNX2	Runt-related transcription factor 2
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STAT1	Signal transducers and activators transcription factor 1
TAZ	Tafazzin
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
TNFR	Receptor for tumor necrosis factor
TRAP	Tartrate resistant acid phosphatase
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
WNT	Wingless-type MMTV integration site family
YAP	Yes-associated protein

CHAPTER 1

INTRODUCTION

Bone: function and health impact from bone disorders

Bone is a dynamic mineralized organ that continually remodels via the turnover of extracellular and cellular components, as regulated by coordinated activities of bone forming cells (osteoblasts) and bone resorbing cells (osteoclasts). Bone by itself, or in concert with cartilage, is a principle structure of the skeletal system which serves a major function in mechanical support. Additionally, the functions of bone involve a) protection of vital organs including brain, heart, lung, and bone marrow, b) locomotion with muscle and tendon attachment, and c) reservoirs for calcium and phosphate mineral contents (Cohen, 2006).

Dysregulation of osteoblasts and/or osteoclasts negatively affects bone structure which leads to disturbance of skeletal functions. Several diseases derived from imbalanced bone homeostasis such as osteoporosis and Paget's disease of bone have been global health problems. Osteoporosis is a metabolic bone disease that leads to reduced bone mineral density as a consequence from excessive bone resorption and diminished bone formation. Paget's disease of bone is a localized bone disorder with enlarged but weak bone affected by excessive bone resorption from overactive

osteoclasts and accelerated bone formation in a disorganized pattern from osteoblasts. In the United States, osteoporosis is one major public health problem that affects more than 44 million American people. Paget's disease of bone is the second most common bone disease with approximately one million patients having misshapen and/or fracture of the affected bones (NIAMS, 2011). In each year, over 1.5 million osteoporotic patients suffer from bone fractures and millions more are at risk. Compromised bone formation in the osteoporotic patients causes problems for orthopedic and bone regenerative treatment outcomes (Cornell et al., 2003; LaPorte et al., 2008). The failure of these treatments is a consequence of decreased amount of bone tissues and active bone forming cells (Perrini et al., 2008). Thus, it is compelling for researchers to clarify pathways that enhance bone formation in order to succeed in the recovery of the bone functions.

Another problematic bone disorder arises from critical-size bone defects from congenital anomalies, ablative surgery, fracture or injuries. The bone defect size over a critical limit results in the impairment of the skeletal functions according to incomplete bone reconstruction. The critical-size bone defect exceeds the natural healing potential of body which leads to fibrous tissue formation rather than new bone formation in the defect. Although bone tissues contain regenerative capacity, compromised conditions from the impaired functions of bone cells or from the critical-size defects can restrain bone reconstruction outcome. Successful treatments of the compromised bone defects by conventional or advanced therapeutic modalities, like bone grafting and tissue engineering techniques, depends on the potential of osteoblasts in synthesizing sufficient bone mass to restore the bone functions (Bruder

and Fox, 1999). Manipulation of coordinating factors that favors osseous healing environments can improve the recruitment of osteogenic cells thus enhancing new bone tissue formation (Salgado et al., 2004; Ward et al., 2010). Although a number of the regulatory molecules of bone have been identified, there is a gap of knowledge regarding complex biological pathways applicable to bone regeneration. Therefore, deciphering the factors that influence bone formation is essential for developing therapeutic approaches for bone defects which will ultimately increase success rates of the treatments.

Bone developmental and regeneration processes

Osteogenesis is a process that results in the formation of bone tissues. To maintain physiological bone structures, osteogenesis is required for increase of bone mass during development and for compensation of bone loss from bone resorption or injuries (Katagiri and Takahashi, 2002; Zaidi, 2007). The mechanisms for osteogenesis occur into two different patterns. One is intramembranous ossification which originates from neural crest cells that form mesenchymal condensation and differentiate directly into osteoblasts in craniofacial bones. The other is endochondral ossification, a developmental process that requires cartilagenous templates before bone replacement in axial and limb skeletons. In the endochondral mechanism, mesenchymal cells first differentiate into chondroblasts to generate the cartilage template then develop to hypertrophic chondrocytes. The chondrocytes are subsequently replaced by osteoblasts which derive from the same mesenchymal cell origin together with vascular ingrowth (Neve et al., 2011). After bone tissues are formed, some osteoblasts are embedded in mineralized bone matrices and become

osteocytes which function in transmitting signals in response to mechanical forces (Jiang et al., 2007). Some osteoblasts become inactive lining cells called pericytes at bone surfaces as a reservoir of bone forming cells. Concurrently to the bone formation process, osteoclasts develop and function in remodelling bone structures into proper shapes and resorbing bone tissues to create bone marrow spaces (Neve et al., 2011). During the developmental state, the bone formation rate is higher than the bone resorption rate while bones in mature state require balanced functions between osteoblasts and osteoclasts to maintain a tissue integrity (Cohen, 2006; Datta et al., 2008).

The mechanisms of the bone formation during osseous healing processes are overlapped with those of the bone development in both intramembranous and endochondral ossifications (Gerstenfeld et al., 2003). The expression of the molecular markers of chondrogenesis and osteogenesis during bone repair corresponds to the markers that are presented throughout bone developmental processes (Grimes et al., 2011). One different aspect between bone developmental and healing processes is an initial inflammatory reaction which is apparent in the beginning of osseous healing. Previous studies suggested that the initial inflammatory response plays a role in priming the subsequent bone regeneration process by triggering a release of enhancing factors for bone formation. However, chronic inflammation has an opposite effect, causing a failure in bone formation (Crisostomo et al., 2008; Mountziaris et al., 2011). Another factor that regulates the bone reparative processes is dependent on the mechanical environment. Non-stabilized bone fractures heal via endochondral ossification while stabilized bone fractures and cortical bone defects are restored

through intramembranous healing (Behonick et al., 2007; Lu et al., 2004). During the endochondral healing, granulation tissues are formed at the early inflammatory phase with the aggregation of growth factors and osteoblastic stimulating molecules (Itagaki et al., 2008). Stem cells from bone marrow and lining cells at periosteum, and cells from other sources are triggered by these changes of microenvironment, then migrate into the lesion site and differentiate into chondrogenic and osteogenic cells afterwards (Schindeler et al., 2008). A later stage is a reparative/regenerative period starting with callus formation, determined by a fibrocartilage formation at post-fracture from day 3 to day 7 then followed by bone replacement at post-fracture day 10 to day 14. The newly formed tissue is finally remodeled by osteoclasts representing functional bone architecture (Gerstenfeld et al., 2003; Kilian et al., 2008). For intramembranous bone healing, bone formation is established following an inflammatory phase without cartilage formation from the beginning of bone regeneration until the end of bone remodeling phase (Thompson et al., 2002).

In tissue engineering-induced bone regeneration, designated approaches are based on the principles of modulating a healing environment to be suitable for bone reconstruction by targeting cells, extracellular matrix, and signaling molecules (Cooper et al., 2001; Scheller et al., 2009). The repairing processes from bone tissue engineering occur in a cascade of events similar to bone fracture healing with acute inflammation in the beginning, followed by bone formation and bone remodeling. For instance, the use of bone morphogenetic proteins 2 (BMP2)-induced bone formation leads to endochondral ossification according to osteoinduction potential of this growth factor (Hou et al., 2007).

Osteoblast formation and function in osteogenesis

Osteoblasts play a central role in osteogenesis during skeletal development, bone homeostasis, bone repair and regeneration as these cells serve functions in bony structure formation and remodeling. Osteoblasts arise from mesenchymal origin and share a common progenitor with chondrocytes, myoblasts and adipocytes (Pittenger et al., 1999; Prockop et al., 2003; Song et al., 2006). During osteogenesis, osteoblasts are developed from mesenchymal stem cells through multiple stages from proliferation, differentiation, matrix maturation, and mineralization. Mesenchymal stem cells differentiate into osteochondro-progenitor cells which are able to transform to chondrogenic or osteogenic cells. These progenitor cells develop to particular terminally differentiated cells according to the specification of cell fate (called lineage commitment) via expression of specific signaling molecules during differentiation (Long, 2011). For osteoblastic lineage, the progenitor cells differentiate to proliferating pre-osteoblasts which turn into mature osteoblasts. After mature osteoblasts perform bone matrix production, many osteoblasts succumb to apoptosis. Some of these osteoblasts proceed to terminally differentiated osteocytes residing in calcified bone tissue. Some osteoblasts become inactive lining cells adjacent to periosteum and bone surface for bone turnover purpose (Neve et al., 2011).

During osteoblast formation, a number of molecules are expressed as phenotypic markers of specific developmental stages of osteoblast and involve in bone structure establishment (Long, 2011). Increased levels of type 1 collagen, alkaline phosphatase (ALP) and bone sialoprotein (BSP) are considered as early markers of osteoblast development from proliferation to matrix maturation stages. Osteocalcin

(OCN) is presented during mineralization stage as a late marker for mature osteoblasts. In addition to bone formation, osteoblasts serve a function in osteoclast regulation as a part of bone remodeling control (Neve et al., 2011). Osteoblasts produce receptor activator of nuclear factor kappa-B ligand (RANKL), a required factor for osteoclast differentiation and activation. With stimulation by RANKL and macrophage colony stimulating factor (M-CSF), osteoclasts become mature, as characterized by the presence of tartrate resistant acid phosphatase (TRAP) and cathepsin K, and mediate bone resorption during osteogenesis and bone repair (Yamamoto et al., 2006). Osteoblasts also provide negative feedback control in osteoclast development by production of osteoprotegerin (OPG) to block RANKL-activation of osteoclasts (Neve et al., 2011).

Regulation of osteoblastic differentiation

The control of osteoblastic differentiation is orchestrated by a complex array of environmental factors that are manifested in extracellular matrix such as cytokines, growth factors, and hormones. The extrinsic regulation over osteoblasts is exerted through cellular interaction with these extracellular elements or cell-cell interaction which provokes intrinsic change over aspects of cell fate involving pluripotency, proliferative capacity, and differentiation state (Katagiri and Takahashi, 2002). Intrinsic change during osteoblast differentiation requires sequential expression of several molecules to determine the specification of mesenchymal stem cells into ostoeblastic lineage (Long, 2011). The most frequently investigated osteogenic signaling pathway involves the expression of transcription factor RUNX2 and OSX which contributes to BMP2 regulation of osteoinduction and maintenance of

osteoblast specification during mesenchymal stem cell differentiation (Baek et al., 2010; Ducy et al., 1999; Katagiri and Takahashi, 2002; Nakashima et al., 2002).

BMP2 osteogenic signaling

BMP2 belongs to a transforming growth factor beta (TGFβ) superfamily as a member of Bmps subfamily (Cao and Chen, 2005). Recombinant human BMP2 has been used clinically to promote bone formation via profound action on osteoblastogenesis (Boyne et al., 2005; Katayama et al., 2009). BMP2 is critical to embryonic development and postnatal physiology attributed to its pleiotropic function. Various animal models have been used to reveal the functions of BMP2. Null mutation of *Bmp2* caused embryonic lethality in mice from day 7.5 to day 10.5 due to failure in cardiac development (Zhang and Bradley, 1996). Mice with conditional deletion of *Bmp2* under control of Pair-related homeobox (Prx)-1 expression, a marker in axial skeletons, have retarded bone growth and spontaneous bone fracture in limb skeletons with impaired fracture healing at postpubertal age (Tsuji et al., 2006). Application of BMP2 in either soluble form or by gene delivery induce bone formation in both orthotropic and heterotropic locations (Hou et al., 2007; Hu et al., 2010; Kim et al., 2011).

BMP2 signaling of osteoblast specification is mediated through the activation of BMP type I and type II receptors (BMP-RI, BMP-RII) similar to other BMPs (Nohe et al., 2002). Upon stimulation by BMP2, there are two different signaling pathways depending on the oligomerization patterns of BMP receptors. In the first pathway, BMP2 binds to preformed receptor complex of BMP-RI and BMP-RII. This pathway initiates signal transduction through SMAD1/5/8 phosphorylation by BMP-RI in the

ligand-receptor complex. The phosphorylated SMAD1/5/8 proteins generate complex formation with common partners-SMAD, SMAD4 and translocate into the nucleus to regulate target gene transcription. One SMAD-mediated transcriptional control proceeds through co-activation with RUNX2 transcription factor (Afzal et al., 2005; Lee et al., 2000). The second pathway is signaled by BMP2 involves oligomerization of homomeric BMP-RI complex which subsequently recruits BMP-RII into the complex then activates p38 mitogen activated protein kinase (MAPK) signaling pathway which leads to induction of ALP activity (Nohe et al., 2002).

RUNX2 osteogenic signaling

Runt-related transcription factor 2 (RUNX2) is the earliest marker that contributes to the regulation of osteoblast specification by controlling the expression of osteoblastic related genes such as *COL1A1, BSP*, osteopontin (*OPN*), and *OCN* (Jang et al., 2012). Gene mutation of *RUNX2* indicates its essential role in osteoblast and chondrocyte differentiation. *RUNX2* mutation in human is associated with bone disorder from defect in intramembranous ossification called cleidocranial dysplasia which resembles bone phenotype in *Runx2* haploinsufficient mice (Otto et al., 1997; Zhang et al., 2000). Mice devoid of *Runx2* exhibit cartilaginous skeleton with defect in chondrocytes and lack of osteoblasts (Ducy, 2000; Komori et al., 1997; Nakashima et al., 2002). Overexpression of RUNX2 can provoke osteoblastic phenotype expression as presented by OCN and BSP in non-osteoblastic cells (Ducy et al., 1997).

RUNX2 is a multifunctional transcription factor that interacts with a specific DNA binding site in promoter or enhancer regions of its target genes to induce or suppress transcription activity (Ducy et al., 1997). RUNX2 regulates target genes by

9

involves in chromatin remodeling, reacting at promoter regions of target genes, and independent signaling pathway. The temporal action of RUNX2 involves activation of various osteoblastic genes at specific differentiation stages and is dependent on its association with various co-regulatory proteins and collaborating proteins (Lian and Stein, 2003). An example of RUNX2 and co-regulatory protein complex is RUNX2-SMAD regulation following BMP induction (Afzal et al., 2005). The other co-regulatory proteins that involve in osteoblast regulation include TAZ (Cui et al., 2003), STAT1 (Kim et al., 2003), YAP (Stein et al., 2003), and TWIST (Yousfi et al., 2002).

OSX osteogenic signaling

The zinc finger-containing transcription factor OSX or the present name SP7 appears to act downstream of RUNX2 to commit progenitor cells into osteoblastic differentiation (Nakashima et al., 2002). In situ hybridization of mouse embryonic tissues at different stages demonstrates the localization of *Osx* in cells associated with developing bones. Mice with target deletion of *Osx* develop normal cartilage but no bone formation occurs due to arrested osteoblast differentiation. Runx2 expression in Osx null mice showed comparable levels to wild type mice. Overexpression of Runx2 results in *Osx* induction in C2C12 cells. Runx2 regulates Osx expression through Smad signaling pathway as Osx expression in Runx2 deficient mesenchymal cells is rescued by the presence of Smad1/4. In addition to the Runx2 related mechanism, Osx expression is regulated under Msx homeobox homologue 2 (Msx2)-independent pathway upon Bmp2 stimulation (Matsubara et al., 2008).

Osx contributes to osteoblast differentiation control under overlapping and

non-overlapping mechanisms to Runx2 function. Microarray data from C3H10T1/2 cells reveal different gene profiles upon stimulation by Osx in compared with Runx2. Although both groups share several similar osteoblastic related genes such as *Wnt4, Bglap1, Bmp7*, there are a group of genes induced by Osx but not Runx2 (Matsubara et al., 2008). The function of Osx is directed through its transcriptional activity as found in regulation of type I collagen, bone sialoprotein, osteopontin, osteocalcin, and an osteoinductive factor Nel-like molecule1 (Nell1) (Chen et al., 2011; Harada and Rodan, 2003). The nuclear factor of activated T cells, cytoplasmic 1 (Nfatc1) has been found to promote Osx transcriptional function during osteoblast differentiation (Koga et al., 2005; Winslow et al., 2006). One mechanism in which Osx induces osteoblastic gene expression was defined by the study from osteocalcin transcriptional regulation. Osx binds to *Ocn* promoter region at Sp1/Osx binding site with the enhancement by Specificity protein 1 (Sp1) in increasing Osx affinity (Niger et al., 2011). However there are other pathways involves during osteoblast differentiation. The regulatory mechanism for bone formation remains to be elucidated.

Inflammation and bone homeostasis

Inflammation is an immediate response that occurs during injuries to eliminate noxious stimuli and to initiate healing/repair process. The inflammatory response is tightly associated with immune cells which are provoked to migrate to the lesion site. The detrimental effects of chronic inflammatory responses have been implicated in pathogenesis of several diseases relevant to the imbalance of bone formation and resorption such as periodontitis and rheumatoid arthritis (Makarov, 2001; Nichols et al., 2001). Rarely is inflammation considered positively in bone formation except during

healing process as it represents a critical aspect of wound healing. Impaired osseous wound healing due to reduced osteogenic potential has been illustrated in mice with impaired inflammatory responses from target impairment of genes encoding cyclooxygenase-2, Tnfα, Il6, and Fas-ligand (Mountziaris et al., 2011). Although the regulatory role of inflammatory response in bone homeostasis has been studied perspective of bone resorption through immune cells and osteoclasts (Makarov, 2001; Nichols et al., 2001), little is known about direct relationship between inflammation and bone formation specifically in cells responsible for osteogenesis.

NFκB pathway

Nuclear factor κB (NFκB) is a family of dimeric transcription factors that mediates a wide range of cellular activities regarding its prominent capacity in gene expression control (Perkins, 2007). NFκB has been considered as a key determinant for inflammatory response owing to its profound role in inflammatory-related genes regulation (Li and Verma, 2002). In mammalian cells, NFκB complexes exist in different forms of heterodimers or homodimers of NFκB subunit members which include RelA (p65), RelB, c-Rel, p50, and p52. All NFκB family members share similar Rel homology domain at N-terminus for sequence specific DNA binding, nuclear localization, and dimerization. RelA (p65), RelB, and c-Rel contain non-homologous transcriptional activation domain in their C-terminus to serve regulatory functions in the expression of target genes. The p50 and p52 subunits, without transcriptional activation domain, are synthesized from posttranslational cleavage of precursor p105 and p100 proteins which are encoded from *nfκb1* and *nfκb2* genes respectively. The p105 and p100 contain ankyrin repeated domain in their C-terminus

12

similar to IκBα proteins which allow these precursor proteins to inhibit NFκB activation (Perkins, 2007).

NFκB activation and signaling pathways

In an unstimulated state, NFκB dimers accumulate in the cytoplasm in association with inhibitory proteins which interfere with nuclear localization and DNA binding processes of NFκB (Hayden and Ghosh, 2004). The principle NFκB inhibitors include IκBα, IκBβ, and IκBε. These inhibitory proteins bind to NFκB at conserved nuclear localization sequences in the N-terminus. Alternative NFκB inactivation can be directed through p100 and p105 due to their homologous multiple ankyrin repeats resembling the IκB proteins structure (Perkins, 2007). Each inhibitor protein has preferential binding capacity to different NFκB dimers. For instance, IκBα is a major inhibitor for p65/p50 heterodimer, the most common NFκB complex in mammalian cells while IκBε predominantly binds to p65/p65 and c-Rel/p65 dimers. IκBβ preferentially engages to p65/p50 similar to IκBα (Boyce et al., 2010). NFκB complexes with IκBα and IκBε can shuttle between cytoplasm and nucleus but the nuclear export process is more active which causes predominant NFκB localization in cytoplasm. Additionally, IκBα also plays a role in deactivating NFκB by shuttling it back to cytoplasm (Huang et al., 2000; Lee and Hannink, 2002). Conversely, the inhibitor IκBβ retains NFκB in the cytoplasm due to its inability to promote cytoplasm-nucleus transport (Malek et al., 2001).

The functions of NFκB are usually mediated through direct regulation of target gene expression in response to stimulation from different sources, especially inflammatory cytokines. Upon activated by various stimuli, such as TNFα, processing

13

of inhibitory proteins occurs through enzymatic events and leads to degradation or structural modification of the inhibitory proteins thus enables NFκB to relocate into nucleus where NFκB modulates the target gene expression (Perkins, 2007). Several NFκB activation pathways have been discovered and extensively studied. Major pathways consist of classical canonical and non-canonical pathways which predominantly depend on IκB kinase (IKK) function. Canonical pathway is mediated typically by p65/p50 in associated with IKK. Canonical pathway can be activated by TNFα, bacterial product lipopolysaccharide, IL1, and RANKL as an osteoclast regulator. Non-canonical pathway involves RelB/p52 which originally forms as a RelB/p100 inactive complex (Shih et al., 2011). Induction of noncanonical pathway is influenced by both IKK and NFκB-inducing kinase (NIK) following stimulation by certain stimuli such as RANKL, BAFF, LPS, CD40, and lymphotoxin receptors. Atypical NFκB activation pathway is regulated by different enzymes including tyrosine kinase and casein kinase-2 which act on IκBα phosphorylation following specific stimuli such as UV-light exposure for tyrosine kinase and hypoxia or hydrogen peroxide for casein kinase-2 (Boyce et al., 2010; Perkins, 2007).

There are three core IKK subunits including IKKα, IKKβ, and IKKɣ (NEMO; NFκB essential modulator). IKKα and IKKβ contain kinase domain in their N-terminus. IKKɣ, lacking of kinase domain, contains multiple regulatory subunits which contribute to the initiation of canonical NFκB activation. In canonical pathway, stimulus-responsive activation of trimeric IKK complex of IKKα, IKKβ, and IKKɣ leads to phosphorylation, ubiquitination, and proteolysis induction of IκBα (Boyce et al., 2010). Genetic studies have indicated that IKKβ plays an important role in IκBα

phosphorylation in canonical pathway activation (Gerondakis et al., 1999). In noncanonical pathway, NIK is a key regulator of the activation process via direct phosphorylation and induction of IKKα-mediated phosphorylation of p100. Subsequently, the p52 formation from p100 is achieved through 26S-proteosomal relating process then RelB/p52 dimer translocates into nucleus for further functions (Shih et al., 2011).

Transcriptional control functions of IKK/NFκB system are varied depending on the interaction of NFκB with other co-regulators which determines cell type- and stimulus-specific regulation of the target genes. After NFκB accumulates in the nucleus, NFκB interacts with co-activator or co-repressor which directs the modes of transcription control in either activation or suppression. Additionally, NFκB can bind with heterologous transcription factors which influence promoter targeting and selectivity (Perkins, 2007).

NFκB involvement in physiological processes

NFκB exerts a regulatory role in various physiological processes from inflammation, immunity, proliferation, differentiation to cell survival through intracellular signaling network with transcriptional and post-transcriptional regulation (Perkins, 2007; Sitcheran et al., 2003). Genetic approaches in animal studies have been used to identify the functions of each NFκB subunit and pathway in organ and tissue specific manners. Table 1 summarizes transgenic and mutant mice models for investigating the function of major components in NFκB pathway *in vivo* (Gerondakis et al., 1999; Li and Verma, 2002).

Table1 Phenotype of mutant mice for NFκB related components

Mutated gene	Phenotype
NFκB subunits	
p65	Embryonic lethal at E15 – E16 from liver apoptosis
p65 and c-rel	Embryonic lethal at E13 - E13.5 from liver apoptosis
p65 and nfκb1	Embryonic lethal at E13 - E13.5 from liver apoptosis
relB	Postnatal lethal due to organ inflammation from T-cell infiltration, defects in acquired and innate immunities, deficiency in dendritic cell development
c-rel	Impaired T and B cell responses
nfκb1 (p105 and p50)	Defect in B lymphocyte proliferation and activation
nfκb2 (p100 and p52)	Disruption of spleen and lymph node architecture, impaired T cell response
nfκb1 and nfκb2	Bone growth retardation, craniofacial abnormalities with osteopetrotic appearance, defects in osteoclasts and B cell development
nfκb1 and RelB	Postnatal lethal in 3-4 weeks due to organ inflammation, defect in B cell development
nfκb1 and c-rel	Defects in lymphocyte activation, diminished humoral immunity
IκB proteins	
IκBα	Postnatal lethal in 7-10 days from severe dermatitis and granulocytosis
IκBα knockout and IκBβ knock-in	Rescue phenotype of IκBα knockout mice
IκBε	Decreased CDD44$^-$CD25$^+$ T cells
IKK complexes	
IKKα	Neonatal lethal from morphological and skeletal defects due to impaired keratinocyte differentiation, defect in B cell development
IKKβ	Embryonic lethal at E12.5 – E14.5 from liver apoptosis
IKKα and IKKβ	Embryonic lethal at E11.5 – E12.5 from liver apoptosis
IKKγ	Embryonic lethal at E11.5 – E12.5 from liver apoptosis
Related proteins	
NIK	Disruption of spleen and lymph node architecture, lack of lymph nodes and Peyer's patches

Mice with genetic manipulation revealed unique and overlapping functions of individual Nfκb subunits and other components in NFκB pathway. All Nfκb subunits except p65 have profound effect on immune system. The function of p65 involves protective role in hepatocyte survival (Beg et al., 1995) which also presents in *Ikkβ* and *Ikkγ* null mice according to tight correlation of p65/p50 and Ikk complex in the

canonical pathway. Protective function of p65 is attributed to its potential in activation of anti-apoptotic genes *A1/Bfl-1*, *Xiap*, and *Bcl-xL* (Wang et al., 1996; Wang et al., 1999). Embryonic lethality in *p65* deficient mice impeded the studies of p65 functions in other organs in postnatal stage. Cells/tissues specific gene manipulation emerges growing knowledge of the significance of p65 in specific organs/tissues (Geisler et al., 2007; Steinbrecher et al., 2008; Tang et al., 2010). Therefore decoding the role of p65 in other organs can be accomplished to reveal new aspects of NFκB function.

NFκB involvement in bone biology

The fundamental participation of NFκB in bone biology has been addressed in both physiological and pathological conditions of osteoclasts and osteoblasts (Boyce et al., 2010; Novack, 2011). Aberrant NFκB activation, typically canonical pathway, is implicated in pathogenesis of several bone inflammatory diseases such as rheumatoid arthritis, inflammatory arthritis, osteoarthritis, and periodontitis (Nichols et al., 2001; Novack, 2011). NFκB is constitutively activated at lesion sites where inflammation is elevated. The pathogenic effect of NFκB is described by its potent transcriptional activation and responsive effect on inflammatory mediators from immune cells, osteoclasts, chondrocytes or osteoblasts. A list of involved mediators includes TNFα, IL1, IL6, IL8, cyclooxygenase 2 (COX2), matrix metalloproteinases (MMPs), and inducible nitric oxide synthase (iNOS) (Li and Verma, 2002; Novack, 2011). In addition to inflammatory related diseases, the pathogenesis of degenerative diseases osteoporosis and Paget's disease of the bone also show the correlation with NFκB activation in osteoclasts.

NFκB involvement during bone repair/regeneration is not well studied. A gene

profile study in osseointegration of implants in humans indicates a prominent IκB/NFκB cascade at early healing stage of day 4 postimplantation which correlates NFκB with initial inflammatory phase in bone healing process (Ivanovski et al., 2011). Owing to the profound effect of NFκB in inflammation, studies regarding inflammatory effects on bone fracture healing may infer the role of NFκB in bone repair/regeneration (Mountziaris et al., 2011). Chronic inflammation causes damaging effect on bone regeneration whereas lack of inflammatory responses at initial stage of bone healing impairs regenerative potential of bone tissues which is the outcome of decreased osteoblasts function. It is imperative that NFκB may contribute to early stage of bone formation through osteoblasts regulation. However, the function of NFκB in osteoblasts at early repair/regeneration stages remains unclear.

The role of NFκB in osteoclastogenesis and bone resorption has been defined extensively. Mice with target deletion of *nfκb1* and *nfκb2* display defects in osteoclast development which impair bone resorption activities as shown by osteopetrotic phenotype and failure of tooth eruption (Franzoso et al., 1997). The osteoclast phenotypes in these *nfκb1/nfκb2* knockout mice are similar to the findings in *Rankl* null mice which is a required factor for osteoclast development and activation (Xing et al., 2002). RANKL induces NFκB activation in canonical pathway through TRAF6 and noncanonical pathways through NIK by activating IKKβ and IKKα (Chaisson et al., 2004; Liu et al., 2004). The study in *p65$^{-/-}$Tnfr$^{-/-}$* mice indicates that p65 subunit is involved in osteoclast development via apoptosis prevention but not osteoclast induction (Vaira et al., 2008).

The role of NFκB in bone formation from the perspective of

osteoblastogenesis is less defined than for osteoclasts. Bone formation is a complex process that involves multiple pathways orchestrated in time- and stage-specific manners. In addition to the osteoclast defect, the *nfκb1/nfκb2* knockout mice exhibit dwarfism phenotype with reduced *Bmp2* mRNA level in growth plate of long bones (Feng et al., 2003). Bone growth retardation in this model was explained as consequence of compromised chondrogenesis through direct effect on *Bmp2* induction which is attributed to p65-mediated transactivation. BMP2 can induce both osteoblast and chondrocyte differentiation (Jang et al., 2011; Matsubara et al., 2008). Conflicting results from posttranscriptional function of NFκB illustrate that NFκB inhibits chondrogenesis and myogenesis by silencing *Sox9* and *MyoD*, key determinants of chondrocyte and myoblast development respectively (Sitcheran et al., 2003). A recent study has identified the role of the canonical NFκB pathway in cells in osteoblastic lineage at mature stage by using transgenic mice that express a dominant negative Ikkγ (IKKγ-DN) driven by *Bglap2*, the osteocalcin promotor (Chang et al., 2009). Young mice with this Nfκb inhibition in differentiated osteoblasts showed increased trabecular bone mass as a result of increased osteoblast function. Additional function of NFκB in mature osteoblasts is implicated in osteoporosis pathogenesis as adult *Bglap2*-IKKγ-DN mice are resistance to ovarectomy-induced osteoporotic bone loss. Despite the function of NFκB in differentiated osteoblasts is defined, a mechanism during the early stages of bone formation may be critical to bone development. The importance of NFκB in osteoblast differentiation requires elucidation.

NFκB may be significant in the intrinsic modulation of osteoblastic activity. The underlying mechanisms in which NFκB controls bone formation are partially

defined. The discoveries of pathways that NFκB directly regulates osteoblast as well as the regulatory mechanisms for NFκB function in osteogenesis will ultimately provide us fundamental knowledge in defining physiological process of bone development as well as developing novel bone regeneration approaches. The hypothesis of this dissertation is p65 mediated NFκB affects bone formation via osteoblast regulation.

References (Chapter 1)

Afzal F, Pratap J, Ito K, Ito Y, Stein JL, van Wijnen AJ, et al. (2005). Smad function and intranuclear targeting share a Runx2 motif required for osteogenic lineage induction and BMP2 responsive transcription. *J Cell Physiol* 204(1)*:*63-72.

Baek WY, de Crombrugghe B, Kim JE (2010). Postnatally induced inactivation of Osterix in osteoblasts results in the reduction of bone formation and maintenance. *Bone* 46(4)*:*920-8.

Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D (1995). Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature* 376(6536)*:*167-70.

Behonick DJ, Xing Z, Lieu S, Buckley JM, Lotz JC, Marcucio RS, et al. (2007). Role of matrix metalloproteinase 13 in both endochondral and intramembranous ossification during skeletal regeneration. *PLoS One* 2(11)*:*e1150.

Boyce BF, Yao Z, Xing L (2010). Functions of nuclear factor kappaB in bone. *Ann N Y Acad Sci* 1192(367-75.

Boyne PJ, Lilly LC, Marx RE, Moy PK, Nevins M, Spagnoli DB, et al. (2005). De novo bone induction by recombinant human bone morphogenetic protein-2 (rhBMP-2) in maxillary sinus floor augmentation. *J Oral Maxillofac Surg* 63(12)*:*1693-707.

Bruder SP, Fox BS (1999). Tissue engineering of bone. Cell based strategies. *Clin Orthop Relat Res* 367 Suppl)*:*S68-83.

Cao X, Chen D (2005). The BMP signaling and in vivo bone formation. *Gene* 357(1)*:*1-8.

Chaisson ML, Branstetter DG, Derry JM, Armstrong AP, Tometsko ME, Takeda K, et al. (2004). Osteoclast differentiation is impaired in the absence of inhibitor of kappa B kinase alpha. *J Biol Chem* 279(52)*:*54841-8.

Chang J, Wang Z, Tang E, Fan Z, McCauley L, Franceschi R, et al. (2009). Inhibition of osteoblastic bone formation by nuclear factor-kappaB. *Nat Med* 15(6)*:*682-9.

Chen F, Zhang X, Sun S, Zara JN, Zou X, Chiu R, et al. (2011). NELL-1, an osteoinductive factor, is a direct transcriptional target of Osterix. *PLoS One* 6(9)*:*e24638.

Cohen MM, Jr. (2006). The new bone biology: pathologic, molecular, and clinical correlates. *Am J Med Genet A* 140(23)*:*2646-706.

Cooper LF, Harris CT, Bruder SP, Kowalski R, Kadiyala S (2001). Incipient analysis of mesenchymal stem-cell-derived osteogenesis. *J Dent Res* 80(1)*:*314-20.

Cornell CN, Lane JM, Poynton AR (2003). Orthopedic management of vertebral and long bone fractures in patients with osteoporosis. *Clin Geriatr Med* 19(2)*:*433-55.

Crisostomo PR, Wang Y, Markel TA, Wang M, Lahm T, Meldrum DR (2008). Human mesenchymal stem cells stimulated by TNF-alpha, LPS, or hypoxia produce growth factors by an NF kappa B- but not JNK-dependent mechanism. *Am J Physiol Cell Physiol* 294(3)*:*C675-82.

Cui CB, Cooper LF, Yang X, Karsenty G, Aukhil I (2003). Transcriptional coactivation of bone-specific transcription factor Cbfa1 by TAZ. *Mol Cell Biol* 23(3)*:*1004-13.

Datta HK, Ng WF, Walker JA, Tuck SP, Varanasi SS (2008). The cell biology of bone metabolism. *J Clin Pathol* 61(5)*:*577-87.

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G (1997). Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* 89(5)*:*747-54.

Ducy P, Starbuck M, Priemel M, Shen J, Pinero G, Geoffroy V, et al. (1999). A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. *Genes Dev* 13(8)*:*1025-36.

Ducy P (2000). Cbfa1: a molecular switch in osteoblast biology. *Dev Dyn* 219(4)*:*461-71.

Feng JQ, Xing L, Zhang JH, Zhao M, Horn D, Chan J, et al. (2003). NF-kappaB specifically activates BMP-2 gene expression in growth plate chondrocytes in vivo and in a chondrocyte cell line in vitro. *J Biol Chem* 278(31)*:*29130-5.

Franzoso G, Carlson L, Xing L, Poljak L, Shores EW, Brown KD, et al. (1997). Requirement for NF-kappaB in osteoclast and B-cell development. *Genes Dev* 11(24)*:*3482-96.

Geisler F, Algul H, Paxian S, Schmid RM (2007). Genetic inactivation of RelA/p65 sensitizes adult mouse hepatocytes to TNF-induced apoptosis in vivo and in vitro. *Gastroenterology* 132(7)*:*2489-503.

Gerondakis S, Grossmann M, Nakamura Y, Pohl T, Grumont R (1999). Genetic approaches in mice to understand Rel/NF-kappaB and IkappaB function: transgenics and knockouts. *Oncogene* 18(49)*:*6888-95.

Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA (2003). Fracture healing as a post-natal developmental process: molecular, spatial, and temporal

aspects of its regulation. *J Cell Biochem* 88(5)*:*873-84.

Grimes R, Jepsen KJ, Fitch JL, Einhorn TA, Gerstenfeld LC (2011). The transcriptome of fracture healing defines mechanisms of coordination of skeletal and vascular development during endochondral bone formation. *J Bone Miner Res* 26(11)*:*2597-609.

Harada S, Rodan GA (2003). Control of osteoblast function and regulation of bone mass. *Nature* 423(6937)*:*349-55.

Hayden MS, Ghosh S (2004). Signaling to NF-kappaB. *Genes Dev* 18(18)*:*2195-224.

Hou LT, Liu CM, Liu BY, Chang PC, Chen MH, Ho MH, et al. (2007). Tissue engineering bone formation in novel recombinant human bone morphogenic protein 2-atelocollagen composite scaffolds. *J Periodontol* 78(2)*:*335-43.

Hu WW, Ward BB, Wang Z, Krebsbach (2010). PH Bone regeneration in defects compromised by radiotherapy. *J Dent Res* 89(1)*:*77-81.

Huang TT, Kudo N, Yoshida M, Miyamoto S (2000). A nuclear export signal in the N-terminal regulatory domain of IkappaBalpha controls cytoplasmic localization of inactive NF-kappaB/IkappaBalpha complexes. *Proc Natl Acad Sci U S A* 97(3)*:*1014-9.

Itagaki T, Honma T, Takahashi I, Echigo S, Sasano Y (2008). Quantitative analysis and localization of mRNA transcripts of type I collagen, osteocalcin, MMP 2, MMP 8, and MMP 13 during bone healing in a rat calvarial experimental defect model. *Anat Rec (Hoboken)* 291(8)*:*1038-46.

Ivanovski S, Hamlet S, Salvi GE, Huynh-Ba G, Bosshardt DD, Lang NP, et al. (2011). Transcriptional profiling of osseointegration in humans. *Clin Oral Implants Res* 22(4)*:*373-81.

Jang WG, Kim EJ, Kim DK, Ryoo HM, Lee KB, Kim SH, et al. (2012). BMP2 regulates osteocalcin expression via Runx2 mediated ATF6 gene transcription. *J Biol Chem* 287(2)*:*905-15.

Javed A, Barnes GL, Jasanya BO, Stein JL, Gerstenfeld L, Lian JB, et al. (2001). runt homology domain transcription factors (Runx, Cbfa, and AML) mediate repression of the bone sialoprotein promoter: evidence for promoter context-dependent activity of Cbfa proteins. *Mol Cell Biol* 21(8)*:*2891-905.

Jiang JX, Siller-Jackson AJ, Burra S (2007). Roles of gap junctions and hemichannels in bone cell functions and in signal transmission of mechanical stress. *Front Biosci* 12*:*1450-62.

Katagiri T, Takahashi N (2002). Regulatory mechanisms of osteoblast and osteoclast differentiation. *Oral Dis* 8(3)*:*147-59.

Katayama Y, Matsuyama Y, Yoshihara H, Sakai Y, Nakamura H, Imagama S, et al. (2009). Clinical and radiographic outcomes of posterolateral lumbar spine fusion in humans using recombinant human bone morphogenetic protein-2: an average five-year follow-up study. *Int Orthop* 33(4)*:*1061-7.

Kilian O, Wenisch S, Karnati S, Baumgart-Vogt E, Hild A, Fuhrmann R, et al. (2008). Observations on the microvasculature of bone defects filled with biodegradable nanoparticulate hydroxyapatite. *Biomaterials* 29(24-25)*:*3429-37.

Kim IS, Lee EN, Cho TH, Song YM, Hwang SJ, Oh JH, et al. (2011). Promising efficacy of Escherichia coli recombinant human bone morphogenetic protein-2 in collagen sponge for ectopic and orthotopic bone formation and comparison with mammalian cell recombinant human bone morphogenetic protein-2. *Tissue Eng Part A* 17(3-4)*:*337-48.

Kim S, Koga T, Isobe M, Kern BE, Yokochi T, Chin YE, et al. (2003). Stat1 functions as a cytoplasmic attenuator of Runx2 in the transcriptional program of osteoblast differentiation. *Genes Dev* 17(16)*:*1979-91.

Koga T, Matsui Y, Asagiri M, Kodama T, de Crombrugghe B, Nakashima K, et al. (2005). NFAT and Osterix cooperatively regulate bone formation. *Nat Med* 11(8)*:*880-5.

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, et al. (1997). Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* 89(5)*:*755-64.

LaPorte DM, Murphy MS, Moore JR (2008). Distal humerus nonunion after failed internal fixation: reconstruction with total elbow arthroplasty. *Am J Orthop (Belle Mead NJ)* 37(10)*:*531-4.

Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, Komori T, et al. (2000). Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. *Mol Cell Biol* 20(23)*:*8783-92.

Lee SH, Hannink M (2002). Characterization of the nuclear import and export functions of Ikappa B(epsilon). *J Biol Chem* 277(26)*:*23358-66.

Li Q, Verma IM (2002). NF-kappaB regulation in the immune system. *Nat Rev Immunol* 2(10)*:*725-34.

Lian JB, Stein GS (2003). Runx2/Cbfa1: a multifunctional regulator of bone formation. *Curr Pharm Des* 9(32):2677-85.

Liu W, Xu D, Yang H, Xu H, Shi Z, Cao X, et al. (2004). Functional identification of three receptor activator of NF-kappa B cytoplasmic motifs mediating osteoclast differentiation and function. *J Biol Chem* 279(52):54759-69.

Long F (2011). Building strong bones: molecular regulation of the osteoblast lineage. *Nat Rev Mol Cell Biol* 13(1):27-38.

Lu C, Huang S, Miclau T, Helms JA, Colnot C (2004). Mepe is expressed during skeletal development and regeneration. *Histochem Cell Biol* 121(6):493-9.

Makarov SS (2001). NF-kappa B in rheumatoid arthritis: a pivotal regulator of inflammation, hyperplasia, and tissue destruction. *Arthritis Res* 3(4):200-6.

Malek S, Chen Y, Huxford T, Ghosh G (2001). IkappaBbeta, but not IkappaBalpha, functions as a classical cytoplasmic inhibitor of NF-kappaB dimers by masking both NF-kappaB nuclear localization sequences in resting cells. *J Biol Chem* 276(48):45225-35.

Matsubara T, Kida K, Yamaguchi A, Hata K, Ichida F, Meguro H, et al. (2008). BMP2 regulates Osterix through Msx2 and Runx2 during osteoblast differentiation. *J Biol Chem* 283(43):29119-25.

Mountziaris PM, Spicer PP, Kasper FK, Mikos AG (2011). Harnessing and modulating inflammation in strategies for bone regeneration. *Tissue Eng Part B Rev* 17(6):393-402.

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, et al. (2002). The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* 108(1):17-29.

Neve A, Corrado A, Cantatore FP (2011). Osteoblast physiology in normal and pathological conditions. *Cell Tissue Res* 343(2):289-302.

NIAMS NIoAaMasd (2011). Osteoporosis and related bone diseases.

Nichols TC, Fischer TH, Deliargyris EN, Baldwin AS, Jr. (2001). Role of nuclear factor-kappa B (NF-kappa B) in inflammation, periodontitis, and atherogenesis. *Ann Periodontol* 6(1):20-9.

Niger C, Lima F, Yoo DJ, Gupta RR, Buo AM, Hebert C, et al. (2011). The transcriptional activity of osterix requires the recruitment of Sp1 to the osteocalcin proximal promoter. *Bone* 49(4):683-92.

Nohe A, Hassel S, Ehrlich M, Neubauer F, Sebald W, Henis YI, et al. (2002). The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. *J Biol Chem* 277(7)*:*5330-8.

Novack DV (2011). Role of NF-kappaB in the skeleton. *Cell Res* 21(1)*:*169-82.

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, et al. (1997). Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* 89(5)*:*765-71.

Perkins ND (2007). Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat Rev Mol Cell Biol* 8(1)*:*49-62.

Perrini S, Natalicchio A, Laviola L, Cignarelli A, Melchiorre M, De Stefano F, et al. (2008). Abnormalities of insulin-like growth factor-I signaling and impaired cell proliferation in osteoblasts from subjects with osteoporosis. *Endocrinology* 149(3)*:*1302-13.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* 284(5411)*:*143-7.

Prockop DJ, Gregory CA, Spees JL (2003). One strategy for cell and gene therapy: harnessing the power of adult stem cells to repair tissues. *Proc Natl Acad Sci U S A* 100 Suppl 1(11917-23.

Salgado AJ, Coutinho OP, Reis RL (2004). Bone tissue engineering: state of the art and future trends. *Macromol Biosci* 4(8)*:*743-65.

Scheller EL, Krebsbach PH, Kohn DH (2009). Tissue engineering: state of the art in oral rehabilitation. *J Oral Rehabil* 36(5)*:*368-89.

Schindeler A, McDonald MM, Bokko P, Little DG (2008). Bone remodeling during fracture repair: The cellular picture. *Semin Cell Dev Biol* 19(5)*:*459-66.

Shih VF, Tsui R, Caldwell A, Hoffmann A (2011). A single NFkappaB system for both canonical and non-canonical signaling. *Cell Res* 21(1)*:*86-102.

Shui C, Spelsberg TC, Riggs BL, Khosla S (2003). Changes in Runx2/Cbfa1 expression and activity during osteoblastic differentiation of human bone marrow stromal cells. *J Bone Miner Res* 18(2)*:*213-21.

Sitcheran R, Cogswell PC, Baldwin AS, Jr. (2003). NF-kappaB mediates inhibition of mesenchymal cell differentiation through a posttranscriptional gene silencing mechanism. *Genes Dev* 17(19)*:*2368-73.

Song L, Webb NE, Song Y, Tuan RS (2006). Identification and functional analysis of candidate genes regulating mesenchymal stem cell self-renewal and multipotency. *Stem Cells* 24(7)*:*1707-18.

Stein GS, Lian JB, Stein JL, van Wijnen AJ, Choi JY, Pratap J, et al. (2003). Temporal and spatial parameters of skeletal gene expression: targeting RUNX factors and their coregulatory proteins to subnuclear domains. *Connect Tissue Res* 44 Suppl 1(149-53.

Steinbrecher KA, Harmel-Laws E, Sitcheran R, Baldwin AS (2008). Loss of epithelial RelA results in deregulated intestinal proliferative/apoptotic homeostasis and susceptibility to inflammation. *J Immunol* 180(4)*:*2588-99.

Tang T, Zhang J, Yin J, Staszkiewicz J, Gawronska-Kozak B, Jung DY, et al. (2010) Uncoupling of inflammation and insulin resistance by NF-kappaB in transgenic mice through elevated energy expenditure. *J Biol Chem* 285(7)*:*4637-44.

Thompson Z, Miclau T, Hu D, Helms JA (2002). A model for intramembranous ossification during fracture healing. *J Orthop Res* 20(5)*:*1091-8.

Tsuji K, Bandyopadhyay A, Harfe BD, Cox K, Kakar S, Gerstenfeld L, et al. (2006). BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. *Nat Genet* 38(12)*:*1424-9.

Vaira S, Alhawagri M, Anwisye I, Kitaura H, Faccio R, Novack DV (2008). RelA/p65 promotes osteoclast differentiation by blocking a RANKL-induced apoptotic JNK pathway in mice. *J Clin Invest* 118(6)*:*2088-97.

Wang CY, Mayo MW, Baldwin AS, Jr. (1996). TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. *Science* 274(5288)*:*784-7.

Wang CY, Guttridge DC, Mayo MW, Baldwin AS, Jr. (1999). NF-kappaB induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. *Mol Cell Biol* 19(9)*:*5923-9.

Ward BB, Brown SE, Krebsbach PH (2010). Bioengineering strategies for regeneration of craniofacial bone: a review of emerging technologies. *Oral Dis* 16(8)*:*709-16.

Winslow MM, Pan M, Starbuck M, Gallo EM, Deng L, Karsenty G, et al. (2006). Calcineurin/NFAT signaling in osteoblasts regulates bone mass. *Dev Cell* 10(6)*:*771-82.

Xing L, Bushnell TP, Carlson L, Tai Z, Tondravi M, Siebenlist U, et al. (2002). NF-kappaB p50 and p52 expression is not required for RANK-expressing osteoclast progenitor formation but is essential for RANK- and cytokine-mediated osteoclastogenesis. *J Bone Miner Res* 17(7)*:*1200-10.

Yousfi M, Lasmoles F, Marie PJ (2002). TWIST inactivation reduces CBFA1/RUNX2 expression and DNA binding to the osteocalcin promoter in osteoblasts. *Biochem Biophys Res Commun* 297(3):641-4.

Zaidi M (2007). Skeletal remodeling in health and disease. *Nat Med* 13(7):791-801.

Zhang H, Bradley A (1996). Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. *Development* 122(10):2977-86.

Zhang YW, Yasui N, Ito K, Huang G, Fujii M, Hanai J, et al. (2000). A RUNX2/PEBP2alpha A/CBFA1 mutation displaying impaired transactivation and Smad interaction in cleidocranial dysplasia. *Proc Natl Acad Sci U S A* 97(19):10549-54.

CHAPTER 2

HAPLOINSUFFICIENCY OF NFκB P65 MODULATES BONE

DEVELOPMENT THROUGH OSTEOBLAST REGULATION

Introduction

Osteogenesis is a process of bone formation that is initiated by osteoblast function and regulated by coupled activities of osteoblasts and osteoclasts. The mechanisms for osteogenesis during bone development are separated into two distinct processes. In limb skeletons, osteogenesis takes place in endochondral ossification manner which begins with cartilage template before bone replacement by osteoblasts. Another mechanism is intramembranous ossification which forms bone directly from mesenchymal cell differentiation into osteoblasts as found in parts of craniofacial bones and clavicles. Both mechanisms terminally engage in calcified extracellular matrix synthesis by osteoblasts (Katagiri and Takahashi, 2002; Rodda and McMahon, 2006).

Successful bone formation requires sufficient number of functional osteoblasts to establish extracellular structural components of bone tissues. During osteogenesis, mesenchymal stem cells differentiate into osteoprogenitor cells before developing into proliferating pre-osteoblasts which turn into mature osteoblasts that perform bone

production (Baek et al., 2010; Nakashima et al., 2002). Coordination of a multitude of different extrinsic factors from paracrine, autocrine, and endocrine origins influences intrinsic regulation of osteoblast development and maturation (Aubin, 2001). Only a few molecules have been exemplified their mechanistic potential to promote bone formation through direct effect on osteoblasts such as PTH, BMPs, Wnt (Cao and Chen, 2005; Guo and Cooper, 2007; Weber et al., 2006). The underlying mechanisms which regulate osteoblast development and function remain partially defined.

Inflammation is a fundamental process that impacts bone homeostasis in physiological and pathological conditions of bone. Various cellular activities corresponding to inflammatory reaction are directed by a transcription factor NFκB (Perkins, 2007). Although the effect of NFκB on osteoclastogenesis is well defined, little is known about direct roles of NFκB in osteoblast differentiation. Previous studies have suggested the roles of NFκB in skeletal growth and development through osteogenesis. Suppression of p65 activation by pharmacological NFκB inhibitor, PDTC and BAY, results in reduced *in vitro* growth of metatarsal growth plate (Wu et al., 2007). The negative influence of p65 NFκB subunit in lineage selection during mesenchymal differentiation towards other lineages suggests a favorable effect on lineage specification of osteogenic pathway. The p65 dependent NFκB activity suppresses myogenesis and chondrogenesis through post-transcriptional gene silencing of *MyoD* and *Sox9* genes (Sitcheran et al., 2003). NFκB and p65 subunit alone have been shown to inhibit adipogenesis by blocking DNA binding potential of the key adipogenic transcription factor PPARɣ (Suzawa et al., 2003). Moreover, the influence of p65 on *Bmp2* expression, a potent osteogenic growth factor, has been demonstrated

at transcriptional regulation level with direct binding of p65 on the *Bmp2* promoter region (Feng et al., 2003). Mice lacking *p50* and *p52*, the major p65 dimeric pairs, exhibit bone growth retardation with reduced amount of *Bmp2* in growth plate of long bones (Feng et al., 2003; Franzoso et al., 1997). Collective evidence from these findings suggest that p65 mediated NFκB activity may be critical for osteogenesis during bone development. However, embryonic lethality of *p65* null mice due to liver apoptosis and the superimposed function of NFκB in osteoclasts precluded the work on defining *in vivo* function of p65 mediated NFκB in osteogenic cells (Beg et al., 1995; Vaira et al., 2008).

To explore the specific role of p65 mediated NFκB on osteogenesis during bone development, we exploited conditional gene targeting approach to abrogate p65 function in osteogenic cells *in vivo* using Cre/loxP recombination strategy. Mice lacking one *p65* allele in osteogenic cells displayed smaller skeleton. Decreased mineralization from impaired osteoblast differentiation and elevated apoptosis affected the qualitative and quantitative properties of bones in these mice. These findings provide *in vivo* evidence that p65 mediated NFκB is required for maintaining osteogenic cells in normal physiological condition of bone homeostasis.

Materials & methods

Animals

Generation of mice with p65 haploinsufficiency

Mice with targeted deletion of *p65* gene in osteoprogenitors were generated using a Cre-LoxP system. Mice in which exons 5-8 of *p65* (exons encoding most of

31

Rel homology domain) were flanked with loxP sites (p65$^{fl/fl}$) were obtained from Dr. Albert S Baldwin, University of North Carolina at Chapel Hill (Steinbrecher et al., 2008). Transgenic mice that expressed GFP linked Cre-recombinase under the control of the *Osx* promoter (Osx-Cre) (Rodda and McMahon, 2006) were purchased from the Jackson Laboratory. The p65$^{fl/fl}$ mice were crossed with Osx-Cre mice to create mice in which one allele of *p65* gene was deleted in osteoprogenitor cells (p65$^{fl/+}$Osx-Cre). Animals were housed under specific pathogen-free conditions with filtered air and fed with autoclaved diet and water. All experiments were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committees of the University of North Carolina. The genotypes of studied mice were identified by PCR amplification of genomic DNA isolated from tail biopsies or ear clip with proteinase K digestion protocol. The *Cre* transgene was analyzed using two primer sets according to the Jackson Laboratory protocol for Cre PCR reaction: CreF (GCG GTC TGG CAG TAA AAA CTA TC), CreR (GTG AAA CAG CAT TGC TGT CAC TT), internal control F (CTA GGC CAC AGA ATT GAA AGA TCT), internal control R (GTA GGT GGA AAT TCT AGC ATC ATC C). The wild-type *p65* and *p65loxP* allele were identified using the following primer pair: p65loxP F (CGA CTT TGG GTT GGA GGG TTA CAG AAG GC) and p65loxP R (TGG TCT GGA TTC GCT GGC TAA TGG C). The wild-type and *p65loxP* alleles were represented by 450-bp and 510-bp PCR products respectively. The presence of both bands represents a heterozygous genotype for *p65loxP* allele. The recombination of *p65* allele which verified target deletion of the *p65loxP* allele was detected by a PCR approach using primers: recombination F (TCT TCT CAA CTC CAG GGG AAT AGG), recombination R

(CAC CAT TGT TTT CTC ACT TGG CAC). The 1113-bp product band represented successful *p65loxP* recombination (Steinbrecher et al., 2008).

Whole mount skeletal staining

Skeletal structures of newborn mice at day1 and day2 were determined by alizarin red S and alcian blue staining as described previously (McLeod, 1980). Briefly, animals were deskinned, eviscerated, and fixed in ethanol for at least 5 days and then in acetone for 2 days. The skeleton was stained with alizarin red S and alcian blue solution for 3 days at 37°C before being cleared in 1% KOH and taken through gradient steps to 100% glycerol.

Microcomputed tomography (µCT)

Images of mineralized skeletal structures from newborn (P2), young (P8), and adult mice (11 weeks) were acquired using a desktop microtomographic imaging system (Skyscan 1074HR, Aartselaar, Belgium) at a resolution of 20.5 µm/pixel. Standardized scanning and image reconstruction settings were used. Humeri of P2 animals were assessed for skeletal dimension and morphology with the following parameters: length, diameter, circumference, and cortical thickness at mid-diaphysis region. Trabecular regions of proximal tibiae and cortical regions of mid-diaphysis of tibiae from P8 mice were used for the analysis of bone length, bone mineral densities, and bone morphology. For trabecular bone, the measured parameters were bone mineral density (BMD), bone volume fraction (BV/TV), bone specific surface (BS/BV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and trabecular number (Tb.N). For cortical bone, the determined parameters included cortical BMD, total cross sectional bone area (B.Ar), mean total cross sectional bone perimeter

(B.Pm), and mean polar moment of inertia (MMI). Craniofacial skeletons of week 11 gender-matched mice were assessed and analyzed for premaxillary deviation. Hydroxyapatite phantoms (250 mg/cc and 750 mg/cc; CIRS, Inc., Norfold, VA) were use as references for BMD calculation.

Cellular and histological characterization

Histological evaluation

The specimens were fixed in 4% phosphate-buffered paraformaldehyde, decalcified with buffered 0.2 mM EDTA (pH 7.4), embedded in paraffin, and serial sectioned at 5-μm thickness. Sections were stained with hematoxylin-eosin for morphological examination or stained for other analyses. (Nikon Eclipse50i, Nikon, Melville, NY)

Primary cells isolation and cell culture

Osteoprogenitor cells were isolated from calvariae of 8-day old mice by serial digestion in Dulbecco's modified Eagle's medium (DMEM, Gibco Invitrogen) containing 0.25% trypsin a, 0.1% EDTA, and 0.8 mg/ml type II collagenase (Sigma, St. Louis, MO). Calvariae were digested for 30 min at 37°C with constant agitation. The digestion solution was collected, washed with fresh medium, and digested five additional times. Cell suspensions from the digestion were centrifuged for 5 min at 300x g then resuspended and cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin/streptomycin (regular medium) for 24 hours. Medium was changed every three days. To induce osteoblastic differentiation, cells were cultured in regular medium supplemented with 50 μg/ml L-ascorbic acid, 10 mM β-glycerophosphate, and 10^{-7}M dexamethasone (osteogenic supplement).

Bone marrow cells were flushed from femurs and tibiae of 9-week old mice with α-MEM. Erythrocytes were lysed with 0.8% ammonium chloride. Bone marrow cells were cultured in triplicate in MesenCult™ MSC media with mesenchymal stem cell stimulatory supplements (Stem Cell Technologies, Vancouver, BC, Canada) with 100 U/ml of penicillin and 100 μg/ml of streptomycin in 6-well plates. Cells were allowed to adhere to the plastic support for 24 hours before the first medium change into the conditions with or without osteogenic supplement. The murine mesenchymal precursor cell line C2C12 was obtained from ATCC (Manassas, VA) and maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin/streptomycin.

Immunoblotting analysis

Total calvarial cell lysates (10 μg protein/lane) and protein extracted from flushed tibia/femoral bones of adult mice (20 μg protein/lane) were fractionated in 10% SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with primary antibodies, anti-beta actin, and horseradish peroxidase conjugated goat anti-rabbit antibody (Amersham Biosciences, Pittsburgh, PA). The primary antibodies were rabbit polyclonal antibodies against p65, RelB, c-Rel, p100/p52 (Cell signaling Technology number 4764, 4954, 4174, 4882) and p105/p50 (Santa Cruz Biotechnology number SC7178). The immunocomplexes were detected by chemiluminescence using ECLPlus (Amersham Biosciences). The densities of the bands representing target proteins and beta-actin proteins were quantified using UN-SCANT-IT gel software (Silk Scientific, Orem, UT).

Quantitative real-time PCR

Isolated calvarial cells were seeded in 48-well plates at a density of 1.2 x 10^4

cells/well. To determine the RNA expression of osteogenic differentiation markers, cell layers were maintained in regular media or osteogenic media for 7 days. Total RNA was extracted from calvarial cells by TriZol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized using SuperScriptIII first-strand synthesis supermix for qRT-PCR kit (Invitrogen). Relative quantification of mRNA abundance was performed by real time RT-PCR procedure using ABI prism 7500 system. Selective markers included *Runx2, Osx, Alp, Bsp,* and *Bmp2* (ABI assay No. Mm00501578_ml, Mm00504574_ml, Mm00475831_ml, Mm00492555_ml, Mm01340178_ml respectively). All reactions were amplified using Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Assays were performed in duplicate and the results were normalized to rodent GAPDH expression (ABI assay No. 4308313). The fold changes were calculated using the values obtained from the $p65^{fl/+}$ in regular media group as a calibrator then determined by means of $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Alkaline phosphatase activity

Alkaline phosphatase activity was measured in total lysates from calvarial cell layers after incubating in regular medium or medium with osteogenic supplement for 2 days. Cells were washed with PBS and harvested with 0.1% triton-X100 in PBS before evaluation for Alp activity using p-nitrophenyl phosphate substrate (Sigma, St.Louis, MO, USA; Asahina et al., 1996). Protein concentration was assessed by BCA protein assay (Pierce). The enzymatic activity was calculated as µM/minute/protein concentration from standard p-nitrophenol curve then normalized with protein concentration.

CFU-F/CFU-OB assays and TRAP staining

After culturing in differential/growth medium for 14 days, cell layers explanted from mouse bone marrow were washed with PBS and then fixed in 100% cold methanol for 10 minutes. The cultured fibroblastic colonies were stained with 2.5% crystal violet staining in 70% methanol for 5 minutes at room temperature then rinsed with water. Formation of CFU-OB was detected by alkaline phosphatase activity staining (Sigma, St. Louis, MO, USA) after incubation at 37^0C for 30 minutes, and washing with PBS for 5 times. The colonies (>50 cells/colony) were counted under inverted microscope. GFP representing Cre recombinase enzyme expression was monitored in the cells cultured for CFU-OB assay using a fluorescent microscope (Olympus-IX51, Olympus, Central Valley, PA).

Osteoclastogenic potential was confirmed with *in vitro* TRAP staining as described previously (Yan et al., 2007). Briefly, bone marrow cells (1 x 10^5 cells/well) were seeded onto 48-well plates. The cells were maintained in αMEM containing 10% FBS, 100 U/ml penicillin/streptomycin supplemented with 20 ng/ml recombinant mM-CSF (R&D Minneapolis, MN) and 60 ng/ml recombinant human soluble RANKL (PeproTech Inc, Rocky Hill, NJ) for 6 days. The supplemented medium was changed every 3 days. After fixation in 10% formalin, cells were stained with 0.1 mg/ml of naphthol AS-MS phosphate and 0.6 mg/ml fast red violet LB salt in 0.1 M sodium acetate buffer containing 50 mM sodium tartrate. The numbers of TRAP positive cells with three or more nuclei were counted at 100X magnification. The number of osteoclasts *in vivo* was quantified by TRAP positive staining of the cells in tibia/femoral bone sections of 8-week old mice. The pictures were taken using a light

microscope (Nikon Eclipse50i) with a digital camera (Nikon digital camera DXM1200F, Nikon, Melville, NY). The numbers of TRAP positive cells were counted using the ImageJ software (Yan et al., 2007).

Cell proliferation and apoptosis analyses

For cell proliferation, calvarial cells at density 1×10^4 cells per well were plated in triplicate onto a 96-well plate and cultured in regular medium for up to 7 days. Cell proliferation rate was assessed by MTS assay (Promega, Madison, WI) at day 1, 3, 5, and 7, according to the manufacturer's protocol. The absorbance values representing the amounts of formazan compound produced by metabolically active cells were measured using a Model 550 microplate reader (Biorad) at 490 nm wavelength.

To evaluate the roles of NFκB in osteogenesis regarding osteoblast survival protection, calvarial cells derived from studied mice (1×10^4 cells/well of 96-well plate) were assessed using cell viability and caspase3/7 fluorimetric assays (Promega) in the presence and absence of 1 nM staurosporine. The absorbance reflecting viable cell numbers and caspase3/7 activity was determined with a fluorescent microplate reader (Spectramax M2, Molecular Devices, CA). *In situ* apoptosis of bone cells was detected by TUNEL staining (Trevigen, Gaithersburg, MD) on sections from tibiae of day8 studied mice. At least two sections from each bone were performed for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (Tdt) nick end labeling according to the manufacturer's protocol. A positive control was performed by incubating the tissue section with nuclease solution prior to the labeling procedure. A negative control without Tdt was also included in the experiment. TUNEL positive

cell numbers were determined by direct cell counting in subchondral area.

Luciferase reporter assay and plasmids/virus

The Ad.NFκBLuc adenovirus was kindly provided by Dr. John F Engelhardt (University of Iowa). The p65-CMV and IκB-SR-CMV expression vectors were obtained from Dr. Albert S Baldwin (University of North Carolina at Chapel Hill). The mBmp2-luc plasmids were kind gifts from Dr. Di Chen (University of Missouri).

For NFκB transactivational activity assessment, calvarial cells derived from the studied mice were transduced with Ad.NFκBLuc adenovirus (MOI=20) for 2 hours in serum free medium then 20% FBS supplemented medium was added and maintained to total of 24-hour transduction period (Sanlioglu et al., 2001). The medium was replaced with regular growth media containing 100 ng/ml of TNFα or PBS control for 4 hours prior to luminescence evaluation. Luciferase signal quantification was performed using luciferase assay (Promega) in a Lumat LB 9507 (Berthold Technologies, BadWildbad, Germany). Luciferase activity was normalized to the protein concentration and reported as fold induction.

For evaluation of NFκB capacity on *Bmp2* induction, C2C12 cells were seeded onto 48-well plates one day before transfection. A variety of mouse Bmp2-luc constructs were co-transfected with p65-CMV vector in the presence or absence of IκB-SR-CMV vector. The plasmid cDNA3.1 (Invitrogen) was used as an empty vector to equalize the amount of plasmid transferred into each reaction. All transfections included a constitutively expressed Renilla luciferase plasmid (pRL-TK), using SureFECT reagent (SABiosciences, Frederick, MD). Twenty four hours after transfection, cells were harvested and luciferase activities were measured using Dual

Luciferase assay (Promega). The luciferase signal was normalized to Renilla luciferase activity.

Statistical analysis

All experiments were performed in duplicate or triplicate. The data were calculated and presented as mean $\pm$ S.D. Statistical analyses were performed using Student's t-test or analysis of variance or non-parametric test. Significant difference was considered when $p<0.05$.

Results

Generation of mice with p65 haploinsufficiency in osteogenic cells and characterization of the gross phenotype

Defining the physiological role of p65 in osteoblasts in animal models has been difficult due to the embryonic lethality in mice lacking p65 subunit and its redundant effect on osteoclast differentiation and function. To clarify the function of p65 NFκB in osteogenesis, mice with *p65* haploinsufficiency in osteoprogenitors (p65$^{fl/+}$Osx-Cre) were examined. Transgenic mice expressing Cre recombinase under the control of *Osx* promoter (Osx-Cre) were bred with loxP-flanked *p65* mice (p65 $^{fl/fl}$) to generate mice with *p65* haploinsufficiency in osteoprogenitor cells (p65$^{fl/+}$Osx-Cre). The p65$^{fl/+}$ littermates were used as controls since they are indistinguishable from wild type C57BL/J mice (Steinbrecher et al., 2008). The specificity of Osx-Cre function, determined by *p65* recombination gene product, was present in bone but not in heart, kidney, spleen, and liver (Fig.1C). Targeted disruption of one *p65* allele was verified by diminished p65 protein levels. A 20-40% reduction in whole cell extracted p65 levels was seen in isolated calvarial cells from 8-day-old mutant mice compared to

controls. Lower p65 protein (p65/β-actin ratio of p65$^{fl/+}$ versus p65$^{fl/+}$Osx-Cre 2.62 $\pm$ 0.76 and 1.25 $\pm$ 0.66) was also observed from total protein isolated from long bones of adult mutant mice (Fig.1D). Immunoblot data of other NFκB subunits illustrated comparable levels between p65$^{fl/+}$ control and p65$^{fl/+}$Osx-Cre mice, indicating that the reduced amount of p65 from haploinsufficiency does not cause compensatory response of other NFκB subunits at physiological condition.

The p65$^{fl/+}$Osx-Cre progeny were born in an expected genetic ratio. Mice were viable and fertile. To determine whether NFκB affected osteogenesis, whole mounted stained skeletons of the mutant newborns were compared with the control skeletons. Morphologically, p65$^{fl/+}$Osx-Cre mice exhibited noticeably reduced sizes with proportionally smaller skeletons since birth (Fig.1A). Their body sizes remained smaller at 8 days and 11 weeks old. Their mean body weights were significantly lower in comparison with the controls for both genders (Fig.1E). When comparing between genders, the weight differences between mutants and controls were larger in male mice (Male 25-47% vs Female 18-37% at 8 to 3 weeks of age respectively, $p < 0.001$).

As Osx-Cre is present in both endochondral and intramembranous bony elements (Rodda and McMahon, 2006), detectable bone phenotypes were represented in craniofacial and long bones of p65$^{fl/+}$Osx-Cre mice (Fig.2). Skeletal staining revealed less bone mineralization, and reduced skeletal dimension particularly in craniofacial bones in p65$^{fl/+}$Osx-Cre neonates. Reduced skeleton sizes along with impaired bone deposition in frontal, parietal, nasal bones were observed in postnatal day2 (P2) stages (Fig.2A). The craniofacial phenotypes in p65$^{fl/+}$Osx-Cre mice remained until adulthood with smaller skulls and premaxillary deviation (Fig.2B).

41

Mean degrees of deviation measured by angle between premaxillary suture and frontal suture were 3.79 ± 1.53 in p65^{fl/+}Osx-Cre and 0.63 ± 0.69 in p65^{fl/+} controls (Fig.2C; $p<0.001$). In endochondral bones, overall reduction of skeletal dimensions with metaphyseal flaring was observed in scapula, humeri, tibiae, and femurs of P2 mutants. Growth plates of these bones were also proportionally longer when compared with those from P2 controls (Fig.2D).

MicroCT analysis confirmed quantitative reduction in bone dimension and architecture in p65^{fl/+}Osx-Cre mice. Dimension and cortical thickness of humeri from P2 mutant mice were significantly smaller than those from controls (Table 2, $p<0.05$). Similar phenotypes were detected in later stage (P8) as observed in femur length (p65^{fl/+} 4.010 ± 0.23 mm, p65^{fl/+}Osx-Cre 3.647 ± 0.26 mm., $p<0.05$), tibia length and perimeter (Fig.3, $p<0.05$). Cortical tibia bone morphology of P8 mutant mice was significantly different from the controls. Cortical bone area and mean polar moment of inertia were lower in p65^{fl/+}Osx-Cre mice ($p<0.05$). Tibia trabecular bone of the mutant mice also indicated altered architecture in compared with the controls. Lower trabecular number was observed in proximal regions of p65^{fl/+}Osx-Cre tibiae ($p<0.05$) with less trabecular thickness and trabecular separation. However, bone mineral densities from cortical and proximal trabecular tibia bone were not significantly different between mutants and controls.

The p65^{fl/+} femurs from P8 mice showed typical cortical and trabecular compartments with normal cellular organization. In the mutant p65^{fl/+}Osx-Cre mice, thinner cortical and less dense trabeculae appeared in the ossification zone (Fig.2E). Cell density at the chondroosseous junction was lower in the mutant mice, implying to

the reduced number of osteogenic cells. Taken together, deletion of one *p65* allele in osteogenic cells affected skeletal dimension and architecture from early postnatal stage to adulthood.

Targeted disruption of p65 single allele impaired osteoblast differentiation

To address the direct effect of NFκB disruption in osteogenic cells, calvarial-derived osteoprogenitors from young (P8) p65$^{fl/+}$Osx-Cre and p65$^{fl/+}$ littermates were isolated and characterized. Reduced skeletal dimension of p65$^{fl/+}$Osx-Cre could be a consequence of either reduced numbers of bone forming cells or decreased differentiation potential of these cells. The proliferation rate of the isolated calvarial cells, determined with MTS assay, showed no significant difference between cells with one *p65* allele and controls (Fig.4). Cellular assays comparing p65$^{fl/+}$Osx-Cre with the controls demonstrated reduced differentiation markers in both mRNA and early osteoblastic activity. Cultured p65$^{fl/+}$Osx-Cre calvarial cells had markedly reduced expression levels of osteoblast-specific mRNAs including *Runx2, Osx, Alp,* and *Bsp* (Fig.5D). Alp activity, an early osteoblast differentiation marker, significantly decreased in calvarial cells from p65$^{fl/+}$Osx-Cre mice (Fig.5C, $p<0.05$).

Reduced osteogenic potential in p65$^{fl/+}$Osx-Cre mice was verified by comparison of bone-forming capacity of MSCs from these mutants versus p65$^{fl/+}$controls. Formation of CFU-F/CFU-OB was examined in freshly isolated bone marrow stromal cells from 9-week old mutant and control mice. Consistent with calvarial data, CFU-F from p65$^{fl/+}$Osx-Cre and control mice were comparable (Fig.5A; $p=0.276$) while CFU-OB from p65$^{fl/+}$Osx-Cre MSCs were fewer for than controls ($p<0.05$). The CFU-OB/CFU-F ratio from the mutants was 34% and 54% from the

controls, implicating that deletion of one p65 allele affected dynamic of the MSCs population. The GFP emission in CFU-OB of $p65^{fl/+}$Osx-Cre was observed after 12 days in culture which indicated the expression of cre-recombinase enzyme during osteoblastic differentiation (Fig.5B). Additionally, osteoclastogenic potential of $p65^{fl/+}$Osx-Cre and control bone marrow stromal cells were assessed following mCSF and sRANKL induction. The numbers of TRAP staining positive cells from both groups were not different ($p65^{fl/+}$ versus $p65^{fl/+}$Osx-Cre 376 ± 51 VS 365 ± 43; $p>0.05$) and no significant difference was seen in osteoclast numbers from *in vivo* TRAP staining ($p65^{fl/+}$ versus $p65^{fl/+}$Osx-Cre; Chondro-osseous junction 31 ± 16.9 VS 21 ± 1.4; Trabecular region 60 ± 15.5 VS 71.5 ± 16.3 cells; $p>0.05$), indicating that there was no confounding effect from osteoclast formation in this model (Fig.6).

Effect of p65 disruption on osteogenic cell survival

To determine the role of NFκB/p65 in osteoblast survival, *in vitro* and *in vivo* apoptosis/survival tests were exploited. Caspase3/7 activity from cultured $p65^{fl/+}$Osx-Cre calvarial cells was higher than controls in both normal and staurosporine induced apoptosis conditions (Fig.7A; $p<0.05$). Cell survival from $p65^{fl/+}$Osx-Cre, as analyzed by fluorometric cell viability measurement, was lower than controls in both conditions. *In situ* apoptosis evaluation showed consistent results with the *in vitro* findings. More TUNEL positive cells were observed in subchondral area of tibia bones of 8-day-old $p65^{fl/+}$Osx-Cre mice (Fig.7B, 70.5 ± 12.21 VS 20 ± 7.45; $p=0.001$). Collectively, the elevated apoptosis in osteogenic cells with lack of one *p65* allele suggested a protective role of p65 in osteogenic cell survival which may partially explain how NFκB influences osteogenesis.

Roles of p65 in osteoblast differentiation through transactivation of Bmp2 genes

The impact of p65 NFκB on *BMP2* expression was suggested during osteoblast differentiation in human mesenchymal stem cells (Hess et al., 2009). Two NFκB responsive elements function in the promoter region of *Bmp2* gene (Feng et al., 2003). To examine the diminution of p65/NFκB modulating osteogenesis, NFκB transactivation activities in primary calvarial cells were quantified by luciferase assay following transduction of luciferase adenovirus containing NFκB responsive element. Less luciferase reporter signal in p65$^{fl/+}$Osx-Cre calvarial cells demonstrated reduction of NFκB transcriptional activity upon 4 hours of TNFα stimulation (Fig.8A, p<0.001). To confirm the potential NFκB transactivation effect on Bmp2 induction, a simple transfection approach in which modulation of p65 NFκB by p65 and IκB-SR (a constitutively negative NFκB signaling regulator) plasmids were conducted in a mouse stem-like cell line, C2C12 cells. Overexpression of p65 resulted in elevated induction of *Bmp2* promoter-driven luciferase expression. Conversely, coexpression of p65 and IκB-SR showed significantly blunted luciferase production to basal level (Fig.8B). These observations were corroborated with decreased *Bmp2* mRNA levels in primary *p65* haploinsufficient calvarial cells in compared with the controls (Fig.8C). These results suggested that reduced osteogenesis from NFκB signaling reduction may be partly the consequence of decreased *Bmp2* expression.

Figure 1 Characteristic of p65$^{fl/+}$ versus p65$^{fl/+}$Osx-Cre phenotype. Reduced amounts of p65 protein in osteogenic cells resulted in smaller body sizes in heterozygotes carrying one functional *p65* allele. A) Whole mount skeletal preparations of p65$^{fl/+}$ (wild type control) and p65$^{fl/+}$Osx-Cre newborns at postnatal day2. B) PCR genotyping of *p65* flanked loxP and *Cre* allele genes: p65$^{fl/fl}$, homozygous p65 loxP; p65$^{fl/+}$, heterozygous p65 loxP; WT, wild type. C) PCR genotyping of *p65* recombination demonstrated tissue specific mutation of *p65* gene in bone from p65$^{fl/fl}$Osx-Cre and p65$^{fl/+}$Osx-Cre mice. D) Immunoblotting of NFκB protein levels from total lysates of isolated calvarial cells and from tibia/femoral bones obtained from p65$^{fl/fl}$Osx-Cre and p65$^{fl/+}$Osx-Cre mice. n=3 animals/group. Experiments were done independently in triplicate. E) Mean body weight of p65$^{fl/+}$ and p65$^{fl/fl}$Osx-Cre mice from age of 3 weeks to 8 weeks old. n=10/group *, p<0.001 versus p65$^{fl/+}$ male mice; #, p<0. 001 versus p65$^{fl/+}$ female mice.
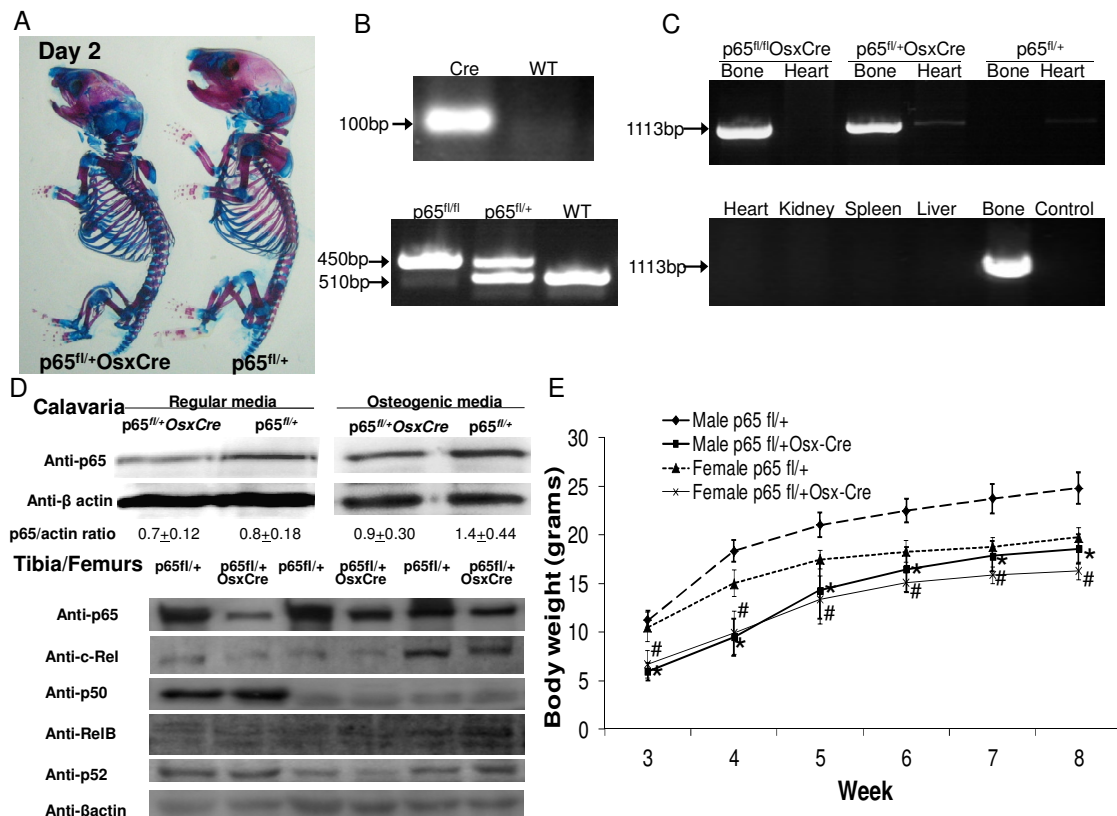

46

Figure 2 Gross phenotype. Conditional removal of *p65* allele in osteogenic cells affects skeletal development. A) Alizarin red/alcian blue staining of skulls of P2 $p65^{fl/+}$ control and $p65^{fl/+}$Osx-Cre mutant pups. Dorsal and ventral views of skulls of P2 control and mutant mice showed less mineralization in premaxilla, nasal and parietal bones. n=10/group B) Gross structure of skulls from week-11 male mutant mice in compared with controls C) Comparison of angle between premaxillary suture and frontal suture from gender matched week-11 $p65^{fl/+}$ and $p65^{fl/+}$Osx-Cre mice. Data are shown as mean$\pm$SD. n= 10-11 animals/group. *, p <0.05 versus $p65^{fl/+}$ samples. D) Higher magnification of forelimbs, hindlimbs, and tibia. Black arrowheads indicate metaphyseal flaring in the long bones of $p65^{fl/+}$Osx-Cre pups. E) The proximal growth plate of femurs from P8 $p65^{fl/+}$ and $p65^{fl/+}$Osx-Cre mice stained with hematoxylin and eosin. n=3/group
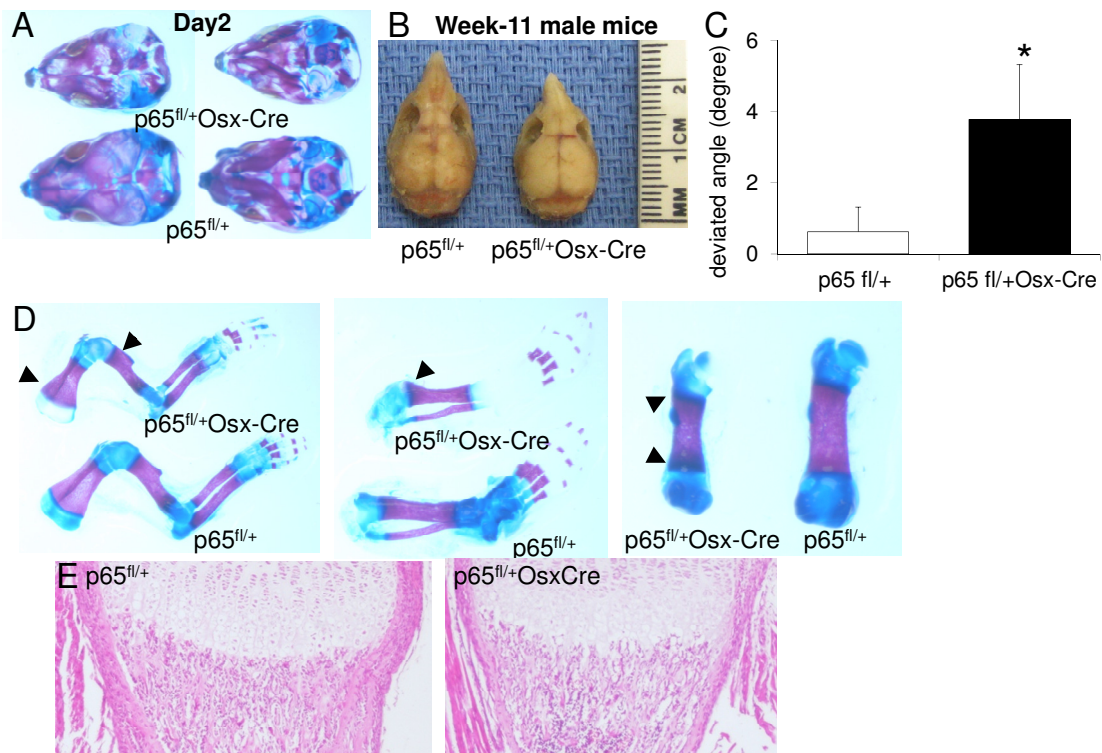
Table 2 μCT measurements of humerus bones in gender-match day2 p65$^{fl/+}$ and p65$^{fl/+}$Osx-Cre pups

μCT Measurements	p65$^{fl/+}$ Osx-Cre	p65$^{fl/+}$
Bone length	2.523±0.083*	2.673±0.099
Diameter	0.512±0.029*	0.588±0.038
Cortical thickness	0.134±0.019*	0.185±0.026
Circumference	1.754±0.068*	2.006±0.068

*$p<0.05$ versus p65$^{fl/+}$ controls, n = 6-7 animals per group.

Figure 3 μCT analysis of femur bones from day 8 mice. Comparison of trabecular and cortical bone morphology and BMD in the region of proximal tibia bone from gender-matched p65^{fl/+} control and p65^{fl/+}Osx-Cre mice. Data are shown as mean ± SD. n=8 animals/group. *, p <0.05 versus p65^{fl/+} samples.
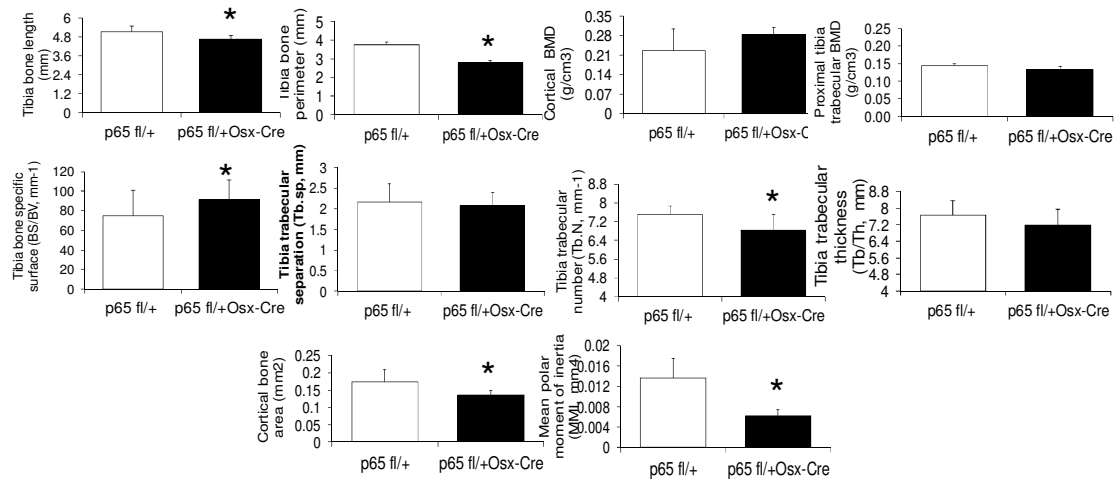
Figure 4 Cell proliferation. Proliferation pattern of calvarial cells derived from 8-day old p65^{fl+} and p65$^{fl/+}$Osx-Cre mice. Absorbance at 490 nm was measured to detect formazan produced by live cells at indicated time points. Data are presented as mean $\pm$ SD.
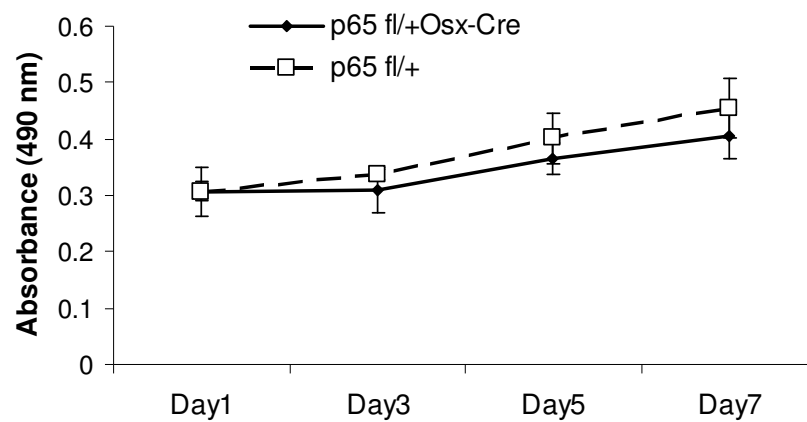
Figure 5 Osteoblast differentiation analyses. *In vitro* differentiaion assays provide evidence of reduced osteoinductive potential in p65^{fl/+}Osx-Cre mice. A) CFU-Fs and CFU-OBs in the bone marrow from femur/tibia bones of 9-week old mice of the indicated genotypes. Mean CFU-F colonies/1.5x10^6 BMCs. Mean CFU-OB colonies/2.5x10^6 BMCs. n= 8-10 animals per group/condition B) CFU-OBs staining obtained from p65^{fl/+} Osx-Cre mutants and controls. Colonies from CFU-OBs of p65^{fl/+} Osx-Cre samples expressed GFP attributed to the presence of Osx-Cre recombinase after 12-day incubation in osteogenic media. C) Alkaline phosphatase activity of calvarial cells from 8-day old wild type and mutant mice after 2 days culture was assessed by p-nitrophenol assay. D) Relative expression levels of early osteoblastic gene markers after 10-day osteogenic media induced differentiation on calvarial cells from studied mice. Differentiation was assessed by realtime PCR for *Runx2, Osx, Alp, Bsp*. n=5 animals per group/condition. Data are shown as mean $\pm$ SD. *, p<0.005 versus p65^{fl/+} control.
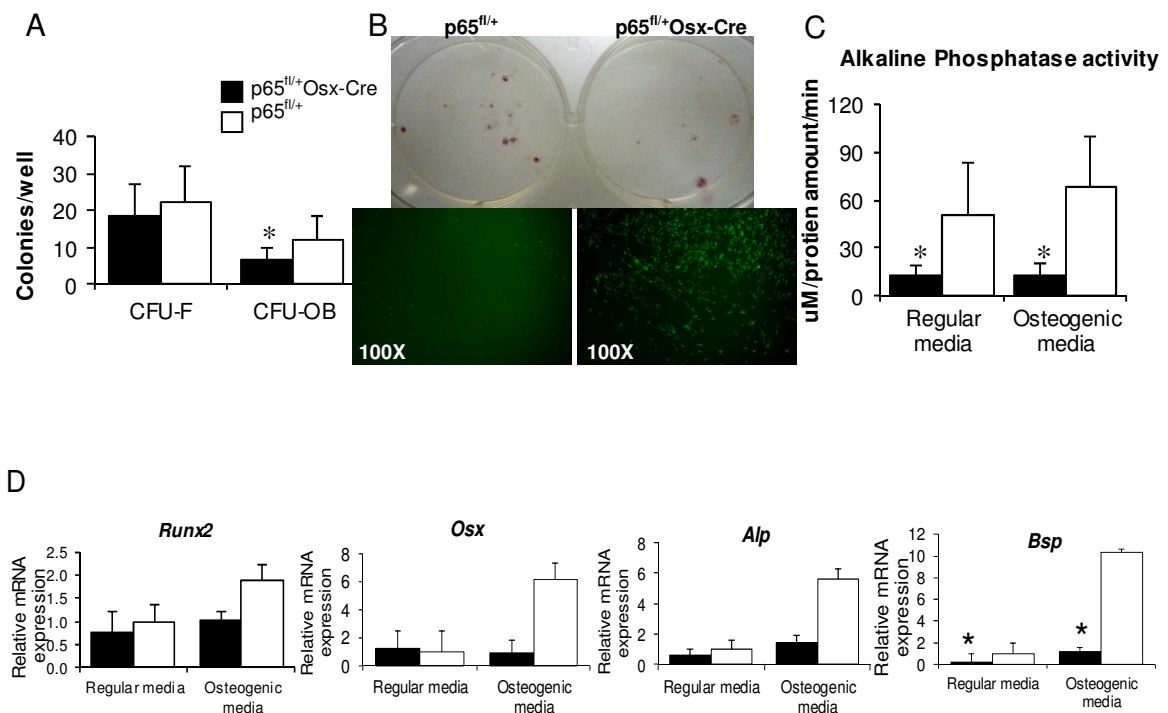
Figure 6 Osteoclastogenic potential analyses. Haploinsufficiency of *p65* in osteoblasts does not alter osteoclasts population. A) TRAP staining of osteoclasts derived from bone marrow cells isolated from p65^{fl/+}Osx-Cre mutant and B) control mice. C) Mean TRAP positive cells/1.0x10^{6} BMCs. n= 2 animals per group in quadricate. Data are shown as mean $\pm$ SD. p>0.05 versus p65^{fl/+} control. D) TRAP staining of sections from femurs of 8-week old p65^{fl/+}Osx-Cre and E) p65^{fl/+} mice. n=3 animals/group.
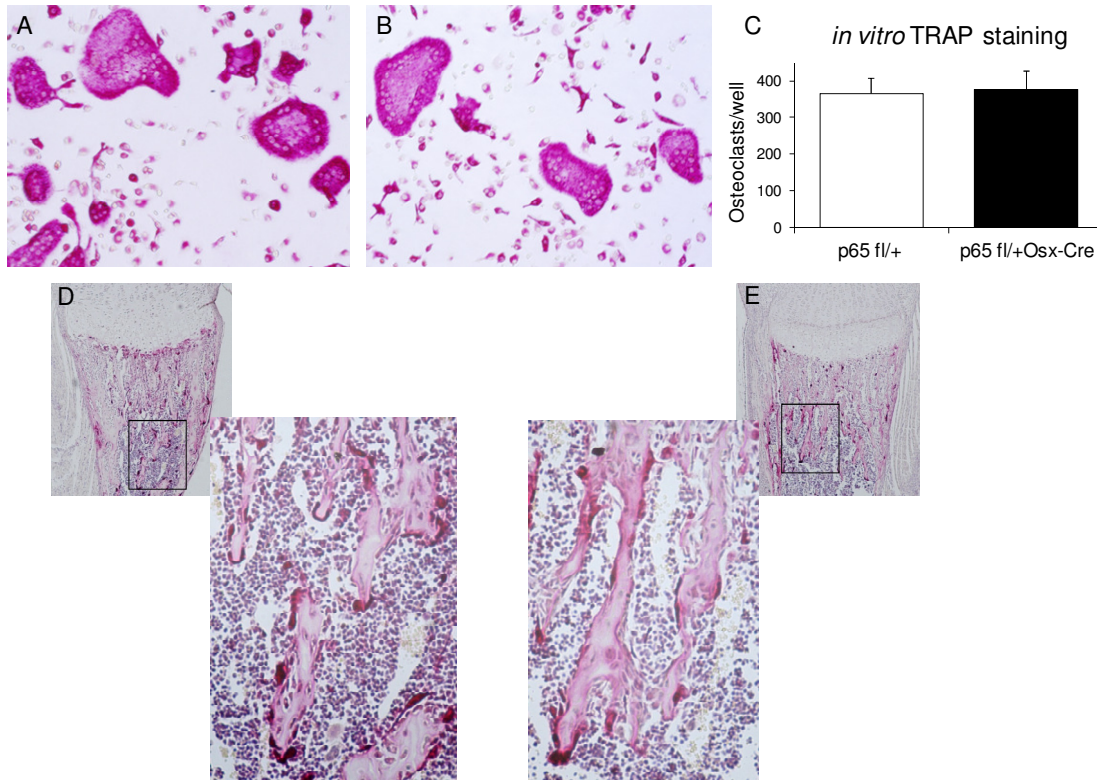
Figure 7 Apoptosis analyses. Lack of a functional *p65* allele results in increased apoptosis in osteoblasts. A) Caspase-3/7 activity and cell viability (Fluorescent RFU) in calvaria cells isolated from wild type p65$^{fl/+}$ control and mutant mice. Cells were challenge with 1 μM Staurosporine-induced apoptosis or normal saline for 24 hours prior to measurement with fluorometric analysis. B) In situ apoptosis was determined in tibia bones by counting TUNEL positive cells in the metaphysis areas which showed higher apoptotic cells in 8-day old p65$^{fl/+}$ Osx-Cre mice. n=3 animals/group. Data are shown as mean $\pm$ SD. * $p<0.05$, # $p<0.005$ versus p65$^{fl/+}$ control. Scale bar represent 10 μM.
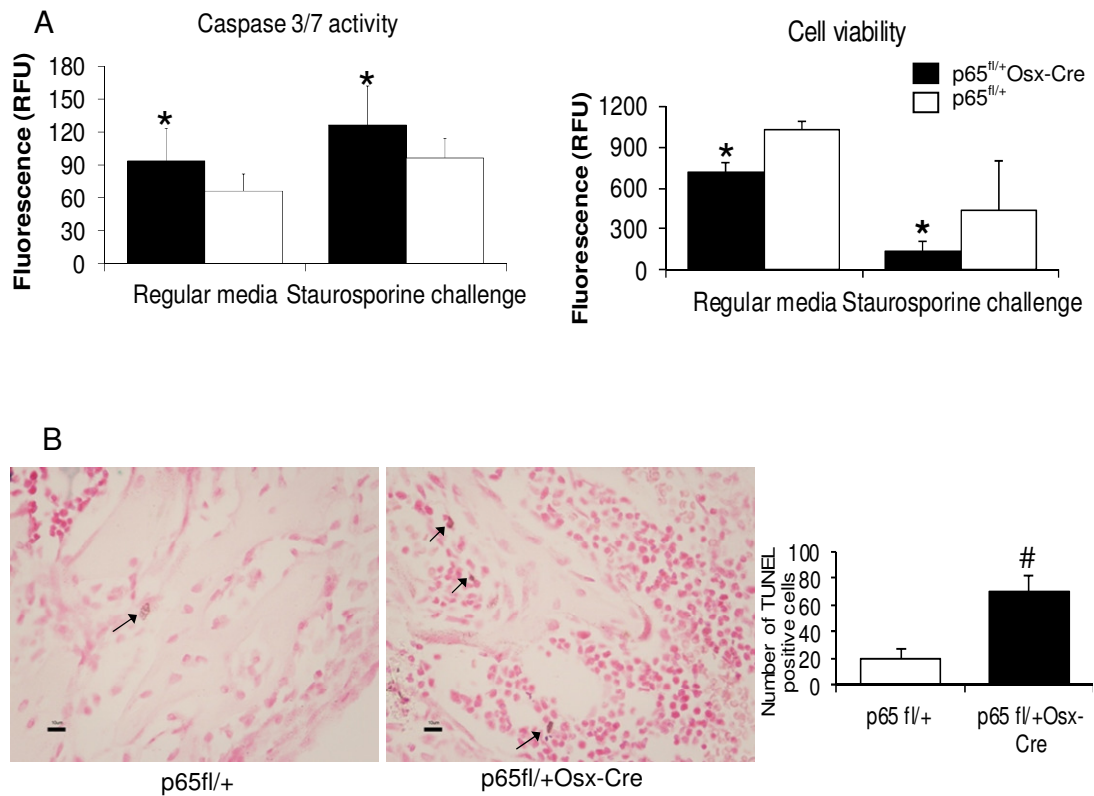
Figure 8 Effect of p65 on the induction of Bmp2 promoter activities. A) Transactivational activity of NFκB in calvarial cells from studied animals, assessed with luciferase reporter assay following 24-hour transduction of Ad.NFκBLuc then 4-hour challenge of 100 ng/ml TNFα or PBS. B) Luciferase reporter assays were performed in C2C12 cells using co-transfection of osteoblastic-related responsive luciferase reporters and p65 plasmid with or without stabilized mutant IκBα (IκB-SR) plasmid. The target constructs included Bmp2 promoters with different deletions of promoter sizes (-150/+165, -2457/+165, and -2712/+165). Cells were transiently transfected for 16 hours and incubated in normal growth medium for 24 hours prior to measurement. The luciferase activity was calculated as relative fold change by normalization with Renilla luciferase activity. C) Relative *Bmp2* gene expression from calvarial cells from day-8 old wild type and mutant p65$^{fl/+}$ Osx-Cre mice. n=5 animals per group/condition. *, $p<0.005$ versus p65$^{fl/+}$ control.

54
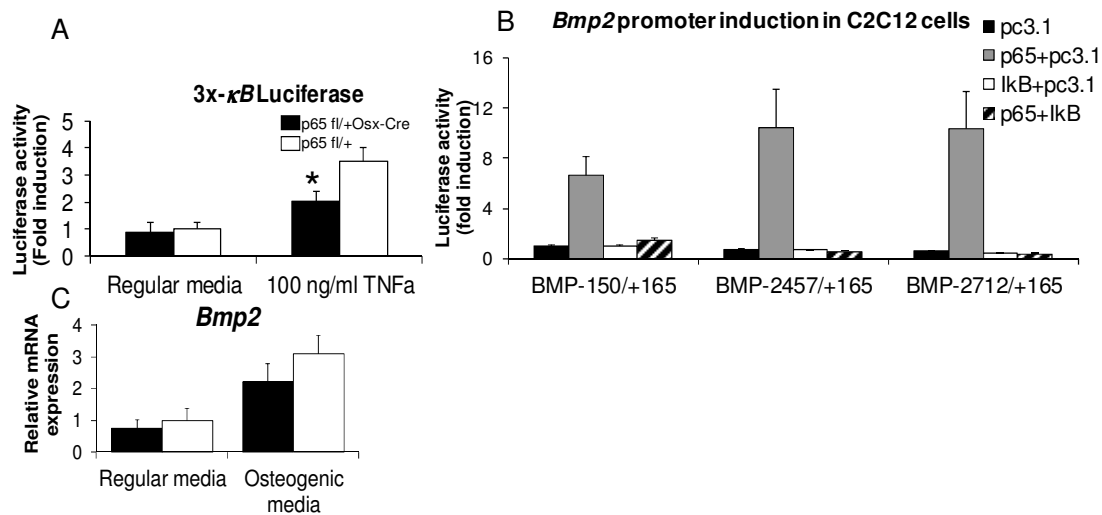
Discussion

NFκB plays pivotal roles in controlling a wide range of genes influencing diverse cellular activities including cell proliferation, differentiation, and apoptosis (Shih et al., 2011). Modulating NFκB activity is one therapeutic target in inflammatory bone diseases based upon the role of NFκB in osteoclast regulation (Jimi et al., 2004; Makarov, 2001; Nichols et al., 2001). P65 is a major functional subunit of NFκB family. The structure of p65 consists of 2 major domains, amino-terminal Rel homology domain serving for DNA-binding and dimerization and carboxyl-terminal transactivation domain serving for transcription regulation (Perkins, 2007). Activation of p65 NFκB is mediated primarily by phosphorylation of IκB in IKK dependent pathway. The trimeric IKK complex typically consists of three subunits including two key catalytic subunits, IKKα and IKKβ, which interact with IKKɣ regulatory subunit (Chen and Greene, 2004; Perkins, 2007; Shih et al., 2011). IKK complex inhibits most, but not all, activities of p65 NFκB as illustrated by activation of p65 NFκB by casein kinase-II and typrosine kinase dependent pathways (Perkins, 2007).

Growing evidence suggests a role for p65 NFκB during skeletal growth and development attributable to osteoblasts regulation. The expression of the p65 NFκB subunit is abundant while p50 and p52 subunits show lesser expression in growth plate of long bones where ossification occurs (Wu et al., 2007). Inhibition of p65 NFκB activity by specific NFκB inhibitors, PDTC and BAY, hampers longitudinal growth in rat metatarsal bone culture. *In vitro* activation of p65 mediated NFκB pathway by constitutively active IKKα promotes osteogenic differentiation of human

mesenchymal stem cells (Hess et al., 2009). However, the previous conclusions were based on indirect regulation of p65 NFκB function with redundant effects from other NFκB subunits. This present study demonstrates that p65 mediated NFκB activity is a critical factor in maintaining *in vivo* bone development and remodeling. Suppression of p65 NFκB activity impairs early osteoblast differentiation and survival of osteoprogenitor cells.

Ikkα^{-/-} mice have abnormal skeletal and craniofacial morphogenesis with delayed mineralization in palatal bones as accounted for unique function of Ikkα in epidermal differentiation (Hu et al., 1999; Li et al., 1999; Sil et al., 2004). In contrary, the current study demonstrated similar skeletal phenotypes but reflected direct effects of NFκB disruption on osteoprogenitors. Lack of one *p65* allele affects bone development with impact on bone dimension and architecture. Skeletal staining of p65^{fl/+}Osx-Cre mice exhibited osteopenic phenotype in longitudinal and craniofacial bone growth (Fig.2). Histological and μCT analysis revealed reduction of bone dimension as well as alteration of bone trabecular number in p65^{fl/+}Osx-Cre mice (Fig.2E, Fig.3 and Table2). These bone phenotypes do not reflect nonspecific effect of Cre-recombinase enzyme in osteogenic cells as reported by indistinguishable microCT data between Osx-Cre mice and wild type controls from previous studies (Shimada et al., 2008). Indirect evidence of the effect of p65 mediated NFκB on skeletal growth was indicated with reduced size and lower weight in *p65* haploinsufficient mice as compared with controls. A report regarding growth retardation with short stature in one patient identified a heterozygous mutation of *IκBα* gene which suggests the effect of impaired NFκB function in skeletal growth (Janssen et al., 2004).

The transactivational activity of NFκB is primarily attributed to the function of p65 subunit (Steinbrecher et al., 2008). Earlier studies indicated that p65 and p65/p50 are transcriptional activators of Osx, a specific osteogenic regulator which implicated the role of p65 in osteoblast differentiation (Lu et al., 2006). The present study illustrates that the presence of one *p65* allele failed to support sufficient p65 protein and activity, thereby affecting the osteogenesis process. In osteoinduced condition, osteoprogenitor cells lacking one *p65* allele had 40% decrease of total p65 protein level in compared with the control counterparts (Fig.1D) which was concomitant with 42% diminished NFκB transactivation activity (Fig.8A). Diminished NFκB activity from absence of one *p65* allele impaired differentiation with less effect on proliferation. Osteogenic cells including MSCs and osteoprogenitor cells from p65$^{fl/+}$Osx-Cre mice clearly presented impaired osteogenic potential. Markedly fewer CFU-OB in comparison with CFU-F of p65$^{fl/+}$Osx-Cre MSCs implicated the role of NFκB in mesencymal stem cells differentiation. Less Alp activity with reduced mRNA abundance of selected ostegenic markers of p65$^{fl/+}$Osx-Cre calvarial cells denoted impaired early differentiation in committed osteogenic cells. Differing evidence has been suggested in mice carrying defective *Ikkγ* in early differentiated osteoblasts (Col1α1-IKK-DN) (Chang et al., 2009). Difference in bone phenotypes between p65$^{fl/+}$Osx-Cre mice as described here and Col1α1-IKK-DN mice which exhibit enhanced bone formation may be attributed to residual activation of p65 NFκB from IKK independent pathway or from compensatory function of other NFκB subunits (Perkins, 2007; Shih et al., 2011; Steinbrecher et al., 2008). NFκB perturbation in terminally differentiated stage of osteoblasts provides differing results

57

in maintaining bone homeostasis, with increased osteoblast function in osteoporotic model of *Bglap2*-IKK-DN mice (Chang et al., 2009). Additional studies are essential to elucidate the function of p65 NFκB in bone homeostasis according to cell-and stage-specific characteristic.

The other pivotal role of p65 NFκB is anti-apoptotic function (Beg et al., 1995; Steinbrecher et al., 2008). Mice devoid of *p65* died in embryonic stage from severe liver apoptosis (Beg et al., 1995). Inhibition of p65 in chondrogenic cell line with PDTC, BAY, and *p65* siRNA led to apoptotic mediated cell death as shown by upregulation of *Bcl-2/Bax* gene under caspase3 dependent pathway (Wu et al., 2007). Accumulating findings in our *in vitro* and *in vivo* experiments support a protective role of p65 NFκB in maintaining osteoblasts during normal physiological stage. Reduced caspase3/7 activity in osteoprogenitor lacking one *p65* allele illustrated that p65 NFκB mediated anti-apoptotic function through caspase3/7 dependent pathway. Osteoprogenitors with reduced p65 NFκB activity had elevated apoptosis compared with controls in both normal and straurosporine challenged conditions. MSCs from p65$^{fl/+}$Osx-Cre mice were affected from NFκB perturbation during osteoblast differentiation process. Decreased NFκB and increased apoptosis shift the skeletal balance to a reduced anabolic state. Decreased osteoblast survival may contribute to the observed phenotype in p65$^{fl/+}$Osx-Cre mice. During bone destruction/injuries stage, reestablishement of new osteoblasts is required to counteract bone loss or resorption (Zaidi, 2007). Further work therefore is required to define the consequence of reduced p65 level on MSC and osteoprogenitor recruitment and survival during healing and regeneration stage in order to find optimal condition or underlying

mechanism which support osteoblast activity in bone regeneration.

A mechanistic basis for reduced skeletal growth in p65$^{fl/+}$Osx-Cre mice may also include the lack of skeletal activators driven by NFκB. BMP2 is a key factor in skeletal development. BMP2 is well characterized and is an efficient inducer of RUNX2, a transcription activator controlling osteogenesis (Carter et al., 2008; King and Cochran, 2002; Lian and Stein, 2003; Matsubara et al., 2008). The promoter region of the *Bmp2* gene contains two NFκB responsive elements where p65 and p50 subunits interact with (Feng et al., 2003). Reduced p65 NFκB activity results in decreased *Bmp2* mRNA expression in primary chondrocytes which interrupts metatarsal bone growth (Wu et al., 2007). Consistent with previous finding, p65$^{fl/+}$Osx-Cre osteoprogenitor cells with reduced p65 NFκB activity exhibit less *Bmp2* gene expression which may contribute to delayed bone formation presented in their skeletal phenotype and lower CFU-OB. This confirms a reduction in *BMP2* mRNA level following IKK/NFκB modulation in human mesenchymal stem cells (Hess et al., 2009). Alteration in *Bmp2* induction with p65 and IκB overexpression in this study implicates a primary pathway that p65 mediates *Bmp2* transcription through typical NFκB activity. Additionally, these results may describe the mechanism in which lack of one *p65* allele causes delayed osteoblast development in our animals through abrogation of NFκB transcriptional activity on *Bmp2* induction. Although inhibition of NFκB and its upstream regulators is considered as one factor in controlling the severity of inflammatory bone diseases (Chang et al., 2009), our result indicates that lack of NFκB mediated gene expression in osteoprogenitors negatively influences physiological bone formation process. It is possible that NFκB may

regulate other molecules to induce bone formation. Exploring the affected target genes from osteoprogenitor lacking on *p65* allele will enable us to define signaling pathways that NFκB is involved during bone formation.

In conclusion, a specific role of p65 NFκB in bone development is suggested by alteration of skeletal size and craniofacial development in mice with *p65* haploinsufficiency in osteoprogenitors. Disruption of *p65* NFκB in osteoprogenitors causes detrimental effects in early stage of osteogenesis at multiple levels of regulation through differentiation and apoptosis protection. The osteogenic regulatory roles of p65 NFκB is mediated, at least in part, by *Bmp2* induction. The role of p65 NFκB on osteoblasts during bone repair and regeneration requires further examination.

References (Chapter 2)

Asahina I, Sampath TK, Hauschka PV (1996). Human osteogenic protein-1 induces chondroblastic, osteoblastic, and/or adipocytic differentiation of clonal murine target cells. *Exp Cell Res* 222(1)*:*38-47.

Aubin JE (2001). Regulation of osteoblast formation and function. *Rev Endocr Metab Disord* 2(1)*:*81-94.

Baek WY, de Crombrugghe B, Kim JE (2010). Postnatally induced inactivation of Osterix in osteoblasts results in the reduction of bone formation and maintenance. *Bone* 46(4)*:*920-8.

Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D (1995). Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature* 376(6536)*:*167-70.

Cao X, Chen D (2005). The BMP signaling and in vivo bone formation. *Gene* 357(1)*:*1-8.

Carter TG, Brar PS, Tolas A, Beirne OR (2008). Off-label use of recombinant human bone morphogenetic protein-2 (rhBMP-2) for reconstruction of mandibular bone defects in humans. *J Oral Maxillofac Surg* 66(7)*:*1417-25.

Chang J, Wang Z, Tang E, Fan Z, McCauley L, Franceschi R, et al. (2009). Inhibition of osteoblastic bone formation by nuclear factor-kappaB. *Nat Med* 15(6)*:*682-9.

Chen LF, Greene WC (2004). Shaping the nuclear action of NF-kappaB. *Nat Rev Mol Cell Biol* 5(5)*:*392-401.

Feng JQ, Xing L, Zhang JH, Zhao M, Horn D, Chan J, et al. (2003). NF-kappaB specifically activates BMP-2 gene expression in growth plate chondrocytes in vivo and in a chondrocyte cell line in vitro. *J Biol Chem* 278(31)*:*29130-5.

Franzoso G, Carlson L, Xing L, Poljak L, Shores EW, Brown KD, et al. (1997). Requirement for NF-kappaB in osteoclast and B-cell development. *Genes Dev* 11(24)*:*3482-96.

Guo J, Cooper LF (2007). Influence of an LRP5 cytoplasmic SNP on Wnt signaling and osteoblastic differentiation. *Bone* 40(1)*:*57-67.

Hess K, Ushmorov A, Fiedler J, Brenner RE, Wirth T (2009). TNFalpha promotes osteogenic differentiation of human mesenchymal stem cells by triggering the NF-kappaB signaling pathway. *Bone* 45(2)*:*367-76.

Hu Y, Baud V, Delhase M, Zhang P, Deerinck T, Ellisman M, et al. (1999). Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. *Science* 284(5412)*:*316-20.

Janssen R, van Wengen A, Hoeve MA, ten Dam M, van der Burg M, van Dongen J, et al. (2004). The same IkappaBalpha mutation in two related individuals leads to completely different clinical syndromes. *J Exp Med* 200(5)*:*559-68.

Jimi E, Aoki K, Saito H, D'Acquisto F, May MJ, Nakamura I, et al. (2004). Selective inhibition of NF-kappa B blocks osteoclastogenesis and prevents inflammatory bone destruction in vivo. *Nat Med* 10(6)*:*617-24.

Katagiri T, Takahashi N (2002). Regulatory mechanisms of osteoblast and osteoclast differentiation. *Oral Dis* 8(3)*:*147-59.

King GN, Cochran DL (2002). Factors that modulate the effects of bone morphogenetic protein-induced periodontal regeneration: a critical review. *J Periodontol* 73(8)*:*925-36.

Li Q, Lu Q, Hwang JY, Buscher D, Lee KF, Izpisua-Belmonte JC, et al. (1999). IKK1-deficient mice exhibit abnormal development of skin and skeleton. *Genes Dev* 13(10)*:*1322-8.

Lian JB, Stein GS (2003). Runx2/Cbfa1: a multifunctional regulator of bone formation. *Curr Pharm Des* 9(32)*:*2677-85.

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25(4)*:*402-8.

Lu X, Gilbert L, He X, Rubin J, Nanes MS (2006). Transcriptional regulation of the osterix (Osx, Sp7) promoter by tumor necrosis factor identifies disparate effects of mitogen-activated protein kinase and NF kappa B pathways. *J Biol Chem* 281(10)*:*6297-306.

Makarov SS (2001). NF-kappa B in rheumatoid arthritis: a pivotal regulator of inflammation, hyperplasia, and tissue destruction. *Arthritis Res* 3(4)*:*200-6.

Matsubara T, Kida K, Yamaguchi A, Hata K, Ichida F, Meguro H, et al. (2008). BMP2 regulates Osterix through Msx2 and Runx2 during osteoblast differentiation. *J Biol Chem* 283(43)*:*29119-25.

McLeod MJ (1980). Differential staining of cartilage and bone in whole mouse fetuses by alcian blue and alizarin red S. *Teratology* 22(3)*:*299-301.

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, et al. (2002). The novel zinc finger-containing transcription factor osterix is required for osteoblast

differentiation and bone formation. *Cell* 108(1):17-29.

Nichols TC, Fischer TH, Deliargyris EN, Baldwin AS, Jr. (2001). Role of nuclear factor-kappa B (NF-kappa B) in inflammation, periodontitis, and atherogenesis. *Ann Periodontol* 6(1):20-9.

Perkins ND (2007). Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat Rev Mol Cell Biol* 8(1):49-62.

Rodda SJ, McMahon AP (2006). Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. *Development* 133(16):3231-44.

Sanlioglu S, Williams CM, Samavati L, Butler NS, Wang G, McCray PB, Jr., et al. (2001). Lipopolysaccharide induces Rac1-dependent reactive oxygen species formation and coordinates tumor necrosis factor-alpha secretion through IKK regulation of NF-kappa B. *J Biol Chem* 276(32):30188-98.

Shih VF, Tsui R, Caldwell A, Hoffmann A A (2011) single NFkappaB system for both canonical and non-canonical signaling. *Cell Res* 21(1):86-102.

Shimada M, Greer PA, McMahon AP, Bouxsein ML, Schipani E (2008). In vivo targeted deletion of calpain small subunit, Capn4, in cells of the osteoblast lineage impairs cell proliferation, differentiation, and bone formation. *J Biol Chem* 283(30):21002-10.

Sil AK, Maeda S, Sano Y, Roop DR, Karin M (2004). IkappaB kinase-alpha acts in the epidermis to control skeletal and craniofacial morphogenesis. *Nature* 428(6983):660-4.

Sitcheran R, Cogswell PC, Baldwin AS, Jr. (2003). NF-kappaB mediates inhibition of mesenchymal cell differentiation through a posttranscriptional gene silencing mechanism. *Genes Dev* 17(19):2368-73.

Steinbrecher KA, Harmel-Laws E, Sitcheran R, Baldwin AS (2008). Loss of epithelial RelA results in deregulated intestinal proliferative/apoptotic homeostasis and susceptibility to inflammation. *J Immunol* 180(4):2588-99.

Suzawa M, Takada I, Yanagisawa J, Ohtake F, Ogawa S, Yamauchi T, et al. (2003). Cytokines suppress adipogenesis and PPAR-gamma function through the TAK1/TAB1/NIK cascade. *Nat Cell Biol* 5(3):224-30.

Vaira S, Alhawagri M, Anwisye I, Kitaura H, Faccio R, Novack DV (2008). RelA/p65 promotes osteoclast differentiation by blocking a RANKL-induced apoptotic JNK pathway in mice. *J Clin Invest* 118(6):2088-97.

Weber JM, Forsythe SR, Christianson CA, Frisch BJ, Gigliotti BJ, Jordan CT, et al. (2006). Parathyroid hormone stimulates expression of the Notch ligand Jagged1 in osteoblastic cells. *Bone* 39(3):485-93.

Wu S, Flint JK, Rezvani G, De Luca F (2007). Nuclear factor-kappaB p65 facilitates longitudinal bone growth by inducing growth plate chondrocyte proliferation and differentiation and by preventing apoptosis. *J Biol Chem* 282(46):33698-706.

Yan D, Gurumurthy A, Wright M, Pfeiler TW, Loboa EG, Everett ET (2007). Genetic background influences fluoride's effects on osteoclastogenesis. *Bone* 41(6):1036-44.

Zaidi M (2007). Skeletal remodeling in health and disease. *Nat Med* 13(7):791-801.

CHAPTER 3

NFκB P65 MODULATES BONE FORMATION IN BMP2-INDUCED
BONE REGENERATION

Introduction

Inflammatory regulation has emerged as a significant factor that determines bone regeneration outcome. Previous studies have demonstrated that several inflammatory mediators play essential roles in initial stages of bone repair/regeneration, while aberrant or chronic inflammation exacerbates detrimental effect in bone formation and bone remodeling as seen in rheumatoid arthritis or periodontitis (Mountziaris et al., 2011; Takayanagi, 2009). Mice with chronic inflammation caused by long term exposure of TNFα exhibit rheumatoid arthritis-like condition which affects bone volume and strength (Alblowi et al., 2009). Abnormal elevated level of IL6 contributed to pathogenesis of polyarticular juvenile idiopathic arthritis with impaired osteoblast function (Caparbo et al., 2009). In contrast, mice with impaired *Tnfα*, *Il6*, and *Cox2* have reduced bone regeneration potential with less mineralization due to compromised osteogenic response (Gerstenfeld et al., 2001; Yang et al., 2007; Zhang et al., 2002). Mice with nonfunctional *Faslg* display limited capability in rhBMP2 induced bone tissue engineering which is attributed to

suppressed *Bsp*, type I collagen and osteopontin mRNA levels (Katavic et al., 2003). In vitro evidences indicate positive roles of TNFα and IL6 in early stage of osteoblast development. In vitro short term stimulation of MSCs with TNFα and/or IL1β enhances osteogenic activities by promoting increase of early and late osteoblastic markers (Hess et al., 2009 Mountziaris et al., 2010). Preculture of preosteoblast MC3T3 cells with IL6 induces *Runx2* and *Ocn* mRNA upregulation (Li et al., 2008). In recognition of the influence of inflammation on bone reconstruction, the molecular mechanism underlying this regulation remains unclear.

NFκB complexes represent the key inflammatory regulators based on their major role in innate and adaptive immunities (Li and Verma, 2002). NFκB is described as a dimeric transcription factor that is involved in activation and control of a variety of genes in response to cellular conditions (Perkins, 2007). Several lines of evidence suggest that NFκB pathway contribute to the control of inflammatory mediator-induced bone formation. The IKK/NFκB p65 pathway is indicated as an underlying mechanism in which TNFα stimulates bone formation in MSCs (Bocker et al., 2008; Crisostomo et al., 2008; Hess et al., 2009). In addition to TNFα, a potential inducer of NFκB activation, Fas ligand induces IL8 production by activating NFκB in a TNFα independent manner (Imamura et al., 2004). Additionally, several inflammatory mediators including COX2, PGE$_2$, and IL6 showed to promote osteogenesis by acting on osteoblasts through the NFκB pathway (Chen et al., 2003; Fukuno et al., 2011; Jung et al., 2003).

BMP2, a member of transforming growth factor-β superfamily, has been widely used in bone tissue engineering owing to its potent osteoinducing capacity

(Choi et al., 2002; Hou et al., 2007; Katayama et al., 2009; Triplett et al., 2009). The clinical outcomes of BMP2-induced bone regeneration are influenced by interplay among multiple factors including the implanted materials, the methods of BMP administration, and the host response which determines cellular activities attributed to bone formation process (Hu et al., 2010; Kim et al., 2005; Kim et al., 2011). The phenomenon of BMP2-mediated bone tissue engineering start from initial inflammation and cell migration, and continue with differentiation, bone replacement and remodeling of implanted materials (Hou et al., 2007). The association of an NFκB-mediated inflammatory response with osteoblast regulation during bone development as presented in chapter 2 suggests the potential effect of NFκB in bone regenerative outcome. To gain insight and understand the role of NFκB in osteogenesis during bone regeneration, the hypothesis was that NFκB modulates bone formation through osteoblast regulation in recombinant human BMP2-induced bone tissue engineering.

Materials & method

In vivo bone formation

To further confirm the involvement of NFκB p65 in osteoblast differentiation *in vivo*, the bone formation capacity during bone regeneration process was stimulated by the implantation of collagen sponges containing 1 ug of recombinant human BMP2 (rhBMP2) into subcutaneous tissues of *cis*-NFκB GFP mice (a kind gift from Dr. Christian Jobin from Lineburger Cancer Center, UNC), p65$^{fl/+}$mice, p65$^{fl/+}$Osx-Cre mice, and Osx-Cre mice. The collagen scaffolds and rhBMP2 were obtained from Infuse$^®$ Bone Graft kit (Medtronic, Minneapolis, MN). The collagen scaffolds were

prepared in a disk shape at 5 mm diameter using a tissue biopsy punch. The rhBMP2 or deionized water as a negative control was loaded into the scaffolds fifteen minutes prior to the implantation procedure. Recombinant human tumor necrosis factor alpha (TNFα) at 100 ng/ml concentration was also applied to some scaffolds as positive controls for NFκB activation analysis. Mice at five to seven weeks old of age were used. At least three mice from each group were used for each condition at each time point. The scaffolds were harvested at 3 days, 7 days, and 2 weeks after implantation in *cis*-NFκB GFP mice and at 2 weeks and 5 weeks after implantation in p65$^{fl/+}$, p65$^{fl/+}$Osx-Cre and Osx-Cre mice. At least two independent experiments were performed. All experimental procedures have been approved by Institution Animal Care and Use Committee at the University of North Carolina at Chapel Hill and been in accordance with NIH animal handling procedures.

For surgical procedures, the animals were anesthetized by intraperitoneal injection with a combination of 100 mg/kg ketamine and 5 mg/kg xylazine (PSS, West Columbia, SC). Following the preparation of the surgical site, a midlongitudinal skin incision at <1.5 cm in length was made at dorsal surface of each mice using a scalpel blade or scissors. Two to four subcutaneous pockets were created by blunt dissection per mouse and a single transplant was placed into each pocket. The surgical wound was closed with resorbable 4 or 5-0 chromic gut sutures (Ethicon, Somerville, NJ). The animals were sacrificed with carbon dioxide asphyxiation followed with cervical dislocation prior to the scaffold retrieval. The samples were fixed in 4% phosphate buffered paraformaldehyde for 24 hours and transferred to 70% ethanol before performing microcomputed tomographic, histological and immunohistochemical

analyses. In some samples, the scaffolds were divided into 2 pieces with a scalpel and were frozen in liquid nitrogen and stored at -80°C for RNA isolation and quantitative real-time PCR analysis.

Microcomputed tomography (μCT)

The images of mineralized structures from the collagen scaffolds retrieved from p65$^{fl/+}$and p65$^{fl/+}$Osx-Cre mice were acquired using a desktop microtomographic imaging system (Skyscan 1074HR, Aartselaar, Belgium) at a resolution of 20.5 μm/pixel. Standardized scanning and image reconstruction settings were used. At least three specimens from each condition at each time point were imaged and assessed for the volume and quality of newly formed mineralized tissues. The measured parameters included bone mineral density (BMD) and bone volume fraction (BV/TV). Gray scale setting at 52 to 255 was used for bone volume and bone mineral density calculation. Hydroxyapatite phantoms (250 mg/cc and 750 mg/cc; CIRS, Inc., Norfold, VA) were used as references for bone mineral density (BMD) calculation.

Histological evaluation

After the scaffold samples were fixed in 4% phosphate-buffered paraformaldehyde and transferred into 70% ethanol, the samples were decalcified with 0.2 mM Ethylenediamine-tetraacetic acid (EDTA, Sigma) at pH 7.4 in deionized water then dehydrated in descending ethanol series from 70%, 95% to 100% before a paraffin embedding procedure. The tissue slides were obtained by serial sectioned cutting at 5-μm thickness. The sections were processed in a standard histological procedure and stained with hematoxylin-eosin for morphological examination or stained for other analyses. At least 6 representative sections from each implant were

evaluated and photographed under a light microscope (Nikon Eclipse50i, Nikon, Melville, NY). The slides from similar sections level were compared histological findings between the target and the control groups.

RNA preparation and Quantitative real-time PCR

In order to determine the mRNA expression patterns of osteogenic differentiation markers and NFκB pathway-related markers, the collagen scaffolds loaded with rhBMP2 or deionized water were harvested from the animals 2 weeks after subcutaneous implantation. The scaffolds were cut in half and total RNA was extracted from tissues formed in one-half piece of the scaffolds using TriZol reagent (Invitrogen, Carlsbad, CA) according to manufacturer protocol. Five hundred ng of the total RNA was reversed transcribed into first strand cDNA using the SuperScriptIII first-strand synthesis supermix for qRT-PCR kit (Invitrogen) in a 20-µl reaction volume. Quantitative real-time PCR was performed using the sequence specific primers and probes for osteogenic markers including *Runx2* (Mm00501578_ml), *Osx* (Mm00504574_ml), *Alp* (Mm00475831_ml), *Bsp* (Mm00492555_ml), type I collagen alpha 1 chain (*Col1A1*; Mm00801666_g1), and osteocalcin (*Bglap1*; Mm_03413826_mH). The NFκB related molecules included *p65* (Mm00501346_m1), *nfkb1* (Mm00476361_m1), and *Iκbα* (Mm00477798_m1). All reactions were amplified using Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA) in ABI prism 7500 system. Assays were performed in duplicate and the results were normalized to rodent *Gapdh* expression (ABI assay No. 4308313). The fold changes were calculated using the values obtained from the $p65^{fl/+}$ animal group in the deionized water-loaded condition in as a calibrator then determined by means of

70

$2^{-\Delta\Delta}$Ct method (Livak and Schmittgen, 2001). For the comparison among groups in the rhBMP2-induced condition, the values from the p65$^{fl/+}$ group with loaded rhBMP2 were used as a calibrator. Statistical analyses were performed using an independent T-test.

Immunohistochemical staining

The tissue sections from the paraffin embedded samples were processed by deparaffinization with xylene substitute (Hemo-D, Fisher) and rehydration using gradient ethanol. The antigens were retrieved with 10 mM sodium citrate buffer at 70°C for 20 minutes. Endogenous hydrogen peroxidases in the tissues were quenched with 3% hydrogen peroxide in methanol followed by three rinses with phosphate buffered saline. The sections were incubated with 2.5% goat serum (Vector lab) diluted in 0.1% bovine serum albumin for 30 minutes at room temperature for nonspecific blocking. The sections were then blotted overnight at 4^{0}C with primary antibodies. After washed three times in PBS, the slides were incubated with a biotinylated secondary antibody for 45 minutes at room temperature. The antibody reaction was amplified and stained using an immunoperoxidase system by the incubation of the slides with the RTU ABC Elite reagents (Vector Laboratories, Burlingame, CA). The antibody complexes were visualized with a perioxidase substrate from DAB substrate kit (Vector Laboratories). The sections were counterstained with hematoxylin and processed for gradient dehydration before mounted with permount medium. The primary antibodies included a rat monoclonal anti-F4/80 (Abcam, catalogue number ab6640) antibody at 1:200 dilution and a rabbit anti-bone sialoprotein antibody (a kind gift from Dr. Larry Fisher, NIH) at 1: 400

dilution. The secondary antibodies were goat anti-rat and goat anti-rabbit IgG antibodies (Vector Laboratories). The secondary antibodies were used at dilution of 1:200 and 1:800 respectively. Control sections were incubated with a nonspecific rat or rabbit IgG instead of the primary antibodies. Photographs of histological stained sections were captured under a Nikon Eclipse 50i microscope.

Assessment of NFκB activation during rhBMP2-induced bone formation

Enhanced green fluorescent protein (eGFP) representing NFκB activation was observed in the tissue sections from BMP2-loaded scaffolds from the *cis*-NFκB GFP mice. The scaffolds were retrieved from the *cis*-NFκB GFP mice after 3 days, 7 days, and 14 days postimplantation. The scaffold samples were fixed in 4% paraformaldehyde for 10-24 hours then transferred to 70% ethanol before further processes. The samples were decalcified by 0.2 mM EDTA in deionized water, washed with tap water and transferred into 30% sucrose in phosphate buffered saline overnight. The samples were embedded in Tissue Tek® optimal cutting temperature compound (Sakura Finetek, Torrance, CA) and 5-μM sections were cut on a cryostat. After rinsing the sections with PBS for 5 minutes 2 times, the slide sections were soaked in 1 mM MgCl2 in PBS for 30 minutes and washed again with PBS for 5 minutes. The sections were mounted in glycerol-based mounting medium before a microscopic analysis (Jiang et al., 2005). The eGFP expression was observed and images were captured using an inverted fluorescent microscope (Nikon Eclipse50i, Nikon, Melville, NY). After the fluorescent evaluation, some slide sections were counterstained with hematoxylin and photographed under the same microscope.

Statistical analysis

The data were presented as mean with standard deviation. Statistical significant differences were determined using an independent Student's t-test or non-parametric test. Statistical significance was defined at p-value less than 0.05.

Result

Effect of p65 haploinsufficiency in bone regeneration potential

To evaluate changes in the bone formation potential as a result of *p65* haploinsufficiency, the heterotopic bone formation model was employed using BMP2-mediated osteoinduction in this model. The capabilities of the target $p65^{fl/+}$Osx-Cre and the control $p65^{fl/+}$ mice to form mineralized tissues were assessed at 2 weeks and 5 weeks postimplantation. MicroCT analysis of the transplants at both time points revealed no mineralization in the scaffolds without rhBMP2. The samples with rhBMP2 demonstrated small amount of calcified tissues at two weeks and more extensive mineralization at five weeks in both $p65^{fl/+}$ and $p65^{fl/+}$Osx-Cre groups. The mineralization was observed mainly at peripheral regions of the scaffolds. Although mineralized matrices were present in the rhBMP2-loaded transplants from the $p65^{fl/+}$Osx-Cre group, quantification by microCT analysis indicated markedly lower mineralized tissue volume than those of controls (p<0.05) as demonstrated by bone volume per tissue volume ratio (Table 2). However, the mineralized tissue densities values of the mineralized scaffolds were comparable between the target $p65^{fl/+}$Osx-Cre and the controls.

Hematoxylin and eosin staining examination confirmed that the microstructures of the scaffolds delivering rhBMP2 from both target and control

animals contained newly formed mineralized tissues when rhBMP2 was applied. In contrary, the negative control scaffolds had only fibrous tissue ingrowth at both two- and five-week time points. Loose fibrous connective tissues with granulocytes were observed surrounding the scaffolds from both conditions. At 2 weeks, only the rhBMP2-loaded scaffolds from the control p65$^{fl/+}$ mice displayed matrices representing woven bone structure at the periphery of the scaffolds. The rhBMP2-loaded scaffolds from the target p65$^{fl/+}$Osx-cre mice showed less amount of the bone like structure with small nodules of mineralization (Fig.9). At 5 weeks in intact mice, the scaffolds containing rhBMP2 developed more bone like tissues in the presence of bone marrow compartments along with partial degradation of the collagen scaffolds. The control group established intact cortical bone and trabecular structures with a large number of hematopoietic cells infiltrated into bone marrow region. The mineralized scaffolds from the target group showed less bony structures but displayed comparable bone marrow structure to the controls. Unorganized mineralized regions were observed in certain areas of the p65$^{fl/+}$Osx-cre scaffolds. The residual scaffold materials remained visible in larger sizes than those from the controls (Fig.12).

Molecular characterization at gene expression levels was performed to determine the effect of p65 haploinsufficiency during the bone regeneration process from the transplanted scaffolds after 2-week implantation. The osteoblast differentiation and inflammatory activities were evaluated by quantifying the mRNA abundance of selected markers using quantitative real-time PCR (Fig. 10). The expression levels of all selected osteoblastic markers from all groups increased significantly upon rhBMP2 stimulation except *Col1a1*. The expression levels of these

markers were comparable between two control groups, the $p65^{fl/+}$ and the Osx-Cre groups. *Runx2* mRNA, a BMP2 target gene responsible for osteochondrogenic differentiation (Nakashima et al., 2002), demonstrated upregulation more than two times in response to the BMP2 osteogenic signal with comparable levels between the control $p65^{fl/+}$ and the target $p65^{fl/+}$OsxCre groups. In contrast, the expression levels of *Osx*, an osteogenic lineage specific marker, was significantly lower in the target group as compared with those in the controls. The levels of *Alp* and *Bsp* mRNA abundance were consistent with the *Osx* result as both genes are downstream targets of the *Osx* transcription factor (Matsubara et al., 2008). Change in *Ocn* mRNA abundance, a late maker of osteoblasts, confirmed the negative effect of *p65* haploinsufficiency on osteoblast differentiation during the regeneration process with markedly lower expression level in the $p65^{fl/+}$OsxCre group. These data indicated reduced osteoblast differentiation from cells migrating into the rhBMP2-loaded scaffolds of the $p65^{fl/+}$OsxCre mice. This result resembles the previous result in the phenotype examination of the calvarial cells from $p65^{fl/+}$OsxCre mice. However, the *Col1A1* mRNA demonstrated higher, although not statistical different, expression level in the target group. This may be relevant to the fibrous encapsulation at the scaffolds of the $p65^{fl/+}$OsxCre group.

Previous studies have suggested that the inflammatory response has an important influence in bone repair and bone regeneration therapies (Mountziaris and Mikos, 2008; Mountziaris et al., 2011). Alteration of NFκB activity by targeting NFκB subunits expression or modulating IκB/NFκB complexes leads to change in inflammatory reaction via the effect on transcription control of cellular responses

(Shih et al., 2011). Therefore the mRNA abundance of *p65*, *nfkb1* which encodes p105/p50, and *Iκbα* was measured in cells from the harvested scaffolds as presented in Figure 11. The mRNA levels of these NFκB related genes, specifically *Iκbα*, in the control group revealed suppression following rhBMP2 stimulation after 2 weeks whereas the expression levels of these genes elevated in the target group, especially the *p65* level, which was higher than the control at the non-stimulated condition. In rhBMP2-induced conditions, *Iκbα* mRNA levels from the p65$^{fl/+}$OsxCre group showed significantly higher levels than the p65$^{fl/+}$ control group, implicating difference of NFκB activity as a consequence of *p65* haploinsufficiency.

To further assess the profile of newly formed tissues in the osteoinduced scaffolds, immunohistochemistry was used to determine the population of active osteogenic cells and mature macrophages which were responsible for bone replacement in the scaffold material. Bsp is an essential molecule for active osteoblasts (Ganss et al., 1999). F4/80 serves as a marker for functioned macrophages but not osteoclasts (Chang et al., 2008). Therefore immunostaining was performed using antibodies against Bsp and F4/80 in this study. Haploinsufficiency of *p65* in osteoprogenitors revealed retarded bone formation in the scaffolds with fewer Bsp positive cells and less F4/80 positive macrophages which played a role in scaffold matrices degradation. In contrast, the control group illustrated several areas with osteogenic cells and large population of macrophages surrounding the collagen scaffolds (Fig. 13 and 14).

cis-NFκB EGFP expression exhibits temporal and spatial pattern of NFκB activation during bone formation

To define the association between NFκB activitiy and bone formation *in vivo*, the *cis*-NFκB EGFP reporter mice were used as a tool to monitor the kinetics of NFκB activation following BMP2-mediated osteoinduction. Frozen sections of the scaffolds disected from the *cis*-NFκB EGFP reporter mice were examined for eGFP expression under a fluorescent microscope (Fig. 15). The eGFP emission representing NFκB activation exhibited the strongest intensity in the TNFα-loaded group at day 3 then decreased gradually from day 7 to day 14. The deionized water-loaded group which served as a negative control showed slightly expression of eGFP only in day 3, reflecting an inflammatory response from a surgical procedure. The addition of rhBMP2 resulted in different eGFP expression patterns with low intensity at day 3. Although sparsely, increased *cis*-NFκB EGFP positive cells were observed at day 7 and day 14 respectively. The same sections from rhBMP2 samples at day 14 were counterstained by hematoxylin to localize morphological structures in the scaffolds. The *cis*-NFκB EGFP positive cells were visualized in the area next to newly formed bone tissues. The result from this experiment implicated NFκB involvement during BMP2-mediated osteogenesis.

Table3 μCT analysis of rhBMP2-induced scaffolds from p65$^{fl/+}$ and p65$^{fl/+}$Osx-Cre mice

Measurement	Week 2		Week 5	
	p65$^{fl/+}$	p65$^{fl/+}$Osx-Cre	p65$^{fl/+}$	p65$^{fl/+}$Osx-Cre
Percent bone volume	11.726±3.36	1.443±0.68*	16.955±5.54	2.999±1.71*
Bone mineral density (g/cm³)	0.087±0.030	0.053±0.021	0.205±0.023	0.192±0.035

*p<0.05 versus p65fl/+, n = 4-5 per group

Figure 9 Hematoxylin and eosin staining of sections from 2-week implanted scaffolds. A) Paraffin-embedded sections of representative implanted scaffolds harvested from p65$^{fl/+}$ and p65$^{fl/+}$Osx-Cre mice. Upper panel displayed different morphology of the scaffolds from each condition. Scale bars indicate 200 μM. Lower panel demonstrated magnification of the structure of the peripheral region of the scaffolds. The scaffolds without rhBMP2 contained surrounding fibrous tissues whereas those with rhBMP2 displayed bone-like structure with less amount in the p65$^{fl/+}$Osx-Cre group. Scale bars indicate 10 μM. N=3 animals per group per condition B) Representative sections of the rhBMP2 loaded scaffolds retrieved from the Osx-Cre mice. Scale bars indicate 10 μM. N=2 animals.
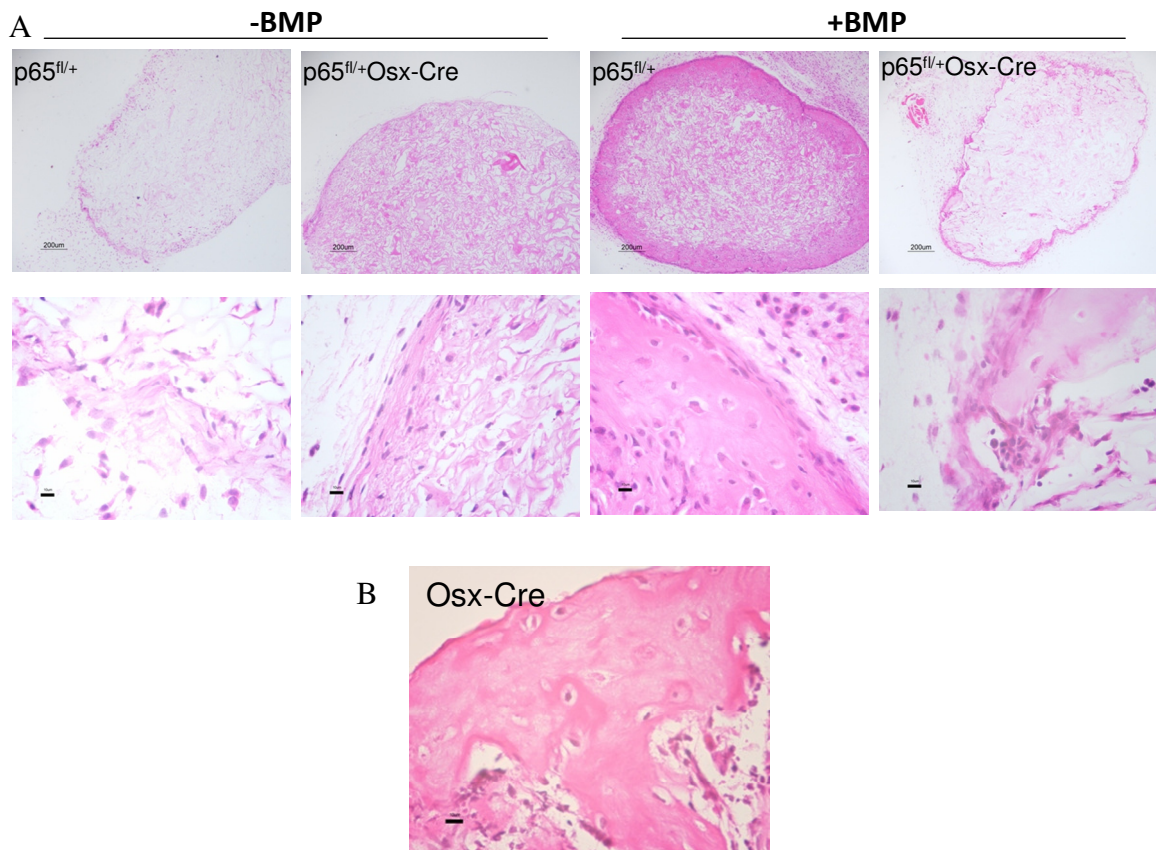
Figure 10 mRNA expression profiles of osteoblastic markers. The expression levels of osteoblastic markers from the dissected scaffolds demonstrated reduced osteogenic potential in p65$^{fl/+}$Osx-Cre mice. Relative expression levels of osteoblastic gene markers after 14 days post-implantation in p65$^{fl/+}$ and p65$^{fl/+}$Osx-Cre mice were assessed by realtime PCR for *Runx2, Osx, Alp, Bsp, Col1a1,* and *Ocn*. n=4 animals per group/condition. Data are shown as mean $\pm$ SD. *, p<0.05 versus p65$^{fl/+}$ in condition without BMP2. #, p<0.05 represents difference between the p65$^{fl/+}$Osx-Cre versus the p65$^{fl/+}$ groups in BMP2 condition. The experiment was performed in duplicate.
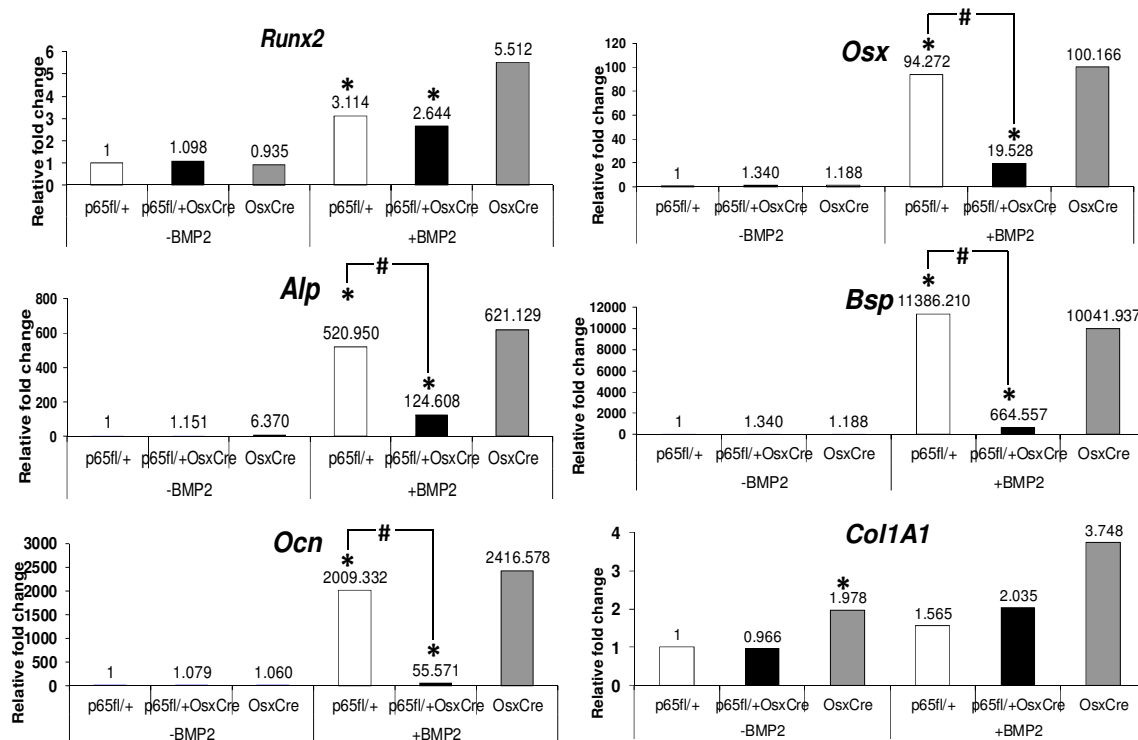
Figure 11 mRNA expression profiles of NFκB pathway related molecules. Relative expression levels of NFκB related molecules from the scaffolds after 14 days post-implantation in p65^{fl/+} and p65^{fl/+}Osx-Cre mice were assessed by realtime PCR for *p65, nfkb1,* and *Iκbα* n=4 animals per group/condition. Data are shown as mean $\pm$ SD. *, $p < 0.05$ versus p65^{fl/+} in condition without BMP2. #, $p < 0.05$ represents difference between p65^{fl/+}Osx-Cre versus p65^{fl/+} group in BMP2 condition. The experiment was performed in duplicate.
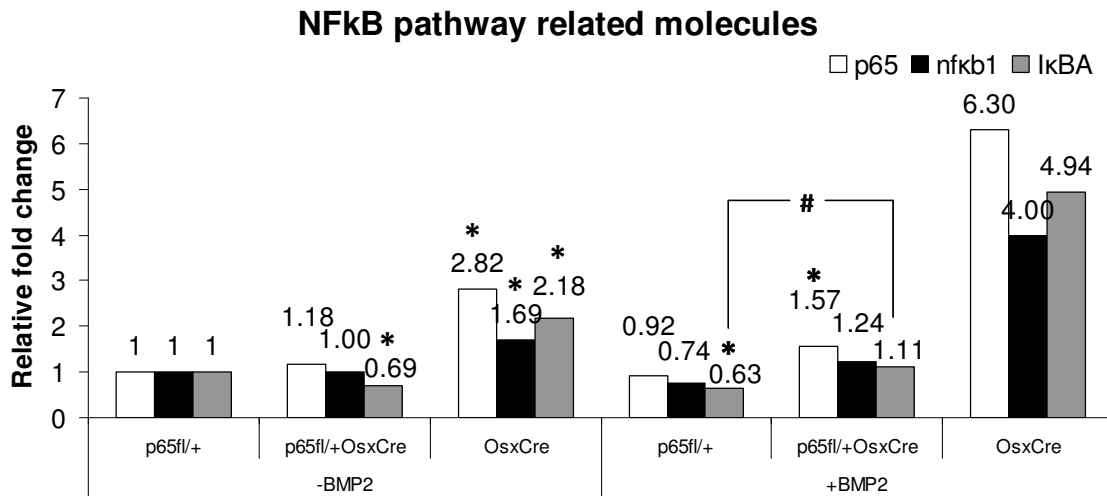

NFkB pathway related molecules

Figure 12 Hematoxylin and eosin staining of sections from 5-week implanted scaffolds. A), B) Paraffin embedded sections of the representative rhBMP2-loaded scaffolds harvested from two different p65$^{fl/+}$ and C), D) p65$^{fl/+}$Osx-Cre mice. Upper panel displayed overall morphology of the scaffolds from each condition. Scale bars indicate 200 μM. Middle panel displayed structures at the peripheral regions of the scaffolds. Scale bars indicate 100 μM. Lower panel demonstrated magnification of the structure at mineralized-bone marrow junctions inside the scaffolds. Scale bars indicate 10 μM. N=4 animals per group per condition.
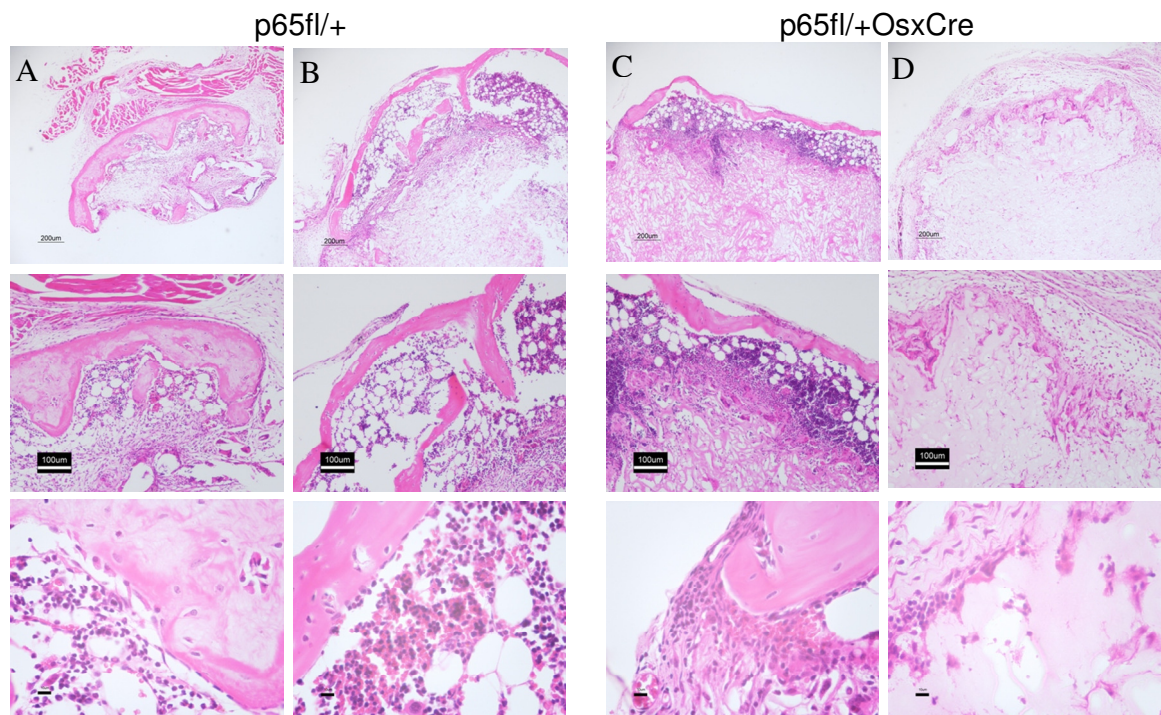
Figure 13 Immunostaining of Bsp in 5-week mineralized scaffolds. A) Bsp immunohistochemical staining of paraffin embedded sections of the representative scaffolds harvested from $p65^{fl/+}$ and B) $p65^{fl/+}$Osx-Cre mice. C) IgG control for Bsp immunohistochemical staining of sections of the scaffolds from $p65^{fl/+}$ and D) $p65^{fl/+}$Osx-Cre mice. Scale bar indicates 10 μM.

Figure 14 Immunostaining of F4/80 in 5-week mineralized scaffolds. A) F4/80 immunohistochemical staining of paraffin embedded sections of the representative scaffolds harvested from $p65^{fl/+}$ and B) $p65^{fl/+}$Osx-Cre mice. C) IgG control for F4/80 immunohistochemical staining of sections of the scaffolds from $p65^{fl/+}$ and D) $p65^{fl/+}$Osx-Cre mice. Scale bar indicates 200 μM.
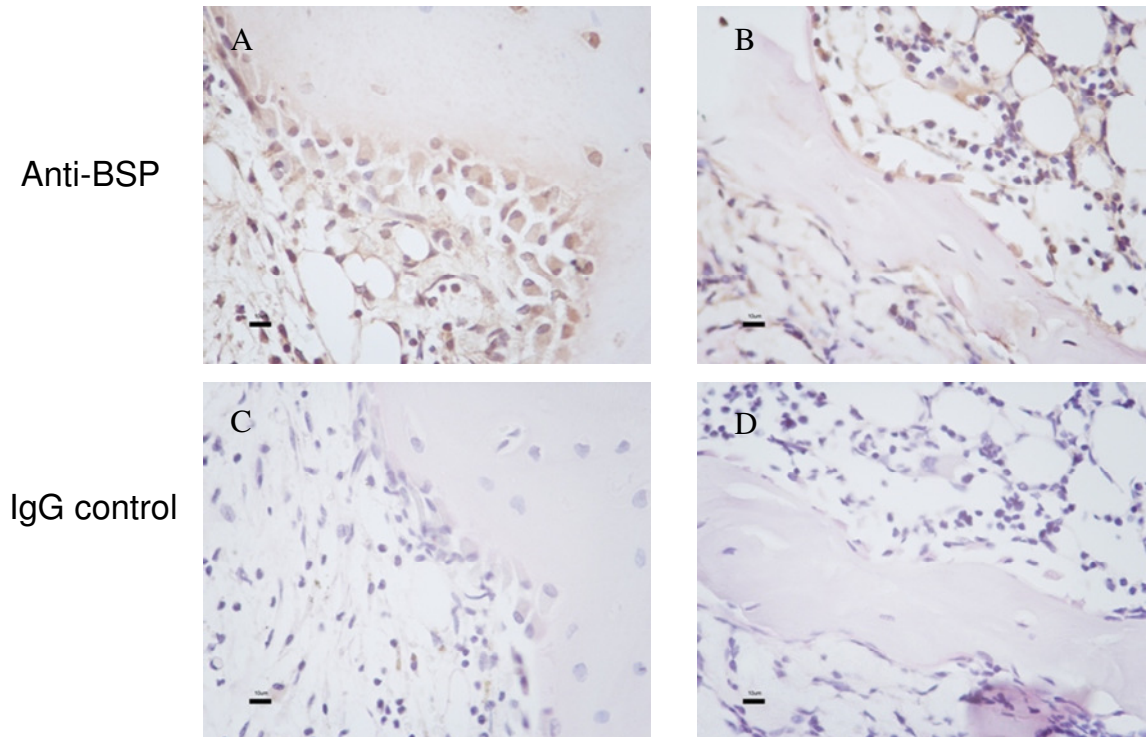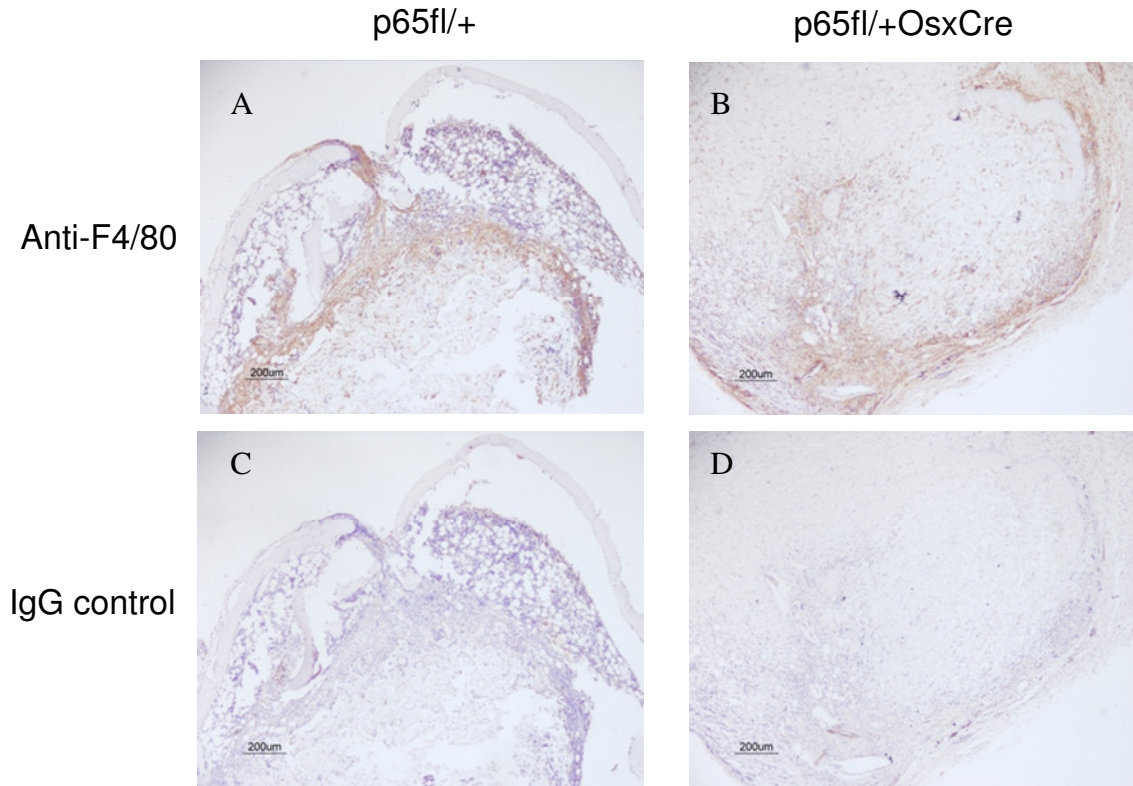

	p65fl/+	p65fl/+OsxCre
Anti-F4/80	A	B
IgG control	C	D

Figure 15 Kinetics of NFκB activation in early stage of bone regeneration.
Enhanced GFP expression was visualized in the sections of harvested scaffolds from *cis*-NFκB EGFP mice. The sections represented the scaffolds pretreated with A) 100 ng of TNFα, B) 1μg of rhBMP2, and C) deionized water from day 3, 7, and 14. Scale bar indicates 200 μM. D) The eGFP positive cells were located in the area adjacent to newly formed bone-like structure in the rhBMP2-loaded scaffolds retrieved at day 14 postimplantation. E) Hematoxylin counterstained of D). Slide sections displayed localization and morphology of bone-like structures in the scaffolds. Scale bars in D) and E) indicate 10 μM. N=3 animals per group.
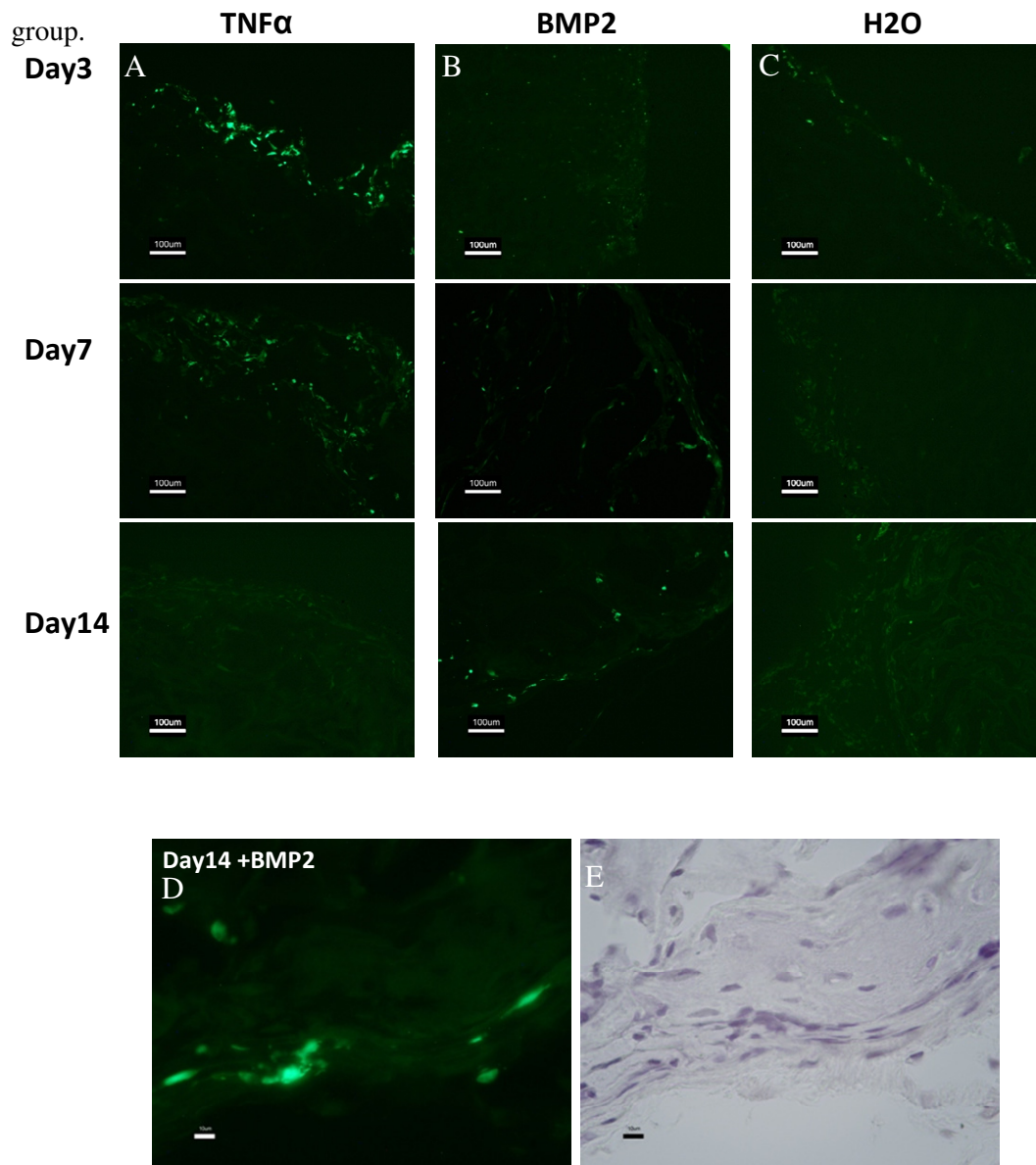
Discussion

NFκB strongly correlates with the initiation and the resolution of diseases of an inflammatory origin due to its profound function in inflammatory gene regulation (Lawrence et al., 2005; Perkins and Gilmore, 2006). Modulation of NFκB activity becomes an attractive target for defining pathogenesis and therapeutic strategies in several inflammatory diseases (Karrasch et al., 2007; Lippert et al., 2009; Steinbrecher et al., 2008). In accordance with the pivotal roles of NFκB in inflammatory regulation, the present study supports the importance of initial inflammatory phase in bone repair/regeneration. *In vitro* evidence demonstrates a stimulatory effect of TNFα in hMSCs as related to osteogenesis through a p65-mediated NFκB pathway (Bocker et al., 2008; Crisostomo et al., 2008; Hess et al., 2009). Absence of *Tnfα* signaling impairs bone repair/regeneration potential in a murine fracture model (Gerstenfeld et al., 2001; Gerstenfeld et al., 2003). Mice lacking downstream NFκB targets such as *Cox2* and *Il6* show delayed bone healing resembling the outcome in TNFα recepter *p55/p75* target deletion mice (Appleby et al., 1994; Libermann and Baltimore, 1990; Simon et al., 2002; Yang et al., 2007; Zhang et al., 2002). Pharmacological COX2 inhibition for 5-7 days immediately after bone fracture interferes with the inflammatory phase of the healing process which leads to disruption of newly formed bone as shown by less bone mineral density or high prevalence of osseous nonunion (Dimmen et al., 2009). Conversely, stimulation of inflammation during early bone healing phase enhanced the bone regeneration outcome. Local administration of TNFα at the beginning stage of bone injury accelerates mineralization after 4 weeks (Glass et al., 2011). Similar results were observed in murine bone fracture healing with short

term exposure of IL1β, or IL6 immediately after bone injury (Ding et al., 2009; Rozen et al., 2007). The role of in NFκB in ectopic bone formation demonstrates a direct effect of NFκB signaling in osteoblastic cells recruited to scaffolds by rhBMP2 in tissue engineering.

The aim of this study was to investigate the relationship between NFκB and bone formation occurring in rhBMP2-mediated tissue engineering. Therapy using rhBMP2 has been considered as an effective approach to promote bone repair and regeneration according to its capability to promote cell migration, differentiation, and bone formation (Kim et al., 2005; Kimura et al., 2010). Despite the transcriptional role of NFκB in BMP2 during bone development was identified (Feng et al., 2003), the effect of NFκB in exogenous BMP2-induced bone regeneration remains inconclusive. The results of phenotype characterization of p65$^{fl/+}$Osx-Cre mice as described in chapter 2 indicate that NFκB p65 modulates bone development through osteoblast regulation in perspective of differentiation control and protective role in apoptosis. In this study, bone regeneration potential in p65$^{fl/+}$Osx-Cre mice was evaluated to define the role of NFκB in an early stage of bone formation in BMP2 delivery system.

The major findings in this study indicated that NFκB p65 regulates BMP2-mediated bone formation by direct modulation of osteoblast differentiation *in vivo*. Osteogenic response in the control mice p65$^{fl/+}$ with normal NFκB activity appeared in a typical pattern of mesenchymal cell infiltration, differentiation, new bone formation in the first 2 weeks then followed with remodeling of the scaffolds and incorporation of hemotopoietic bone marrow at 5 weeks (Fig. 9 and 12). Reduced amount of newly formed bone from H&E staining was observed in the mineralized scaffolds dissected

from p65$^{fl/+}$Osx-Cre mice as early of 2 weeks following BMP2 delivery and remained in the later remodeling stage at 5 weeks. In addition to the histological findings, the microCT assessment confirmed reduced bone formation in p65$^{fl/+}$Osx-Cre mice with apparent smaller mineralized tissue volume in the BMP2-loaded scaffolds. The involvement of NFκB during bone formation was delineated with the *cis*-NFκB GFP mice which are a valid model for monitoring *in vivo* NFκB activation (Magness et al., 2004). Induction of *cis*-NFκB GFP transgene represented dynamic of NFκB activity in response to BMP2 stimulation. NFκB activation appeared in later stages at day 7 and day 14 which concurred with bone formation process (Fig. 15). Additionally, cells with activated NFκB were localized next to newly formed bone tissues, implicating the cells responsible for bone formation. Immunohistochemistry in p65$^{fl/+}$ and p65$^{fl/+}$Osx-Cre samples indicated that prominent population of cells located next to bone tissue were Bsp positive which corresponded to active osteblastic cells (Hunter and Goldberg, 1994). These findings exemplified the correlation of NFκB in osteogenic cells.

The molecular mechanism of BMP2-induced osteoblast differentiation involves sequential activation of Runx2 and Osx transcription factors which play a role as key stimulators of molecules essential for bone tissue formation Col1a1, Alp, Bsp, and Ocn (Balint et al., 2003; Chen et al., 1997; Matsubara et al., 2008). The change in osteogenic gene expression profiles of the scaffolds from p65$^{fl/+}$Osx-Cre mice in the present study suggested that the mechanism in which NFκB modulated osteogenesis in a BMP2-mediated regeneration process contributed to its regulatory roles on osteoblast differentiation. Upregulation of *Runx2* mRNA, an essential

osteochondrogenic transcription factor (Komori et al., 1997) occurred in response to rhBMP2 at comparable levels between target and control groups whereas the expression levels of other downstream specific osteogenic markers *Osx, Bsp, Alp*, and *Ocn* significantly decreased in p65$^{fl/+}$Osx-Cre mice. NFκB may have a selective role in control of osteogenesis; *Runx2* levels were not affected in a dramatic way by the targeted deletion of p65. Although *Osx* expression has been shown to be highly relevant to Runx2 (Nakashima et al., 2002), the transcriptional activation of *Osx* is under distinct regulation. BMP2-mediated *Osx* expression is induced by two distinct pathways through Runx2 and Msx2 regulation. Runx2 positively influences *Osx* mRNA transcription through Smad1/4 signaling upon BMP2 osteinduction. *Runx2* deficient mesenchymal cells reveal the role of Msx2 in Osx with elevated *Osx* expression upon *Msx2* overexpression. Knockdown of *Msx2* leads to reversion of the outcome with suppression of the *Osx* level (Matsubara et al., 2008).

Another explanation is based on NFκB transcriptional role in *Osx* specifically to p65 mediated pathway. The existence of NFκB binding site in Osx promoter was demonstrated by Osx promoter-mediated luciferase expression by p65 and p65/p50 constructs (Lu et al., 2006). Reduced level of *Osx* derived from the mineralized scaffolds of the p65$^{fl/+}$Osx-Cre mice may represent as a consequence of lower NFκB p65 level in osteoblasts. The consistent reduction of *Alp*, *Bsp*, and *Ocn* mRNA abundance (Fig. 10) indicated downstream effect of lower *Osx* expression on osteoblast phenotypes since these osteogenic markers are regulated by the presence of the Osx (Matsubara et al., 2008).

The apparent change of *Bsp* gene expression may be due to a combination of

downstream effect from Osx and direct effect of NFκB on Bsp transcription. The promoter region of *Bsp* gene contains one κB binding site close to TATA box sequence which plays important role in Bsp transcription (Sodek et al., 1996). To better understand the role of NFκB in controlling mesenchymal cell differentiation during bone regeneration, complete gene profiles remain to be clarified.

Comparable osteogenic gene expression levels of the p65$^{fl/+}$ and the Osx-Cre mice confirmed that perturbation of osteoblast development was not an indirect consequence of the Cre-recombinase existence. This result indicated reduced osteoblast differentiation from cells migrating into the scaffolds of the conditional osteoblast-specific *p65* haploinsufficient mice. These data demonstrated similar effect on osteogenesis as shown in the *in vitro* phenotype in chapter 2, illustrating that p65 mediated NFκB pathway is critical in osteogenesis in both development and regeneration processes.

Previous evidence has indicated the significance of balance in basal levels of each NFκB subunit on NFκB activity (Hoffmann et al., 2003; Steinbrecher et al., 2008). The function of p65 mediated NFκB is tightly regulated through IκB/NFκB complexes (Hoffmann et al., 2002). To better understand the compensatory response of NFκB activity in *p65* haploinsufficiency in osteoblasts, this study therefore assessed the alteration of expression levels of NFκB related molecules including *p65, nfkb1*, and *Iκbα*. Despite the *p65* and *nfkb1* mRNA levels in p65$^{fl/+}$Osx-Cre mice were not significantly different from the controls, increased *Iκbα* mRNA level was noted in p65$^{fl/+}$Osx-Cre mice during bone formation (Fig. 11). The major function of *Iκbα* is to inhibit NFκB activation by coordination with other members of IκB proteins to

sequester NFκB in the cytoplasm (Hoffmann et al., 2002; Tergaonkar et al., 2005).

It is plausible that different cell population inhabited in the scaffolds of the control and target groups. The rhBMP2-loaded scaffolds from p65$^{fl/+}$Osx-Cre group may have fewer osteogenic cells but more of other cells types which contain fully functioned NFkB activity. Additionally, less osteogenic cells in the target mice may lead to alteration in wound healing process. Data from immunostaining of F/40 positive cells suggested change in healing process with fewer amount of F/40, marked as macrophages, in the target mice together with larger remaining scaffold matrices (Fig. 14). The delayed tissue/scaffold remodeling suggested that osteoblasts may play a role in recruiting macrophages in the bone regeneration process. Taken together, *p65* haploinsufficiency in osteoblast resulted in delayed bone regeneration at both differentiation and remodeling stages. However, the mechanism in which NFκB affects osteoblast functions during bone regeneration requires further examination.

In conclusion, the present study demonstrates the direct effect of NFκB p65 in tissue-engineering-based bone formation. Lack of one *p65* single allele significantly retarded *in vivo* bone formation in BMP2-delivery system in regard to osteoblast differentiation control. Kinetics of NFκB activation during bone regeneration indicated the interplay between inflammatory response and process of new tissues formation. Together this study provides basic knowledge concerning inflammatory regulation that can impact clinical outcome of bone tissue engineering.

Acknowledgement

I would like to thank Dr. Christian Jobin (Department of Medicine, UNC) for

References (Chapter 3)

Alblowi J, Kayal RA, Siqueira M, McKenzie E, Krothapalli N, McLean J, et al. (2009). High levels of tumor necrosis factor-alpha contribute to accelerated loss of cartilage in diabetic fracture healing. *Am J Pathol* 175(4):1574-85.

Appleby SB, Ristimaki A, Neilson K, Narko K, Hla T (1994). Structure of the human cyclo-oxygenase-2 gene. *Biochem J* 302 (Pt 3)(723-7.

Balint E, Lapointe D, Drissi H, van der Meijden C, Young DW, van Wijnen AJ, et al. (2003). Phenotype discovery by gene expression profiling: mapping of biological processes linked to BMP-2-mediated osteoblast differentiation. *J Cell Biochem* 89(2):401-26.

Bocker W, Docheva D, Prall WC, Egea V, Pappou E, Rossmann O, et al. (2008). IKK-2 is required for TNF-alpha-induced invasion and proliferation of human mesenchymal stem cells. *J Mol Med (Berl)* 86(10):1183-92.

Caparbo VF, Prada F, Silva CA, Regio PL, Pereira RM (2009). Serum from children with polyarticular juvenile idiopathic arthritis (pJIA) inhibits differentiation, mineralization and may increase apoptosis of human osteoblasts "in vitro". *Clin Rheumatol* 28(1):71-7.

Chang MK, Raggatt LJ, Alexander KA, Kuliwaba JS, Fazzalari NL, Schroder K, et al. (2008). Osteal tissue macrophages are intercalated throughout human and mouse bone lining tissues and regulate osteoblast function in vitro and in vivo. *J Immunol* 181(2):1232-44.

Chen D, Harris MA, Rossini G, Dunstan CR, Dallas SL, Feng JQ, et al. (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. *Calcif Tissue Int* 60(3):283-90.

Chen NX, Geist DJ, Genetos DC, Pavalko FM, Duncan RL (2003). Fluid shear-induced NFkappaB translocation in osteoblasts is mediated by intracellular calcium release. *Bone* 33(3):399-410.

Choi SH, Kim CK, Cho KS, Huh JS, Sorensen RG, Wozney JM, et al. (2002). Effect of recombinant human bone morphogenetic protein-2/absorbable collagen sponge (rhBMP-2/ACS) on healing in 3-wall intrabony defects in dogs. *J Periodontol* 73(1):63-72.

Crisostomo PR, Wang Y, Markel TA, Wang M, Lahm T, Meldrum DR (2008).

Human mesenchymal stem cells stimulated by TNF-alpha, LPS, or hypoxia produce growth factors by an NF kappa B- but not JNK-dependent mechanism. *Am J Physiol Cell Physiol* 294(3)*:*C675-82.

Dimmen S, Nordsletten L, Madsen JE (2009). Parecoxib and indomethacin delay early fracture healing: a study in rats. *Clin Orthop Relat Res* 467(8)*:*1992-9.

Ding J, Ghali O, Lencel P, Broux O, Chauveau C, Devedjian JC, et al. (2009). TNF-alpha and IL-1beta inhibit RUNX2 and collagen expression but increase alkaline phosphatase activity and mineralization in human mesenchymal stem cells. *Life Sci* 84(15-16)*:*499-504.

Feng JQ, Xing L, Zhang JH, Zhao M, Horn D, Chan J, et al. (2003). NF-kappaB specifically activates BMP-2 gene expression in growth plate chondrocytes in vivo and in a chondrocyte cell line in vitro. *J Biol Chem* 278(31)*:*29130-5.

Fukuno N, Matsui H, Kanda Y, Suzuki O, Matsumoto K, Sasaki K, et al. (2011) TGF-beta-activated kinase 1 mediates mechanical stress-induced IL-6 expression in osteoblasts. *Biochem Biophys Res Commun* 408(2)*:*202-7.

Ganss B, Kim RH, Sodek J (1999). Bone sialoprotein. *Crit Rev Oral Biol Med* 10(1)*:*79-98.

Gerstenfeld LC, Cho TJ, Kon T, Aizawa T, Cruceta J, Graves BD, et al. (2001). Impaired intramembranous bone formation during bone repair in the absence of tumor necrosis factor-alpha signaling. *Cells Tissues Organs* 169(3)*:*285-94.

Gerstenfeld LC, Cho TJ, Kon T, Aizawa T, Tsay A, Fitch J, et al. (2003). Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption. *J Bone Miner Res* 18(9)*:*1584-92.

Glass GE, Chan JK, Freidin A, Feldmann M, Horwood NJ, Nanchahal J (2011). TNF-alpha promotes fracture repair by augmenting the recruitment and differentiation of muscle-derived stromal cells. *Proc Natl Acad Sci U S A* 108(4)*:*1585-90.

Hess K, Ushmorov A, Fiedler J, Brenner RE, Wirth T (2009). TNFalpha promotes osteogenic differentiation of human mesenchymal stem cells by triggering the NF-kappaB signaling pathway. *Bone* 45(2)*:*367-76.

Hoffmann A, Levchenko A, Scott ML, Baltimore D (2002). The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. *Science* 298(5596)*:*1241-5.

Hoffmann A, Leung TH, Baltimore D (2003). Genetic analysis of NF-kappaB/Rel transcription factors defines functional specificities. *Embo J* 22(20)*:*5530-9.

Hou LT, Liu CM, Liu BY, Chang PC, Chen MH, Ho MH, et al. (2007). Tissue engineering bone formation in novel recombinant human bone morphogenic protein 2-atelocollagen composite scaffolds. *J Periodontol* 78(2)*:*335-43.

Hu WW, Ward BB, Wang Z, Krebsbach PH (2010) Bone regeneration in defects compromised by radiotherapy. *J Dent Res* 89(1)*:*77-81.

Hunter GK, Goldberg HA (1994). Modulation of crystal formation by bone phosphoproteins: role of glutamic acid-rich sequences in the nucleation of hydroxyapatite by bone sialoprotein. *Biochem J* 302 (Pt 1)(175-9.

Imamura R, Konaka K, Matsumoto N, Hasegawa M, Fukui M, Mukaida N, et al. (2004). Fas ligand induces cell-autonomous NF-kappaB activation and interleukin-8 production by a mechanism distinct from that of tumor necrosis factor-alpha. *J Biol Chem* 279(45)*:*46415-23.

Jiang X, Kalajzic Z, Maye P, Braut A, Bellizzi J, Mina M, et al. (2005). Histological analysis of GFP expression in murine bone. *J Histochem Cytochem* 53(5)*:*593-602.

Jung YJ, Isaacs JS, Lee S, Trepel J, Neckers L (2003). IL-1beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. *Faseb J* 17(14)*:*2115-7.

Karrasch T, Kim JS, Muhlbauer M, Magness ST, Jobin C (2007). Gnotobiotic IL-10-/-;NF-kappa B(EGFP) mice reveal the critical role of TLR/NF-kappa B signaling in commensal bacteria-induced colitis. *J Immunol* 178(10)*:*6522-32.

Katavic V, Grcevic D, Lukic IK, Vucenik V, Kovacic N, Kalajzic I, et al. (2003). Non-functional Fas ligand increases the formation of cartilage early in the endochondral bone induction by rhBMP-2. *Life Sci* 74(1)*:*13-28.

Katayama Y, Matsuyama Y, Yoshihara H, Sakai Y, Nakamura H, Imagama S, et al. (2009). Clinical and radiographic outcomes of posterolateral lumbar spine fusion in humans using recombinant human bone morphogenetic protein-2: an average five-year follow-up study. *Int Orthop* 33(4)*:*1061-7.

Kim CS, Kim JI, Kim J, Choi SH, Chai JK, Kim CK, et al. (2005). Ectopic bone formation associated with recombinant human bone morphogenetic proteins-2 using absorbable collagen sponge and beta tricalcium phosphate as carriers. *Biomaterials* 26(15)*:*2501-7.

Kim IS, Lee EN, Cho TH, Song YM, Hwang SJ, Oh JH, et al. (2011) Promising efficacy of Escherichia coli recombinant human bone morphogenetic protein-2 in collagen sponge for ectopic and orthotopic bone formation and comparison with mammalian cell recombinant human bone morphogenetic protein-2. *Tissue Eng Part A* 17(3-4)*:*337-48.

Kimura Y, Miyazaki N, Hayashi N, Otsuru S, Tamai K, Kaneda Y, et al. (2010) Controlled release of bone morphogenetic protein-2 enhances recruitment of osteogenic progenitor cells for de novo generation of bone tissue. *Tissue Eng Part A* 16(4)*:*1263-70.

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, et al. (1997). Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* 89(5)*:*755-64.

Lawrence T, Bebien M, Liu GY, Nizet V, Karin M (2005). IKKalpha limits macrophage NF-kappaB activation and contributes to the resolution of inflammation. *Nature* 434(7037)*:*1138-43.

Li Q, Verma IM (2002). NF-kappaB regulation in the immune system. *Nat Rev Immunol* 2(10)*:*725-34.

Li Y, Backesjo CM, Haldosen LA, Lindgren U (2008). IL-6 receptor expression and IL-6 effects change during osteoblast differentiation. *Cytokine* 43(2)*:*165-73.

Libermann TA, Baltimore D (1990). Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. *Mol Cell Biol* 10(5)*:*2327-34.

Lippert E, Karrasch T, Sun X, Allard B, Herfarth HH, Threadgill D, et al. (2009). Gnotobiotic IL-10; NF-kappaB mice develop rapid and severe colitis following Campylobacter jejuni infection. *PLoS One* 4(10)*:*e7413.

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25(4)*:*402-8.

Lu X, Gilbert L, He X, Rubin J, Nanes MS (2006). Transcriptional regulation of the osterix (Osx, Sp7) promoter by tumor necrosis factor identifies disparate effects of mitogen-activated protein kinase and NF kappa B pathways. *J Biol Chem* 281(10)*:*6297-306.

Magness ST, Jijon H, Van Houten Fisher N, Sharpless NE, Brenner DA, Jobin C (2004). In vivo pattern of lipopolysaccharide and anti-CD3-induced NF-kappa B activation using a novel gene-targeted enhanced GFP reporter gene mouse. *J Immunol* 173(3)*:*1561-70.

Matsubara T, Kida K, Yamaguchi A, Hata K, Ichida F, Meguro H, et al. (2008). BMP2 regulates Osterix through Msx2 and Runx2 during osteoblast differentiation. *J Biol Chem* 283(43)*:*29119-25.

Mountziaris PM, Spicer PP, Kasper FK, Mikos AG (2011) Harnessing and modulating inflammation in strategies for bone regeneration. *Tissue Eng Part B Rev* 17(6)*:*393-402.

Mountziaris PM, Mikos AG (2008). Modulation of the inflammatory response for enhanced bone tissue regeneration. *Tissue Eng Part B Rev* 14(2)*:*179-86.

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, et al. (2002). The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* 108(1)*:*17-29.

Perkins ND, Gilmore TD (2006). Good cop, bad cop: the different faces of NF-kappaB. *Cell Death Differ* 13(5)*:*759-72.

Perkins ND (2007). Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat Rev Mol Cell Biol* 8(1)*:*49-62.

Rozen N, Lewinson D, Bick T, Jacob ZC, Stein H, Soudry M (2007). Fracture repair: modulation of fracture-callus and mechanical properties by sequential application of IL-6 following PTH 1-34 or PTH 28-48. *Bone* 41(3)*:*437-45.

Shih VF, Tsui R, Caldwell A, Hoffmann A A single NFkappaB system for both canonical and non-canonical signaling. *Cell Res* 21(1)*:*86-102.

Simon AM, Manigrasso MB, O'Connor JP (2002). Cyclo-oxygenase 2 function is essential for bone fracture healing. *J Bone Miner Res* 17(6)*:*963-76.

Sodek J, Li JJ, Kim RH, Ogata Y, Yamauchi M (1996). Characterization of the bone sialoprotein (BSP) gene promoter. *Connect Tissue Res* 35(1-4)*:*23-31.

Steinbrecher KA, Harmel-Laws E, Sitcheran R, Baldwin AS (2008). Loss of epithelial RelA results in deregulated intestinal proliferative/apoptotic homeostasis and susceptibility to inflammation. *J Immunol* 180(4)*:*2588-99.

Takayanagi H (2009). Osteoimmunology and the effects of the immune system on bone. *Nat Rev Rheumatol* 5(12)*:*667-76.

Tergaonkar V, Correa RG, Ikawa M, Verma IM (2005). Distinct roles of IkappaB proteins in regulating constitutive NF-kappaB activity. *Nat Cell Biol* 7(9)*:*921-3.

Triplett RG, Nevins M, Marx RE, Spagnoli DB, Oates TW, Moy PK, et al. (2009). Pivotal, randomized, parallel evaluation of recombinant human bone morphogenetic protein-2/absorbable collagen sponge and autogenous bone graft for maxillary sinus floor augmentation. *J Oral Maxillofac Surg* 67(9)*:*1947-60.

Yang X, Ricciardi BF, Hernandez-Soria A, Shi Y, Pleshko Camacho N, Bostrom MP (2007). Callus mineralization and maturation are delayed during fracture healing in interleukin-6 knockout mice. *Bone* 41(6)*:*928-36.

Zhang X, Schwarz EM, Young DA, Puzas JE, Rosier RN, O'Keefe RJ (2002). Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair. *J Clin Invest* 109(11)*:*1405-15.

CHAPTER 4

DISCUSSION

Osteogenesis is a series of complicated bone formation processes that occur during developmental and postnatal stages under multifactorial control of various factors and microenvironment. The physiological maintenance of bone requires bone formation which is established by regulated osteoblast development and function from mesenchymal stem cells. Osteogenesis, the bone formation process, involves segregable cellular phases including proliferation, differentiation, bone matrix synthesis, and mineralization (Neve et al., 2011). Determination of cell fate in osteoblast development is meticulously regulated by extrinsic factors in association with intrinsic responses. Extrinsic regulation of cell fate encompasses components of the microenvironment where a source of osteogenic cells resides including soluble factors, cell-cell communication and cell-extracellular matrix interaction. Intrinsic regulation including transcription factors, transmembrane or intracellular proteins, and chromosomal modification together converge to influence cellular behaviors in response to extracellular microenvironments (Long, 2011).

NFκB provides a comprehensive role in developmental and diseased states as part of intrinsic regulation of cell fate which leads to the transformation of extrinsic factors. In the context of bone pathophysiology, aberrant activation of NFκB induces osteoclast

differentiation and resorption activity through the RANKL dependent pathway (Novack, 2011). NFκB activation also leads to production of inflammatory cytokines such as TNFα, IL1, IL6, IL8 resulting in recruitment of immune cells which in turn secrete these cytokines, thereby promoting persistent NFκB activation (Li and Verma, 2002). Functional roles of NFκB in perspective of bone development are paradoxical to bone pathogenesis in regard to requisite concern on initiation of osseous wound healing and stimulatory effect on mesenchymal stem cell differentiation (Crisostomo et al., 2008; Kon et al., 2001; Mountziaris et al., 2011; Rundle et al., 2006). Ablation of inflammatory mediators that tightly associate with the NFκB pathway, such as TNFα, Cox2, by either genetic or pharmacological manipulation causes bone growth and regeneration retardation due to reduced bone formation capacity (Gerstenfeld et al., 2001; Gerstenfeld et al., 2003; Li et al., 2007; Simon and O'Connor, 2007; Zhang et al., 2002) Enhancing inflammation at the beginning of bone healing by administration of TNFα promotes bone formation at the fracture site (Glass et al., 2011). The promoting effect of TNFα on bone formation has been partially clarified the involvement of NFκB regulation on osteoblast differentiation (Hess et al., 2009).

The aim of this study was to gain more insights into the direct influence of NFκB in osteoblast development which ultimately affected bone homeostasis concerning osteogenesis processes. By employing the genetic manipulating approach, mice with deletion of NFκB p65 single allele in osteoprogenitors, p65^{fl/+}Osx-Cre, have been used to decipher the regulatory role of NFκB on an early stage of osteoblast development during initial skeletal growth and bone regeneration. The association of NFκB and bone development was investigated in the *cis*-NFκB EGFP reporter mice, an *in vivo* model for

NFκB activation analyses (Magness et al., 2004). The bone phenotypes of p65$^{fl/+}$Osx-Cre mice from physiological and BMP2-induced conditions suggested that NFκB is required for both normal skeletal development and bone regeneration. The parallel BMP2 induction experiment in *cis*-NFκB EGFP mice implicated direct association of NFκB signaling in the early stage of bone formation.

Previous studies highlighted the significance of NFκB in endochondral ossification through BMP2-mediated chondrocyte promotion (Feng et al., 2003; Wu et al., 2007). In this study, the results from differentiation assays in Chapter 2 and 3 illustrated that NFκB involves direct regulation of osteoblastic markers expression. Reduced BMP2 mRNA abundance in calvarial cells with p65 haploinsufficiency suggested that BMP2 is also involved in intramembranous bone formation under NFκB regulation. Osteocalcin is a specific marker for mature cells in osteoblastic lineage (Neve et al., 2011). Upregulation of osteocalcin mRNA in the control animals and significant expression reduction of this gene in p65$^{fl/+}$Osx-Cre mice in BMP2-mediated ectopic bone formation clearly indicated that BMP2 directly participates in the NFκB-regulated osteoblast differentiation mechanism. Markedly decreased levels other osteoblastic gene markers, *Osx*, *Alp*, and *Bsp*, in BMP2-induced bone formation verified the critical role of NFκB in early bone regeneration process.

In addition to direct BMP2 and Osx gene regulation (Feng et al., 2003; Lu et al., 2006), a potential role of NFκB in the regulation of other ostegenic transcription factors has been suggested. Previous findings demonstrated that NFκB mediated Msh homeobox homologue 1 (*Msx1*) gene expression which contained three κB binding sites in the promoter region (Bushdid et al., 2001). Msx1 is a homeobox-containing transcription

factor which strongly expressed in developing craniofacial bones. Mutation of *Msx1* causes failure in tooth formation and non-syndromic cleft palate in human and mice (Alappat et al., 2003). *Msx1* deficient mice also display middle ear defects from impaired malleus bones formation (Satokata and Maas, 1994). The diverse functions of NFκB in site-specific bone development need further investigation.

Differences of CFU-F and CFU-OB result from p65$^{fl/+}$Osx-Cre bone marrow cells suggested the relationship between NFκB and stem cell specification. NFκB effect on mesenchymal stem cells differentiation in this study is consistent to previous evidences that demonstrated differential induction effect of NFκB/IKK system on human mesenchymal stem cells (Hess et al., 2009). Mesenchymal stem cells are attractive target for bone regeneration therapy. Mechanisms in which NFκB control mesenchymal cell behaviors should be considered in an effort to better understand the management of stem cell biology for clinical improvement in bone regeneration/repair.

In addition to differential regulation, the protective role of NFκB in apoptosis was reiterated in this study. Profound effects of NFκB p65 in anti-apoptosis have been stated in several studies (Beg et al., 1995; Geisler et al., 2007; Rosenfeld et al., 2000; Steinbrecher et al., 2008). In osteoclasts, p65 provides protective role against apoptosis during osteoclastogenesis (Vaira et al., 2008). Successful osteogenesis requires sufficient number of osteogenic cells especially in bone defects with compromised healing potential. Understanding this function of NFκB may be beneficial to application in stem cell engraftment in bone tissue engineering therapy.

In conclusion, this project has contributed to the understanding of NFκB function in early stages of osteogeneis. Haploinsufficiency of NFκB p65 in osteoprogenitors

impacts bone formation potential at multiple levels of regulation thereby affecting bone

homeostasis.

References (Chapter 4)

Alappat S, Zhang ZY, Chen YP (2003). Msx homeobox gene family and craniofacial development. *Cell Res* 13(6)*:*429-42.

Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D (1995). Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature* 376(6536)*:*167-70.

Bushdid PB, Chen CL, Brantley DM, Yull F, Raghow R, Kerr LD, et al. (2001). NF-kappaB mediates FGF signal regulation of msx-1 expression. *Dev Biol* 237(1)*:*107-15.

Crisostomo PR, Wang Y, Markel TA, Wang M, Lahm T, Meldrum DR (2008). Human mesenchymal stem cells stimulated by TNF-alpha, LPS, or hypoxia produce growth factors by an NF kappa B- but not JNK-dependent mechanism. *Am J Physiol Cell Physiol* 294(3)*:*C675-82.

Feng JQ, Xing L, Zhang JH, Zhao M, Horn D, Chan J, et al. (2003). NF-kappaB specifically activates BMP-2 gene expression in growth plate chondrocytes in vivo and in a chondrocyte cell line in vitro. *J Biol Chem* 278(31)*:*29130-5.

Geisler F, Algul H, Paxian S, Schmid RM (2007). Genetic inactivation of RelA/p65 sensitizes adult mouse hepatocytes to TNF-induced apoptosis in vivo and in vitro. *Gastroenterology* 132(7)*:*2489-503.

Gerstenfeld LC, Cho TJ, Kon T, Aizawa T, Cruceta J, Graves BD, et al. (2001). Impaired intramembranous bone formation during bone repair in the absence of tumor necrosis factor-alpha signaling. *Cells Tissues Organs* 169(3)*:*285-94.

Gerstenfeld LC, Cho TJ, Kon T, Aizawa T, Tsay A, Fitch J, et al. (2003). Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption. *J Bone Miner Res* 18(9)*:*1584-92.

Glass GE, Chan JK, Freidin A, Feldmann M, Horwood NJ, Nanchahal J (2011). TNF-alpha promotes fracture repair by augmenting the recruitment and differentiation of muscle-derived stromal cells. *Proc Natl Acad Sci U S A* 108(4)*:*1585-90.

Hess K, Ushmorov A, Fiedler J, Brenner RE, Wirth T (2009). TNFalpha promotes osteogenic differentiation of human mesenchymal stem cells by triggering the NF-kappaB signaling pathway. *Bone* 45(2)*:*367-76.

Kon T, Cho TJ, Aizawa T, Yamazaki M, Nooh N, Graves D, et al. (2001). Expression of osteoprotegerin, receptor activator of NF-kappaB ligand (osteoprotegerin ligand) and

related proinflammatory cytokines during fracture healing. *J Bone Miner Res* 16(6)*:*1004-14.

Li Q, Verma IM (2002). NF-kappaB regulation in the immune system. *Nat Rev Immunol* 2(10)*:*725-34.

Li X, Wang H, Touma E, Qi Y, Rousseau E, Quigg RJ, et al. (2007). TP508 accelerates fracture repair by promoting cell growth over cell death. *Biochem Biophys Res Commun* 364(1)*:*187-93.

Long F (2011) Building strong bones: molecular regulation of the osteoblast lineage. *Nat Rev Mol Cell Biol* 13(1)*:*27-38.

Lu X, Gilbert L, He X, Rubin J, Nanes MS (2006). Transcriptional regulation of the osterix (Osx, Sp7) promoter by tumor necrosis factor identifies disparate effects of mitogen-activated protein kinase and NF kappa B pathways. *J Biol Chem* 281(10)*:*6297-306.

Magness ST, Jijon H, Van Houten Fisher N, Sharpless NE, Brenner DA, Jobin C (2004). In vivo pattern of lipopolysaccharide and anti-CD3-induced NF-kappa B activation using a novel gene-targeted enhanced GFP reporter gene mouse. *J Immunol* 173(3)*:*1561-70.

Mountziaris PM, Spicer PP, Kasper FK, Mikos AG (2011)Harnessing and modulating inflammation in strategies for bone regeneration. *Tissue Eng Part B Rev* 17(6)*:*393-402.

Neve A, Corrado A, Cantatore FP (2011). Osteoblast physiology in normal and pathological conditions. *Cell Tissue Res* 343(2)*:*289-302.

Novack DV (2011). Role of NF-kappaB in the skeleton. *Cell Res* 21(1)*:*169-82.

Rosenfeld ME, Prichard L, Shiojiri N, Fausto N (2000). Prevention of hepatic apoptosis and embryonic lethality in RelA/TNFR-1 double knockout mice. *Am J Pathol* 156(3)*:*997-1007.

Rundle CH, Wang H, Yu H, Chadwick RB, Davis EI, Wergedal JE, et al. (2006). Microarray analysis of gene expression during the inflammation and endochondral bone formation stages of rat femur fracture repair. *Bone* 38(4)*:*521-9.

Satokata I, Maas R (1994). Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. *Nat Genet* 6(4)*:*348-56.

Simon AM, O'Connor JP (2007). Dose and time-dependent effects of cyclooxygenase-2 inhibition on fracture-healing. *J Bone Joint Surg Am* 89(3)*:*500-11.

Steinbrecher KA, Harmel-Laws E, Sitcheran R, Baldwin AS (2008). Loss of epithelial

RelA results in deregulated intestinal proliferative/apoptotic homeostasis and susceptibility to inflammation. *J Immunol* 180(4)*:*2588-99.

Vaira S, Alhawagri M, Anwisye I, Kitaura H, Faccio R, Novack DV (2008). RelA/p65 promotes osteoclast differentiation by blocking a RANKL-induced apoptotic JNK pathway in mice. *J Clin Invest* 118(6)*:*2088-97.

Wu S, Flint JK, Rezvani G, De Luca F (2007). Nuclear factor-kappaB p65 facilitates longitudinal bone growth by inducing growth plate chondrocyte proliferation and differentiation and by preventing apoptosis. *J Biol Chem* 282(46)*:*33698-706.

Zhang X, Schwarz EM, Young DA, Puzas JE, Rosier RN, O'Keefe RJ (2002). Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair. *J Clin Invest* 109(11)*:*1405-15.

BIBLIOGRAPHY

Alappat S, Zhang ZY, Chen YP (2003). Msx homeobox gene family and craniofacial development. *Cell Res* 13(6)*:*429-42.

Alblowi J, Kayal RA, Siqueira M, McKenzie E, Krothapalli N, McLean J, et al. (2009). High levels of tumor necrosis factor-alpha contribute to accelerated loss of cartilage in diabetic fracture healing. *Am J Pathol* 175(4)*:*1574-85.

Appleby SB, Ristimaki A, Neilson K, Narko K, Hla T (1994). Structure of the human cyclo-oxygenase-2 gene. *Biochem J* 302 (Pt 3)(723-7.

Aubin JE (2001). Regulation of osteoblast formation and function. *Rev Endocr Metab Disord* 2(1)*:*81-94.

Balint E, Lapointe D, Drissi H, van der Meijden C, Young DW, van Wijnen AJ, et al. (2003). Phenotype discovery by gene expression profiling: mapping of biological processes linked to BMP-2-mediated osteoblast differentiation. *J Cell Biochem* 89(2)*:*401-26.

Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D (1995). Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature* 376(6536)*:*167-70.

Bocker W, Docheva D, Prall WC, Egea V, Pappou E, Rossmann O, et al. (2008). IKK-2 is required for TNF-alpha-induced invasion and proliferation of human mesenchymal stem cells. *J Mol Med (Berl)* 86(10)*:*1183-92.

Boyce BF, Yao Z, Xing L (2010). Functions of nuclear factor kappaB in bone. *Ann N Y Acad Sci* 1192(367-75.

Bruder SP, Fox BS (1999). Tissue engineering of bone. Cell based strategies. *Clin Orthop Relat Res* 367 Suppl)*:*S68-83.

Bushdid PB, Chen CL, Brantley DM, Yull F, Raghow R, Kerr LD, et al. (2001). NF-kappaB mediates FGF signal regulation of msx-1 expression. *Dev Biol* 237(1)*:*107-15.

Cao X, Chen D (2005). The BMP signaling and in vivo bone formation. *Gene* 357(1)*:*1-8.

Caparbo VF, Prada F, Silva CA, Regio PL, Pereira RM (2009). Serum from children with polyarticular juvenile idiopathic arthritis (pJIA) inhibits differentiation, mineralization and may increase apoptosis of human osteoblasts "in vitro". *Clin Rheumatol* 28(1)*:*71-7.

Carter TG, Brar PS, Tolas A, Beirne OR (2008). Off-label use of recombinant human bone morphogenetic protein-2 (rhBMP-2) for reconstruction of mandibular bone defects in humans. *J Oral Maxillofac Surg* 66(7)*:*1417-25.

Chang J, Wang Z, Tang E, Fan Z, McCauley L, Franceschi R, et al. (2009). Inhibition of osteoblastic bone formation by nuclear factor-kappaB. *Nat Med* 15(6)*:*682-9.

Chang MK, Raggatt LJ, Alexander KA, Kuliwaba JS, Fazzalari NL, Schroder K, et al. (2008). Osteal tissue macrophages are intercalated throughout human and mouse bone lining tissues and regulate osteoblast function in vitro and in vivo. *J Immunol* 181(2)*:*1232-44.

Chen D, Harris MA, Rossini G, Dunstan CR, Dallas SL, Feng JQ, et al. (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. *Calcif Tissue Int* 60(3)*:*283-90.

Chen NX, Geist DJ, Genetos DC, Pavalko FM, Duncan RL (2003). Fluid shear-induced NFkappaB translocation in osteoblasts is mediated by intracellular calcium release. *Bone* 33(3)*:*399-410.

Choi SH, Kim CK, Cho KS, Huh JS, Sorensen RG, Wozney JM, et al. (2002). Effect of recombinant human bone morphogenetic protein-2/absorbable collagen sponge (rhBMP-2/ACS) on healing in 3-wall intrabony defects in dogs. *J Periodontol* 73(1)*:*63-72.

Cohen MM, Jr. (2006). The new bone biology: pathologic, molecular, and clinical correlates. *Am J Med Genet A* 140(23)*:*2646-706.

Crisostomo PR, Wang Y, Markel TA, Wang M, Lahm T, Meldrum DR (2008). Human mesenchymal stem cells stimulated by TNF-alpha, LPS, or hypoxia produce growth factors by an NF kappa B- but not JNK-dependent mechanism. *Am J Physiol Cell Physiol* 294(3)*:*C675-82.

Dimmen S, Nordsletten L, Madsen JE (2009). Parecoxib and indomethacin delay early fracture healing: a study in rats. *Clin Orthop Relat Res* 467(8)*:*1992-9.

Ding J, Ghali O, Lencel P, Broux O, Chauveau C, Devedjian JC, et al. (2009). TNF-alpha and IL-1beta inhibit RUNX2 and collagen expression but increase alkaline phosphatase activity and mineralization in human mesenchymal stem cells. *Life Sci* 84(15-16)*:*499-504.

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G (1997). Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* 89(5)*:*747-54.

Feng JQ, Xing L, Zhang JH, Zhao M, Horn D, Chan J, et al. (2003). NF-kappaB specifically activates BMP-2 gene expression in growth plate chondrocytes in vivo and in

a chondrocyte cell line in vitro. *J Biol Chem* 278(31)*:*29130-5.

Franzoso G, Carlson L, Xing L, Poljak L, Shores EW, Brown KD, et al. (1997). Requirement for NF-kappaB in osteoclast and B-cell development. *Genes Dev* 11(24)*:*3482-96.

Fukuno N, Matsui H, Kanda Y, Suzuki O, Matsumoto K, Sasaki K, et al. (2011). TGF-beta-activated kinase 1 mediates mechanical stress-induced IL-6 expression in osteoblasts. *Biochem Biophys Res Commun* 408(2)*:*202-7.

Ganss B, Kim RH, Sodek J (1999). Bone sialoprotein. *Crit Rev Oral Biol Med* 10(1)*:*79-98.

Geisler F, Algul H, Paxian S, Schmid RM (2007). Genetic inactivation of RelA/p65 sensitizes adult mouse hepatocytes to TNF-induced apoptosis in vivo and in vitro. *Gastroenterology* 132(7)*:*2489-503.

Gerondakis S, Grossmann M, Nakamura Y, Pohl T, Grumont R (1999). Genetic approaches in mice to understand Rel/NF-kappaB and IkappaB function: transgenics and knockouts. *Oncogene* 18(49)*:*6888-95.

Gerstenfeld LC, Cho TJ, Kon T, Aizawa T, Cruceta J, Graves BD, et al. (2001). Impaired intramembranous bone formation during bone repair in the absence of tumor necrosis factor-alpha signaling. *Cells Tissues Organs* 169(3)*:*285-94.

Gerstenfeld LC, Cho TJ, Kon T, Aizawa T, Tsay A, Fitch J, et al. (2003). Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption. *J Bone Miner Res* 18(9)*:*1584-92.

Glass GE, Chan JK, Freidin A, Feldmann M, Horwood NJ, Nanchahal J (2011). TNF-alpha promotes fracture repair by augmenting the recruitment and differentiation of muscle-derived stromal cells. *Proc Natl Acad Sci U S A* 108(4)*:*1585-90.

Grimes R, Jepsen KJ, Fitch JL, Einhorn TA, Gerstenfeld LC (2011). The transcriptome of fracture healing defines mechanisms of coordination of skeletal and vascular development during endochondral bone formation. *J Bone Miner Res* 26(11)*:*2597-609.

Hayden MS, Ghosh S (2004). Signaling to NF-kappaB. *Genes Dev* 18(18)*:*2195-224.

Hess K, Ushmorov A, Fiedler J, Brenner RE, Wirth T (2009). TNFalpha promotes osteogenic differentiation of human mesenchymal stem cells by triggering the NF-kappaB signaling pathway. *Bone* 45(2)*:*367-76.

Hoffmann A, Levchenko A, Scott ML, Baltimore D (2002). The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. *Science* 298(5596)*:*1241-5.

Hoffmann A, Leung TH, Baltimore D (2003). Genetic analysis of NF-kappaB/Rel transcription factors defines functional specificities. *Embo J* 22(20):5530-9.

Hou LT, Liu CM, Liu BY, Chang PC, Chen MH, Ho MH, et al. (2007). Tissue engineering bone formation in novel recombinant human bone morphogenic protein 2-atelocollagen composite scaffolds. *J Periodontol* 78(2):335-43.

Hu WW, Ward BB, Wang Z, Krebsbach PH (2010). Bone regeneration in defects compromised by radiotherapy. *J Dent Res* 89(1):77-81.

Hunter GK, Goldberg HA (1994). Modulation of crystal formation by bone phosphoproteins: role of glutamic acid-rich sequences in the nucleation of hydroxyapatite by bone sialoprotein. *Biochem J* 302 (Pt 1)(175-9.

Imamura R, Konaka K, Matsumoto N, Hasegawa M, Fukui M, Mukaida N, et al. (2004). Fas ligand induces cell-autonomous NF-kappaB activation and interleukin-8 production by a mechanism distinct from that of tumor necrosis factor-alpha. *J Biol Chem* 279(45):46415-23.

Itagaki T, Honma T, Takahashi I, Echigo S, Sasano Y (2008). Quantitative analysis and localization of mRNA transcripts of type I collagen, osteocalcin, MMP 2, MMP 8, and MMP 13 during bone healing in a rat calvarial experimental defect model. *Anat Rec (Hoboken)* 291(8):1038-46.

Jang WG, Kim EJ, Kim DK, Ryoo HM, Lee KB, Kim SH, et al. (2012). BMP2 protein regulates osteocalcin expression via Runx2 mediated ATF6 gene transcription. *J Biol Chem* 287(2):905-15.

Jiang JX, Siller-Jackson AJ, Burra S (2007). Roles of gap junctions and hemichannels in bone cell functions and in signal transmission of mechanical stress. *Front Biosci* 12(1450-62.

Jiang X, Kalajzic Z, Maye P, Braut A, Bellizzi J, Mina M, et al. (2005). Histological analysis of GFP expression in murine bone. *J Histochem Cytochem* 53(5):593-602.

Jung YJ, Isaacs JS, Lee S, Trepel J, Neckers L (2003). IL-1beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. *Faseb J* 17(14):2115-7.

Karrasch T, Kim JS, Muhlbauer M, Magness ST, Jobin C (2007). Gnotobiotic IL-10-/-;NF-kappa B(EGFP) mice reveal the critical role of TLR/NF-kappa B signaling in commensal bacteria-induced colitis. *J Immunol* 178(10):6522-32.

Katavic V, Grcevic D, Lukic IK, Vucenik V, Kovacic N, Kalajzic I, et al. (2003). Non-functional Fas ligand increases the formation of cartilage early in the endochondral bone induction by rhBMP-2. *Life Sci* 74(1):13-28.

Katayama Y, Matsuyama Y, Yoshihara H, Sakai Y, Nakamura H, Imagama S, et al. (2009). Clinical and radiographic outcomes of posterolateral lumbar spine fusion in humans using recombinant human bone morphogenetic protein-2: an average five-year follow-up study. *Int Orthop* 33(4)*:*1061-7.

Kim CS, Kim JI, Kim J, Choi SH, Chai JK, Kim CK, et al. (2005). Ectopic bone formation associated with recombinant human bone morphogenetic proteins-2 using absorbable collagen sponge and beta tricalcium phosphate as carriers. *Biomaterials* 26(15)*:*2501-7.

Kim IS, Lee EN, Cho TH, Song YM, Hwang SJ, Oh JH, et al. (2011). Promising efficacy of Escherichia coli recombinant human bone morphogenetic protein-2 in collagen sponge for ectopic and orthotopic bone formation and comparison with mammalian cell recombinant human bone morphogenetic protein-2. *Tissue Eng Part A* 17(3-4)*:*337-48.

Kimura Y, Miyazaki N, Hayashi N, Otsuru S, Tamai K, Kaneda Y, et al. (2010). Controlled release of bone morphogenetic protein-2 enhances recruitment of osteogenic progenitor cells for de novo generation of bone tissue. *Tissue Eng Part A* 16(4)*:*1263-70.

King GN, Cochran DL (2002). Factors that modulate the effects of bone morphogenetic protein-induced periodontal regeneration: a critical review. *J Periodontol* 73(8)*:*925-36.

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, et al. (1997). Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* 89(5)*:*755-64.

Kon T, Cho TJ, Aizawa T, Yamazaki M, Nooh N, Graves D, et al. (2001). Expression of osteoprotegerin, receptor activator of NF-kappaB ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing. *J Bone Miner Res* 16(6)*:*1004-14.

Lawrence T, Bebien M, Liu GY, Nizet V, Karin M (2005). IKKalpha limits macrophage NF-kappaB activation and contributes to the resolution of inflammation. *Nature* 434(7037)*:*1138-43.

Li Q, Verma IM (2002). NF-kappaB regulation in the immune system. *Nat Rev Immunol* 2(10)*:*725-34.

Li X, Wang H, Touma E, Qi Y, Rousseau E, Quigg RJ, et al. (2007). TP508 accelerates fracture repair by promoting cell growth over cell death. *Biochem Biophys Res Commun* 364(1)*:*187-93.

Li Y, Backesjo CM, Haldosen LA, Lindgren U (2008). IL-6 receptor expression and IL-6 effects change during osteoblast differentiation. *Cytokine* 43(2)*:*165-73.

Lian JB, Stein GS (2003). Runx2/Cbfa1: a multifunctional regulator of bone formation.

Curr Pharm Des 9(32)*:2677-85.*

Libermann TA, Baltimore D (1990). Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. *Mol Cell Biol* 10(5)*:2327-34.*

Lippert E, Karrasch T, Sun X, Allard B, Herfarth HH, Threadgill D, et al. (2009). Gnotobiotic IL-10; NF-kappaB mice develop rapid and severe colitis following Campylobacter jejuni infection. *PLoS One* 4(10)*:e7413.*

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25(4)*:402-8.*

Long F (2011). Building strong bones: molecular regulation of the osteoblast lineage. *Nat Rev Mol Cell Biol* 13(1)*:27-38.*

Lu X, Gilbert L, He X, Rubin J, Nanes MS (2006). Transcriptional regulation of the osterix (Osx, Sp7) promoter by tumor necrosis factor identifies disparate effects of mitogen-activated protein kinase and NF kappa B pathways. *J Biol Chem* 281(10)*:6297-306.*

Magness ST, Jijon H, Van Houten Fisher N, Sharpless NE, Brenner DA, Jobin C (2004). In vivo pattern of lipopolysaccharide and anti-CD3-induced NF-kappa B activation using a novel gene-targeted enhanced GFP reporter gene mouse. *J Immunol* 173(3)*:1561-70.*

Matsubara T, Kida K, Yamaguchi A, Hata K, Ichida F, Meguro H, et al. (2008). BMP2 regulates Osterix through Msx2 and Runx2 during osteoblast differentiation. *J Biol Chem* 283(43)*:29119-25.*

McLeod MJ (1980). Differential staining of cartilage and bone in whole mouse fetuses by alcian blue and alizarin red S. *Teratology* 22(3)*:299-301.*

Mountziaris PM, Mikos AG (2008). Modulation of the inflammatory response for enhanced bone tissue regeneration. *Tissue Eng Part B Rev* 14(2)*:179-86.*

Mountziaris PM, Spicer PP, Kasper FK, Mikos AG (2011). Harnessing and modulating inflammation in strategies for bone regeneration. *Tissue Eng Part B Rev* 17(6)*:393-402.*

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, et al. (2002). The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* 108(1)*:17-29.*

Neve A, Corrado A, Cantatore FP (2011). Osteoblast physiology in normal and pathological conditions. *Cell Tissue Res* 343(2)*:289-302.*

NIAMS NIoAaMasd (2011). Osteoporosis and related bone diseases.

Niger C, Lima F, Yoo DJ, Gupta RR, Buo AM, Hebert C, et al. (2011). The transcriptional activity of osterix requires the recruitment of Sp1 to the osteocalcin proximal promoter. *Bone* 49(4):683-92.

Perkins ND, Gilmore TD (2006). Good cop, bad cop: the different faces of NF-kappaB. *Cell Death Differ* 13(5):759-72.

Perkins ND (2007). Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat Rev Mol Cell Biol* 8(1):49-62.

Rosenfeld ME, Prichard L, Shiojiri N, Fausto N (2000). Prevention of hepatic apoptosis and embryonic lethality in RelA/TNFR-1 double knockout mice. *Am J Pathol* 156(3):997-1007.

Rozen N, Lewinson D, Bick T, Jacob ZC, Stein H, Soudry M (2007). Fracture repair: modulation of fracture-callus and mechanical properties by sequential application of IL-6 following PTH 1-34 or PTH 28-48. *Bone* 41(3):437-45.

Rundle CH, Wang H, Yu H, Chadwick RB, Davis EI, Wergedal JE, et al. (2006). Microarray analysis of gene expression during the inflammation and endochondral bone formation stages of rat femur fracture repair. *Bone* 38(4):521-9.

Satokata I, Maas R (1994). Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. *Nat Genet* 6(4):348-56.

Schindeler A, McDonald MM, Bokko P, Little DG (2008). Bone remodeling during fracture repair: The cellular picture. *Semin Cell Dev Biol* 19(5):459-66.

Shih VF, Tsui R, Caldwell A, Hoffmann A (2011). A single NFkappaB system for both canonical and non-canonical signaling. *Cell Res* 21(1):86-102.

Simon AM, Manigrasso MB, O'Connor JP (2002). Cyclo-oxygenase 2 function is essential for bone fracture healing. *J Bone Miner Res* 17(6):963-76.

Simon AM, O'Connor JP (2007). Dose and time-dependent effects of cyclooxygenase-2 inhibition on fracture-healing. *J Bone Joint Surg Am* 89(3):500-11.

Sodek J, Li JJ, Kim RH, Ogata Y, Yamauchi M (1996). Characterization of the bone sialoprotein (BSP) gene promoter. *Connect Tissue Res* 35(1-4):23-31.

Steinbrecher KA, Harmel-Laws E, Sitcheran R, Baldwin AS (2008). Loss of epithelial RelA results in deregulated intestinal proliferative/apoptotic homeostasis and susceptibility to inflammation. *J Immunol* 180(4):2588-99.

Takayanagi H (2009). Osteoimmunology and the effects of the immune system on bone. *Nat Rev Rheumatol* 5(12):667-76.

Tergaonkar V, Correa RG, Ikawa M, Verma IM (2005). Distinct roles of IkappaB proteins in regulating constitutive NF-kappaB activity. *Nat Cell Biol* 7(9)*:*921-3.

Thompson Z, Miclau T, Hu D, Helms JA (2002). A model for intramembranous ossification during fracture healing. *J Orthop Res* 20(5)*:*1091-8.

Triplett RG, Nevins M, Marx RE, Spagnoli DB, Oates TW, Moy PK, et al. (2009). Pivotal, randomized, parallel evaluation of recombinant human bone morphogenetic protein-2/absorbable collagen sponge and autogenous bone graft for maxillary sinus floor augmentation. *J Oral Maxillofac Surg* 67(9)*:*1947-60.

Vaira S, Alhawagri M, Anwisye I, Kitaura H, Faccio R, Novack DV (2008). RelA/p65 promotes osteoclast differentiation by blocking a RANKL-induced apoptotic JNK pathway in mice. *J Clin Invest* 118(6)*:*2088-97.

Wu S, Flint JK, Rezvani G, De Luca F (2007). Nuclear factor-kappaB p65 facilitates longitudinal bone growth by inducing growth plate chondrocyte proliferation and differentiation and by preventing apoptosis. *J Biol Chem* 282(46)*:*33698-706.

Yamamoto Y, Udagawa N, Matsuura S, Nakamichi Y, Horiuchi H, Hosoya A, et al. (2006). Osteoblasts provide a suitable microenvironment for the action of receptor activator of nuclear factor-kappaB ligand. *Endocrinology* 147(7)*:*3366-74.

Yan D, Gurumurthy A, Wright M, Pfeiler TW, Loboa EG, Everett ET (2007). Genetic background influences fluoride's effects on osteoclastogenesis. *Bone* 41(6)*:*1036-44.

Yang X, Ricciardi BF, Hernandez-Soria A, Shi Y, Pleshko Camacho N, Bostrom MP (2007). Callus mineralization and maturation are delayed during fracture healing in interleukin-6 knockout mice. *Bone* 41(6)*:*928-36.

Zaidi M (2007). Skeletal remodeling in health and disease. *Nat Med* 13(7)*:*791-801.

Zhang H, Bradley A (1996). Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. *Development* 122(10)*:*2977-86.

Zhang X, Schwarz EM, Young DA, Puzas JE, Rosier RN, O'Keefe RJ (2002). Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair. *J Clin Invest* 109(11)*:*1405-15.