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The roles of nitric oxide synthases (NOS) in endochondral bone formation

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Physiology

THE ROLES OF NITRIC OXIDE SYNTHASES (NOS) IN ENDOCHONDRAL BONE FORMATION

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ABSTRACT

Longitudinal growth of endochondral bones is controlled by the cartilage growth plate. Chondrocyte proliferation and hypertrophy, vascular invasion, formation of ossification centers and cartilage replacement by bone tissue are all important processes required for normal growth. These biological processes have to be tightly regulated or disturbances will lead to skeletal diseases. A large number of genes, growth factors and hormones have been implicated in the regulation of growth plate biology, however, less is known about the intracellular signaling pathways involved. Nitric oxide (NO) has been identified as a regulator of cellular proliferation, differentiation, migration, survival and metabolism in multiple cell types. In bone biology, it has been implicated in bone remodeling and the pathogenesis of osteoarthritis, but the roles of specific nitric oxide synthase (NOS) enzymes in chondrocyte physiology and cartilage development are unclear. The goal of this thesis was to analyze the roles of NOS/NO signaling in specific stages of endochondral bone formation.

We had shown recently that chondrocyte-specific deletion of the *Rac1* gene results in severe dwarfism due to reduced chondrocyte proliferation in mice, but the molecular pathways involved remained unknown. Employing a *Rac1*-deficient monolayer chondrocyte culture, we showed that loss of Rac1 results in severely reduced levels of inducible nitric oxide synthase (iNOS) protein and NO production. Additionally, reduced iNOS expression was found in *Rac1*-decifient mice *in vivo*. Using a tibia organ culture system, we showed that NO donors rescued the antiproliferative effects of Rac1 inhibition. Examination of the growth plate of *iNOS*-deficient mice revealed reduced chondrocyte proliferation and decreased expression of cyclin D1, while ATF3, a

suppressor of cyclin D1 transcription, showed increased expression. Thus, we identified iNOS/NO as a novel mediator of Rac1 signaling and ATF3 as a link between iNOS and chondrocyte cell cycle.

Doe to the skeletal phenotypes we observed in *iNOS*-deficient mice, my next study investigated effects of inactivation of endothelial nitric oxide synthase (*eNOS*) on cartilage development in mice. *eNOS*-deficient mice showed increased lethality and reduced bone growth, delayed ossification and a marked reduction in the number of proliferating chondrocytes. The mechanisms leading to these bone phenotypes appear to be caused by decreased cyclin D1 and increased p57 expressions in mutants, resulting in slower cell cycle progression and earlier cell cycle exit. Additionally, expression of early chondrocyte markers such as Sox9 was reduced and prehypertrophic markers were upregulated in mutant mice.

Because my studies had shown upregulation of nNOS in *eNOS*-null cartilage, next I analyzed the skeletal phenotype of *nNOS*-deficient mice. Transient growth retardation, reduced length of long bones, less trabecular bone and decreased mineralization were shown in *nNOS* KO mice. Reduced proliferating chondrocyte numbers in mutants may in part be due to premature cell cycle exit, shown by reduced cyclin D1 and upregulated p57 expressions. Similar to the other two mutant strains, ATF3 was a link between nNOS and reduced cyclin D1 expression. In addition, I demonstrated increased apoptosis, reduced early chondrocyte markers such as Sox genes and increased prehypertrophic markers RORα and c-Fos in mutant mice. Together, these data suggest that NOS/NO presents a core signaling pathway to regulate chondrocyte proliferation and differentiation through control of cell cycle protein.

KEYWORDS

Endochondral bone formation; cartilage development; chondrocytes; proliferation; differentiation; apoptosis; trabecular bone formation; cell cycle genes; NO; eNOS; iNOS; nNOS; Rac1; cyclin D1; P57; BrdU; PCNA; caspase-3; ATF3; Sox9; Rorα; Hif1α.

CO-AUTHORSHIP

Chapter 2 is authored by Wang, G., Yan, Q., Woods, A., Feng, Q. and Beier, F. and is titled: iNOS/nitric oxide signaling mediates the mitogenic activity of Rac1 during endochondral bone growth. G. Wang performed *Rac1*-deficent monolayer chondrocyte culture experiments and immunohistochemistry of iNOS, nitrotyrosine and ATF3 in *Rac1*-null mice. All other experiments were performed by Q. Yan in the laboratory of Dr. F. Beier. G. Wang and Q. Yan contributed equally to this project. *iNOS*-null mice were provided by Dr. Q. Feng. Q. Yan prepared the manuscript. Dr. F. Beier contributed to study design and the writing of the manuscript. All authors read and approved the submitted version of the manuscript. We are currently revising this manuscript for *J. of Cell Science*.

Chapter 3 is adapted from Yan Q, Feng Q, Beier F. (2010). Endothelial nitric oxide synthase deficiency in mice results in reduced chondrocyte proliferation and endochondral bone growth. *Arthritis & Rheum* 62(7):2013-2022. Figures and text are reproduced with permission from *Arthritis & Rheumatism* (Appendix A). Dr. Q Feng provided *eNOS*-deficient mice. Q. Yan performed all the experiments in the laboratory of Dr. F. Beier and contributed to study design and the writing of the manuscript. All authors read and approved the submitted version of the publication.

Chapter 4 is authored by Yan, Q., Feng, Q. and Beier, F. and is titled: Reduced chondrocyte proliferation, increased apoptosis and premature differentiation in neuronal nitric oxide synthase-deficient mice. The manuscript was written by Q. Yan with

suggestions from Drs. Q. Feng and F. Beier. Dr. Q Feng provided *nNOS*-deficient mice. Q. Yan performed all the experiments in the laboratory of Dr. F. Beier. Dr. F. Beier contributed to study design and the writing of the manuscript. All authors read and approved the submitted version of the manuscript. A version of this chapter has been submitted to *Arthritis Research & Therapy*.

DEDICATION

To the authentic few who know what they do not know and have the courage to seek enlightenment.

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TABLE OF CONTENTS

PAGE

LIST OF TABLES

Table **Page**

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

3D	three-dimensional
ANOVA	analysis of variance
ANP	atrial natriuretic peptide
ATF3	activating transcription factor 3
ATP	adenosine 5'-triphosphate
BMC	bone mineral content
BMD	bone mineral density
BMP	bone morphogenetic protein
BNP	brain natriuretic peptide
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
cAMP	3'-5'-cyclic adenosine monophosphate
CDK	cyclin-dependent kinase
c-Fos	cellular proto-oncogene fos
cGMP	3'-5'-cyclic guanosine monophosphate
cGKII	cGMP-dependent protein kinase II
CNP	C-type natriuretic peptide
Col2a1	type II collagen
Col10a1	type X collagen
COX-2	cyclooxygenase 2
C_T	threshold cycle
Cyclin D1	G1/S-specific cyclin-dependent kinase D1

DAF-FM	4-amino-5-methylamino-2',7'-difluorofluorescein
DAF-FM DA	4-amino-5-methylamino-2',7'-difluorofluorescein diacetate
DCF	2′,7′-dichlorofluorescein
DCF-DA	2′,7′-dichlorofluorescein diacetate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
E	embryonic dat
ECM	extracellular matrix
EDTA	1-(4-aminobenzyl)ethylenediamine-N,N,N'N'-tetra-acetic acid
eNOS	endothelial nitric oxide synthase
EO	endochondral ossification
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GAG	glycosaminoglycan
GAP	GTPase activating protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	guanylyl cyclase
GDP	guanosine 5'-diphosphate
GH	growth hormone
GPCR	G-protein-coupled receptor

G-protein	guanine nucleotide binding-proteins
GSK	glycogen synthase kinase
GTP	guanosine 5’-triphosphate
H_2O_2	hydrogen peroxide
HA	hydroxyapatite
Ibsp	integrin binding sialoprotein
IGF	insulin-like growth factor
IHC	immunohistochemistry
Ihh	indian hedgehog
IL-1β	interleukin-1 beta
iNOS	inducible nitric oxide synthase
KO	knock-out
L-NAME	$N^{(G)}$-nitro-L-arginine methyl ester
MAPK	mitogen activated protein kinase
MEK	MAPK-ERK kinase
Micro-CT	micro-computed tomography
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N-CAM	neural crest adhesion molecule
nNOS	neuronal nitric oxide synthase
NO	nitric oxide

NOC-18	2,2'-(Hydroxynitrosohydrazino) bis-ethanamine
NOS	nitric oxide synthase
OA	osteoarthritis
P57	cyclin-dependent kinase inhibitor 1C
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PFA	paraformaldehyde
PGE_2	prostaglandin E_2
PI3K	phosphatidylinositol 3 kinase
PKA	protein kinase A
PKC	protein kinase C
PLC-γ	phospholipase C gamma
POC	primary ossification center
Ptc-1	patched-1
PTHrP	parathyroid hormone related peptide
RA	Rheumatoid Arthritis
RANKL	receptor activator of NF-kappa-B ligand
RIPA	radioimmuno precipitation assay
RNA	ribonucleic acid
ROI	region of interest
Rorα	retinoic-acid related orphan receptor alpha
RT-PCR	reverse transcription polymerase chain reaction

RUNX2	runt-related transcription factor 2
SDS	sodium dodecyl sulfate
SEM	standard error of mean
SIN-1	3-morpholinosydnonimine
sGC	soluble guanylyl cyclases
SNAP	S-nitroso-N-acetylpenicillamine
SNP	sodium nitroprusside
SOX	SRY (sex determining region Y)-box containing gene
STAT	signal transducer and activator of transcription
TNF-α	tumour necrosis factor alpha
VEGF	vascular endothelial growth factor
WT	wild-type

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 *Introduction*

1.1.1 The function of bones

The skeletal system is one of the major organ systems in the human body and made up of 206 bones (Docherty, 2007a). The most obvious and one of the most important skeletal functions is to provide a framework that supports the human body and protects internal organs such as brain, spinal cord, heart, lungs, liver, and kidneys. There are also many other functions of the skeletal system that are essential for other processes in the human body. The bones in our body are joined and facilitate muscle contractions to move and perform our daily work. The skeleton is also a regulator of metabolism and acts as a reservoir of minerals such as calcium and phosphorous (Karsenty et al., 2009). Additionally, bone marrow is a place for hematopoiesis, where the formation of new blood cells takes place.

1.1.2 The organization of the human skeletal system

The human skeletal system is perpendicularly symmetrical in nature. It consists of the axial and the appendicular skeletons. The axial skeleton includes bones of the vertebral column, thoracic and rib cages, sternum and skull. It is made up of 80 bones (Docherty, 2007b). The main function of the axial skeleton is the protection of organs, like heart, brain and lungs, and maintaining the upright position of the body. The appendicular skeleton is attached to the axial skeleton, including pectoral girdles (shoulder portion), upper limbs, pelvic girdle (hip portion) and lower limbs. Body movements are possible because of these bones (Docherty, 2007b).

1.1.3 Skeletogenesis

The process by which bones are formed is called ossification. Ossification is an extremely complex, life-long process (Docherty, 2007a). Formation of the adult skeleton is achieved through two independent mechanisms: intramembranous and endochondral ossification (Beier, 2005; Olsen et al., 2000). In intramembranous bone formation, mesenchymal cells directly differentiate into osteoblasts. Some of the craniofacial bones are formed this way. Endochondral ossification (EO) is the process responsible for formation of the majority of bones in the vertebrate skeleton (Teixeira et al., 2008; Wagner and Karsenty, 2001). EO starts from mesenchymal cells that condense at the position of future bone, through a complex process of chondrogenesis to form a highly controlled and precisely shaped cartilage template (Ballock and O'Keefe, 2003). The cartilage template grows through chondrocyte proliferation and differentiation to establish the cartilage growth plate that ultimately drives the longitudinal growth of the bone. This process eventually determines the adult height (Fig. 1.1). Long bones such as femur are formed through this process. Finally, a process of growth and remodeling after birth (in a growth and maintenance phase) results in a skeleton which is well adapted to its function as an organ not only for support and protection of internal organs, but also for movement , blood cell formation and regulation of calcium homeostasis.

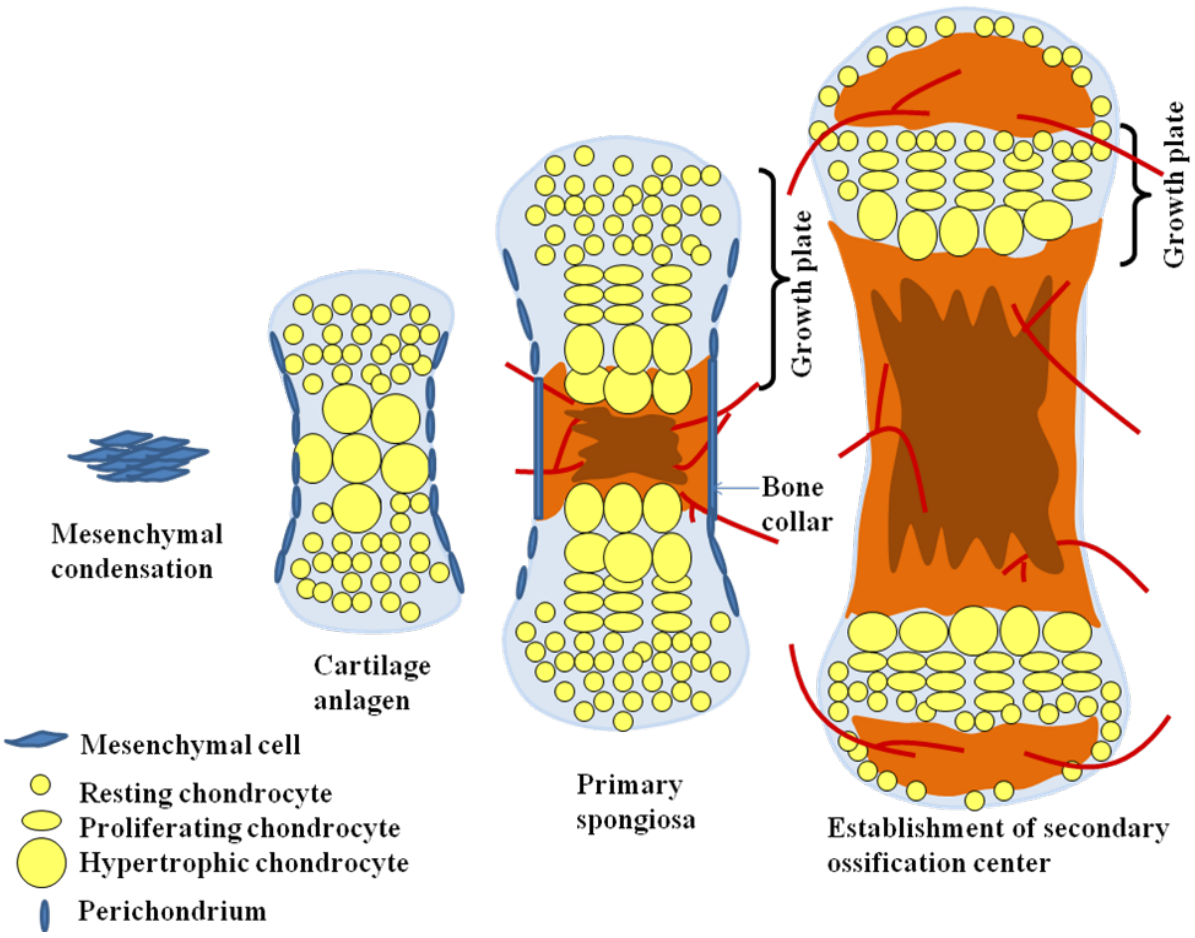
1.2 *Endochondral bone formation*

1.2.1 Chondrogenesis

Endochondral ossification is initiated by aggregation of mesenchymal cells, followed by cell differentiation into chondrocytes through the process of chondrogenesis.

Figure 1.1 Overview of endochondral ossification.

During embryogenesis, mesenchymal cells start to aggregate and condense at the location of future bone. They then differentiate into chondrocyte in the center of these condensations through chondrogenesis. Chondrocytes proliferate and differentiate within these condensations leading to the formation of cartilage anlagen. At the periphery of the condensations, cells flatten, elongate and form the perichondrium. These chondrocytes also keep proliferating to expand the cartilage template. Eventually, cells at the center of this template terminally differentiate into hypertrophic cells. Perichondral cells adjacent to hypertrophic chondrocyte become osteoblasts, forming a bone collar. Hypertrophic chondrocyte direct mineralization of the surrounding cartilage matrix, attract blood vessel invasion, and then undergo apoptosis. Osteoblasts form early trabecular bone adjacent to the hypertrophic chondrocytes, forming the primary spongiosa. Epiphyseal growth plates form at the both ends of the long bone with unique organization of the chondrocytes. At the end of the bone, the secondary ossification center forms through cycles of chondrocyte hypertrophy, vascular invasion and osteoblast activity.


Mesenchymal condensation
Cartilage anlagen
Growth plate
Bone collar
Primary spongiosa
Growth plate
Establishment of secondary ossification center
Mesenchymal cell
Resting chondrocyte
Proliferating chondrocyte
Hypertrophic chondrocyte
Perichondrium

Chondrogenesis is a highly complex process leading to the formation of a cartilage template (Provot and Schipani, 2005). Early condensations can be found in the human fetus at 6.5 week of gestation and at 10.5 days (E10.5) of embryonic mouse development (Kaufman, 1992). At this time point in development, the shape, number and position of future skeletal elements are decided (Goldring et al., 2006). Members of the Sox family of transcription factors (Sox9, Sox5 and Sox6) are required for this early differentiation (Wagner and Karsenty, 2001). Sox9 is one of the earliest markers expressed in cells undergoing condensation. Sox9 transcript is detected in all prechondrogenic mesenchymal condensation as early as 8.5-9.5 days of mouse embryonic development (Kaufman, 1992), and the expression peaks in cartilage at E11.5-14.5 days (Asou et al., 2002). Sox5 and 6 are not found in condensations, but they are co-expressed with Sox9 during differentiation (Wagner and Karsenty, 2001). Cells in the condensations differentiate to chondrocytes and deposit an extracellular matrix specific for cartilage, consisting of molecules such as collagens II, IX, and XI and proteoglycans (e.g. aggrecan). At the border of the condensations, chondrocytes differentiate to flattened elongated cells to form the perichondrium (Kronenberg, 2003). All these events contribute to the establishment of cartilage anlagen for the future bone elements (Fig. 1.1). Cartilage anlagen can be observed at 7 weeks of human and 11.5 days of mouse embryonic development (Olsen et al., 2000; Kaufman, 1992).

1.2.2 Epiphyseal growth plate

Once mesenchymal cells commit to the chondrogenic lineage, the subsequent events of EO occur through the epiphyseal growth plate. Longitudinal bone growth dictates the final height of human bodies and is dependent on the activity of the growth

plates (Provot and Schipani, 2005). The growth plate is established around the end of the first trimester in human fetal development and around embryonic day 15 in mice (Kaufman, 1992). The growth plate is a unique structure where specific chondrocyte populations are organized in distinct zones (e.g. zones of resting, proliferating, and hypertrophic chondrocytes) based on their morphology, cellular activities and gene expression patterns (Ballock and O'Keefe, 2003) (Fig. 1.1). Within these zones, the chondrocytes are organized in distinguishable columnar arrays and express different matrix proteins (Olsen et al., 2000).

The resting chondrocytes are small round cells in a relatively quiescent state and located furthest away from the primary ossification center. It is believed that some of these cells have stem-like properties, and can give rise to the neighboring proliferative zone (Ballock and O'Keefe, 2003). Within this zone of the growth plate, flattened proliferating chondrocytes are organized in a columnar array, and proliferation of chondrocytes occurs in a unidirectional manner, resulting in longitudinal growth of the bone (Hunziker, 1994). Both resting and proliferating chondrocytes express extracellular matrix (ECM) molecules, such as collagen II and aggrecan (Kronenberg, 2003). Throughout chondrogenesis, the balance of signaling by growth factors such as bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) determines the rate of proliferation (Hoffmann and Gross, 2001). Chondrocyte proliferation is also under control of cell-cycle proteins, such as cyclin D1 and A (Beier, 2005; Karsenty and Wagner, 2002; Kronenberg, 2003).

1.2.3 Chondrocyte hypertrophy and extracellular matrix production

Chondrocytes in the center of the cartilage mould then stop proliferating, start to differentiate into prehypertrophic cells, and eventually enlarge to terminally differentiated hypertrophic chondrocytes under the control of several cell cycle genes and different transcription factors (Colnot, 2005; Karsenty and Wagner, 2002; Kronenberg, 2003). The transcription factor RUNX2 (runt related transcript factor 2) is required for this terminal chondrocyte differentiation (Olsen et al., 2000; Provot and Schipani, 2005). It has been shown that chondrocyte hypertrophy is responsible for 60% of long bone growth, and the rest is due to both matrix synthesis and chondrocyte proliferation (Hunziker, 1994; Hunziker and Schenk, 1989; Nilsson et al., 1994).

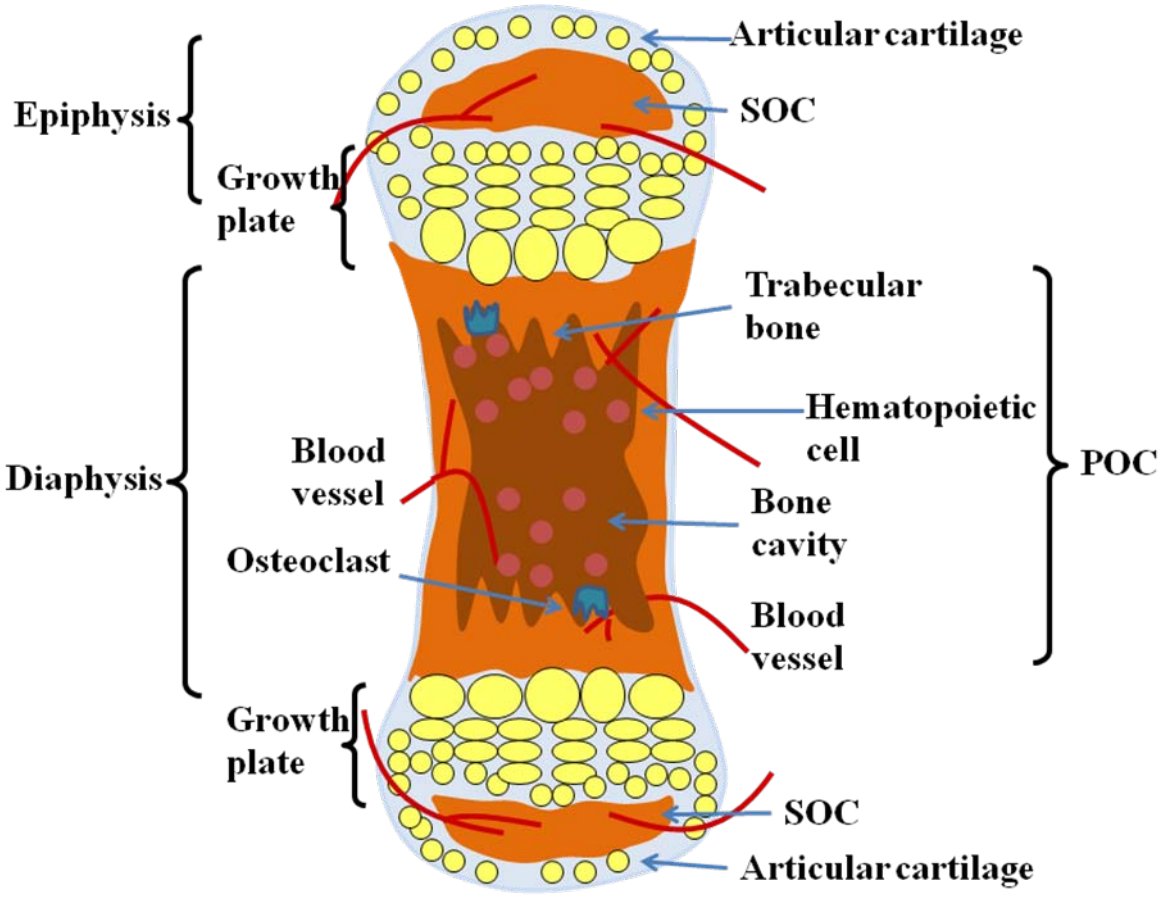
Hypertrophic chondrocytes are the principle regulators of the subsequent events of bone growth (Karsenty and Wagner, 2002; van der Eerden et al., 2003). They change their gene expression pattern to synthesize type X collagen, direct the mineralization of their extracellullar matrix, attract blood vessel invasion through the production of vascular endothelial growth factor (VEGF), activate osteoclasts to digest the matrix, and direct adjacent perichondral cells to become osteoblasts, which then secret a matrix rich in type I collagen and form a bone collar around the center of the cartilage template (Goldring et al., 2006; Kronenberg, 2003) (Fig. 1.1). Hypertrophic chondrocytes then undergo apoptotic cell death (Shum and Nuckolls, 2002). The cartilage matrix provides a scaffold for osteoblasts to lay down bone matrix within it (Ballock and O'Keefe, 2003; Kronenberg, 2003; Lefebvre and Smits, 2005; Lotz et al., 1999; Olsen et al., 2000).

1.2.4 Blood vessel invasion and ossification centers

VEGF and other angiogenic factors secreted by hypertrophic chondrocytes recruit endothelial cells from the surrounding blood circulation, and trigger vascular invasion from the periosteum (once vascularized, the perichondrium becomes the periosteum) (Hunziker, 1994). This invasion brings osteoclasts and hematopoietic cells into the mineralized cartilage. The newly recruited osteoclasts start to degrade the mineralized matrix (Drissi et al., 2005; Hunziker, 1994). Osteoblasts are also recruited from the periosteum, and lay down a new bone-specific matrix on the remains of the hypertrophic cartilaginous matrix. With the formation of the early trabecular bone, the primary spongiosa is established from the middle of the growth plates and eventually develops into a primary ossification center (POC) (Fig. 1.1). Another step in endochondral bone formation is the formation of secondary ossification centers (SOCs), which initiate at a late stage of the development. They start to appear at postnatal day 5 or 6 in mice (Olsen et al., 2000; van der Eerden et al., 2003). These centers form in the middle of epiphyseal cartilage after establishment of the growth plates. After formation of the secondary ossification center, the epiphyseal cartilage is now separated from the growth plate by the SOC and will eventually develop into articular cartilage at the far end of bone, which provides an important cushion during the whole adult life (Hunziker and Schenk, 1989) (Fig. 1.2). The longitudinal growth of the bone continues to depend on the activity of the epiphyseal growth plates between primary and secondary ossification centers (Ballock and O'Keefe, 2003). In humans, it closes during puberty (when primary and secondary ossification centers fuse) and eventually determines adult height (Shum and Nuckolls, 2002).

Figure 1.2 The formation of secondary ossification centers.

The secondary ossification centers (SOC) form in the middle of the epiphyseal cartilage after establishment of growth plates. At this point, the epiphyseal cartilage that is now separated from the growth plate by the SOC will eventually develop into articular cartilage at the far end of bone, which provides an important cushion during the whole adult life. The longitudinal growth of the bone continues to depend on the activity of the epiphyseal growth plates between primary and secondary ossification centers. In humans, the growth plates close during puberty and eventually determine adult height. The mechanisms leading to the formation of the secondary ossification centers are similar to the formation of primary ossification centers. (POC: primary ossification center).



Establishment of secondary ossification center

The mechanisms leading to the formation of the secondary ossification centers are similar to the formation of primary ossification centers. The formation of secondary ossification centers is delayed in cartilage disease, such as spondyloepiphyseal dysplasia (Zelzer and Olsen, 2003).

1.2.5 Bone modeling and remodeling

The last step in bone development is the tissue modeling and remodeling. Bone tissues experience consistent modeling and remodeling during development and in adult life, through the coordinated activity of bone-forming osteoblasts and bone-resorbing osteoclasts (Docherty, 2007a; Karsenty and Wagner, 2002; Olsen et al., 2000; Ortega et al., 2004). Cytokines released from bone resorption by osteoclasts activate osteoblast precursors to lay down cyclically shaped osteoid that is then mineralized to form new osteon (Holmbeck and Szabova, 2006; Olsen et al., 2000). Communication between bone-forming osteoblasts, bone-resorbing osteoclasts and other bone cells regulates bone turnover in balancing the normal remodeling sequence under control of local and systemic bone remodeling pathways (Holmbeck and Szabova, 2006).

1.3 *Systemic control and local regulators of longitudinal growth*

1.3.1 Endocrine factors

The process of longitudinal bone growth is governed by a complex network of endocrine signals, including growth hormone, insulin-like growth factor I, glucocorticoids, thyroid hormone, estrogen, androgen, vitamin D, and leptin (Nilsson et al., 2005). Many of these signals regulate growth plate function, both by acting locally on growth plate chondrocytes and indirectly by modulating other endocrine signals in the

network (Nilsson et al., 1994). Some of the local effects of hormones are mediated by changes in paracrine factors that control chondrocyte proliferation and differentiation (Nilsson et al., 1994). Many human skeletal growth disorders are caused by abnormalities in the endocrine regulation of the growth plate (Nilsson et al., 1994; van der Eerden et al., 2003; Zelzer and Olsen, 2003), some of which are discussed below.

1.3.1.1 Growth Hormone (GH) and insulin-like growth factors (Igfs)

GH is a peptide hormone secreted by the pituitary gland that stimulates growth and cell reproduction. Igfs are peptide hormones secreted from many different cells (Nilsson et al., 2005). GH and Igfs are potent stimulators of longitudinal bone growth (Mushtaq et al., 2004). GH excess, due to pituitary adenomas in childhood, results in gigantism. Conversely, GH deficiency or insensitivity due to GH-receptor mutations or defects in GH signaling pathways markedly impairs postnatal growth (Osafo et al., 2005). Mice lacking the GH gene exhibit normal birth weight, but a reduction in postnatal growth (Osafo et al., 2005). Mice lacking either the Igf-1 or Igf-2 genes show intrauterine growth retardation with birth weights approximately 60% that of wild-type littermates (Wang et al., 2006). The original somatomedin hypothesis stipulates that the effects of GH on linear growth are mediated by stimulating Igf-1 expression, including in chondrocytes (Hunziker, 1994; Nilsson et al., 1994; Nilsson et al., 2005).

1.3.1.2 Glucocorticoids

Glucocorticoids are widely used as anti-inflammatory and immunosuppressive drugs in children (Silvestrini et al., 2000). Long-term, high-dose glucocorticoid treatment often leads to growth failure and osteoporosis (Silvestrini et al., 2000). Similarly, systemic administration of glucocorticoids in mice, rats, and rabbits decreases the rate of

longitudinal bone growth, by inhibiting growth plate chondrocyte proliferation (Silvestrini et al., 2000). In addition, glucocorticoids may stimulate apoptosis of growth plate chondrocytes (Nilsson et al., 1994). The local effects of glucocorticoids on growth plate chondrocyte proliferation may be mediated, in part, by changes in the local Igf-I system (Nilsson et al., 2005; Silvestrini et al., 2000).

1.3.1.3 Thyroid hormone

The thyroid hormones, thyroxine (T4) and triiodothyronine (T3), are tyrosine-based hormones produced by the thyroid gland and primarily responsible for regulation of metabolism. Thyroid hormone is necessary for normal skeletal growth and maturation (Stevens et al., 2000). Hypothyroidism slows longitudinal bone growth and endochondral ossification, while hyperthyroidism accelerates both processes (Nilsson et al., 1994). In hypothyroid animals, there is a decrease in the heights of the proliferative and hypertrophic zones, and a decrease in chondrocyte proliferation, hypertrophy, and vascular/bone cell invasion (Stevens et al., 2000). Additionally, the normal columnar organization of the growth plate is disrupted (Stevens et al., 2000). Some of the skeletal effects of thyroid hormone appear to be due to a direct action on the growth plate (Nilsson et al., 1994).

1.3.2 Local regulatory mechanisms in the growth plate

Proliferation and hypertrophy of growth plate chondrocytes and ECM production drive the longitudinal growth of endochondral bones and eventually determine body length in mammals (Beier, 2005; Kronenberg, 2003; Okazaki and Iwamoto, 2006). Many growth factors and transcription factors control these processes. Bone morphogenetic proteins (BMPs) and the central transcription factor Sox 9 regulate early chondrocyte

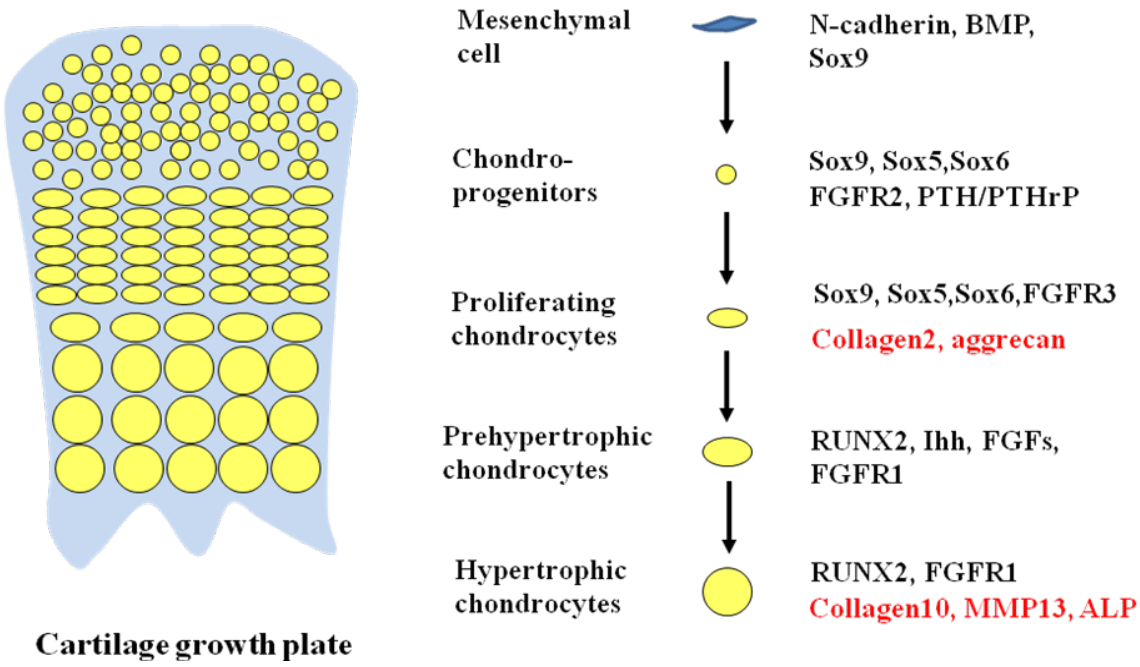
differentiation (Beier, 2005; Karsenty and Wagner, 2002). Chondrocyte proliferation is under control of Indian hedgehog (Ihh), parathyroid hormone-related protein (PTHrP) and Fibroblast growth factor (FGF) signaling, and cell-cycle genes, such as Cyclin D1 and A (Beier, 2005; Kronenberg, 2003). Eventually, chondrocytes differentiate to mature hypertrophic cells under control of the transcription factor RUNX2 among others (Karsenty and Wagner, 2002; Kronenberg, 2003; Shum and Nuckolls, 2002) (Fig. 1.3). VEGF and RANKL mediate angiogenesis and osteoclast activation respectively along with other growth factors (Olsen et al., 2000). Other local factors include transforming growth factor β, and Wnt families (Beier, 2005; Nilsson et al., 2005; Olsen et al., 2000; Teixeira et al., 2008).

1.3.2.1 The Ihh/PTHrP negative feed-back loop

Ihh is a master regulator of bone development, coordinating chondrocyte proliferation, chondrocyte differentiation and osteoblast differentiation (Kronenberg, 2003). During endochondral ossification, Ihh is synthesized by prehypertrophic and early hypertrophic chondrocytes within the growth plates. It binds to its receptor Patched-1 (Ptc-1), causing activation of Smoothened (Smo), which then triggers a cascade leading to gene activation (Kronenberg, 2003; Wagner and Karsenty, 2001). PTHrP is secreted from perichondral cells and resting chondrocytes at the ends of long bone. It acts on receptors on proliferating chondrocytes to keep them in the proliferative pool (Nilsson et al., 2005). The interaction of Ihh and PTHrP leads to the hypothesis that these two paracrine factors together control the number of chondrocytes in the proliferative pool through a feed-back loop (Karp et al., 2000; Kronenberg, 2003). When chondrocytes close to the hypertrophic zone are no longer sufficiently stimulated by PTHrP, they stop

Figure 1.3 Local regulators of growth plate and life cycle of chondrocyte.

Longitudinal growth of the cartilage growth plate is under control of many secreted factors and endogenous regulators such as transcription factors. Each stage of chondrocyte is characterized by express specific molecular markers and matrix proteins (in red): the aggregates of mesenchymal cells express BMPs and Sox family proteins; resting cells express Sox proteins, PTH/PTHrP, FGFR2 and collagen II; proliferative chondrocytes express FGFR3, cell cycle proteins such as Cyclins D1 and A, and collagen II and aggrecan; prehypertrophic cells are under control of the transcription factor RUNX2, and express Ihh and FGFs; hypertrophic cells express collagen X, MMP13 and ALP in bone matrix.



Cartilage growth plate

proliferating and start to synthesize Ihh. Ihh then acts on its receptor on chondrocyte to increase the rate of proliferation and also stimulates the production of PTHrP at the ends of growth plates through an unknown mechanism (Wagner and Karsenty, 2001). Ihh also acts on perichondrial cells to convert these cells into osteoblasts of the bone collar (Karp et al., 2000).

1.3.2.2 Opposing actions of BMPs and FGFs

FGFs are secreted proteins that act through tyrosine kinase receptors. The FGF family of proteins consists of at least 23 structurally related polypeptides that play a critical role in a variety of biological processes. FGF1, 2, 4, 8, 9, and particularly 18 (Provot and Schipani, 2005), and their four receptors are expressed during endochondral bone formation (Ornitz and Marie, 2002). It has been shown that FGF signaling regulates chondrocyte proliferation and differentiation (Karsenty and Wagner, 2002; Kronenberg, 2003; Ornitz and Marie, 2002). FGF receptor 3 (FGFR3) is expressed in proliferating chondrocytes; FGFR1 is expressed in prehypertrophic and hypertrophic chondrocytes and perichondrium; and FGFR2 is expressed in the perichondrium and primary spongiosa (Kronenberg, 2003). FGF18 is expressed in the perichondrium, and acts on FGFR 3 to decrease chondrocyte proliferation, to increase the production of Ihh, and to accelerate the differentiation of hypertrophic chondrocyte into terminal differentiated chondrocytes (Ornitz and Marie, 2002). BMPs are members of the TGF-β family of paracrine factors, which have essential roles at every stage of endochondral bone development (Hoffmann and Gross, 2001). They act on each of these steps, sometimes in a manner opposite to the effects of FGFs (Kronenberg, 2003). They accelerate chondrocyte proliferation, increase Ihh production and stimulate terminal chondrocyte differentiation, as shown by the

expression of osteopontin and other molecular markers (Kronenberg, 2003; Ornitz and Marie, 2002).

1.3.2.3 CNP regulation of endochondral bone growth

The natriuretic peptide family consists of three structurally related peptides: atrial natriuretic peptide (ANP); brain natriuretic peptide (BNP); and c-type natriuretic peptide (CNP) (Pejchalova et al., 2007; Yasoda and Nakao, 2009). These molecules regulate a number of biological processes through intracellular cGMP pathways, by acting through two different membrane-bound receptors: GC-A (NPR-A) and GC-B (NPR-B) (Yasoda and Nakao, 2009). CNP acting through its receptor, NPR-B, plays an important role in endochondral ossification (Teixeira et al., 2008). A number of recent studies of genetic mouse models and organ cultures demonstrated its role *in vivo* and *in vitro* (Agoston et al., 2007; Chikuda et al., 2004; Chusho et al., 2001; Miyazawa et al., 2002; Pejchalova et al., 2007; Teixeira et al., 2008; Yasoda et al., 2004). *CNP*-deficient mice display dwarfism due to impaired endochondral bone formation (Chusho et al., 2001; Pejchalova et al., 2007). The deficient mice have a narrow growth plate with a significant reduction in the hypertrophic zone (Chusho et al., 2001; Miyazawa et al., 2002). *Npr2* (NPR-B) KO mice have a similar bone phenotype as *CNP* KO mice (Bartels et al., 2004; Miyazawa et al., 2002). Metatarsals from fetal rats and tibia isolated from mouse embryos treated with CNP showed increased bone growth (Agoston et al., 2007; Mericq et al., 2000). Chondrocyte proliferation was modestly increased. The most significant characteristic of CNP treatment was increased hypertrophic zone length, due to both increase in size and number of hypertrophic chondrocytes (Agoston et al., 2007; Teixeira et al., 2008).

However, while many extracellular regulators of cartilage development and transcription factors controlling growth plate function have been identified, much less is known about the intracellular signaling pathways. Over the years, nitric oxide (NO) has been identified not only in bone cell metabolism (van't Hof and Ralston, 2001) but also been shown to be involved in endochondral bone formation (Teixeira et al., 2005) and the communication between different cell types in bone (Ralston et al., 1995; van't Hof and Ralston, 1997).

1.4 *Nitric oxide and Bone*

1.4.1 The discovery of NO

Nitric oxide (NO) was first discovered as an endothelial-derived relaxation factor more than two decades ago (Furchgott, 1999; Ignarro, 1999). NO physiology soon became one of the most rapidly growing research areas in biology. The importance of the pioneering work on this small molecule was recognized by the 1998 Nobel Prize in Physiology and Medicine to Drs. Robert F. Furchgott, Louis J. Ignarro, and Ferid Murad.

1.4.2 The physiological functions of NO

NO can function as an intracellular messenger, an autacoid, a paracrine substance, a neurotransmitter, or as a hormone that can be carried to distant sites for the effects (Murad, 1999). The versatile functions of NO come from the chemical properties of the compound. NO is a gaseous uncharged free radical with an unshared electron that makes it easy to react with many molecules in order to regulate biological processes (Moncada et al., 1991). Because it is uncharged, it can freely diffuse to the surrounding cells, making it ideal as a signaling molecule (Teixeira et al., 2008). In most processes, NO

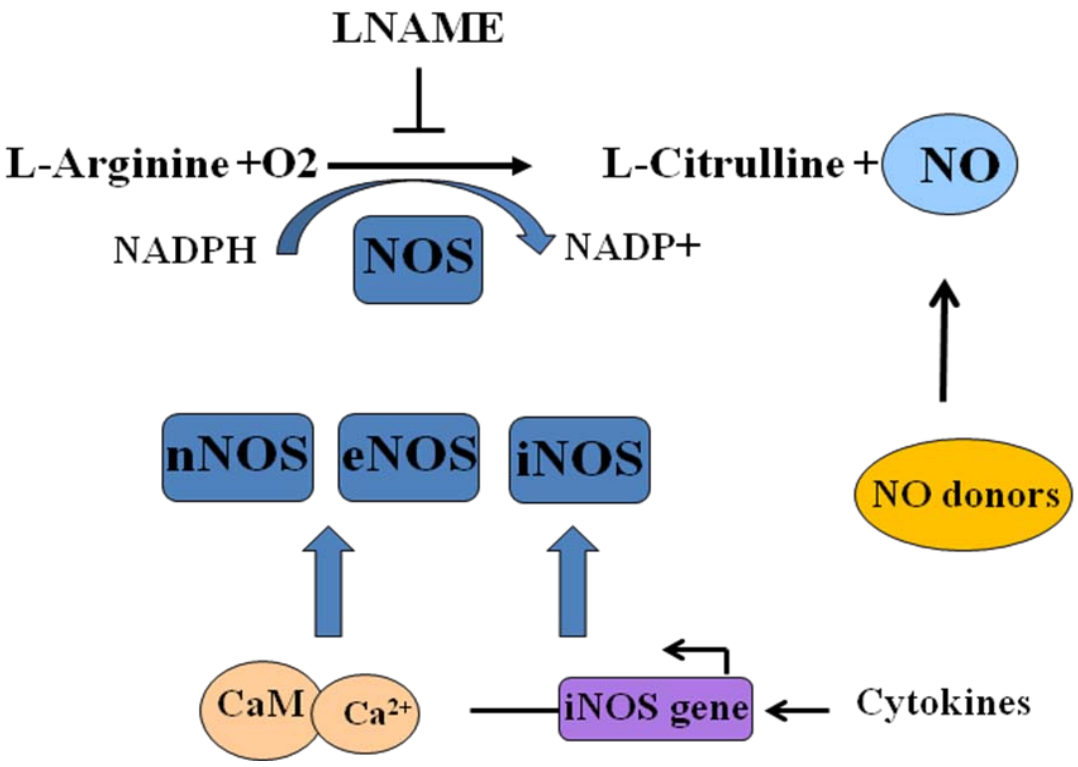
activates soluble guanylyl cyclase (sGC) and causes increased levels of the intracellular secondary messenger cGMP (Moncada et al., 1991; Murad, 1999). However, the reactive properties of NO and the derivatives of NO were found to play important roles in its biological functions too (Lundberg et al., 2008; Marshall et al., 2004; Moncada et al., 1991). For example, NO can interact with superoxide rapidly to form peroxynitrite, which is known to induce smooth muscle cell lipid peroxidation during inflammation (Lundberg et al., 2008). The functions of NO cover almost every biological aspect, for example, in the cardiovascular system it causes vascular relaxation, prevents platelet aggregation, inhibits leukocyte adhesion and scavenges superoxide anions (Ignarro, 1999; Moncada and Higgs, 1993). It is also a neurotransmitter at synaptic endplates in the vagal system (Moncada and Higgs, 1993; Murad, 2006). The ultimate effects of NO are determined by its level, its origin, and by what else in the microenvironment it will react to (Moncada et al., 1991).

1.4.3 NO synthesis and regulation

NO is generated through nitric oxide synthase (NOS) enzymes by oxidizing a guanidino nitrogen of L-arginine to NO and L-citrulline (Moncada and Higgs, 1993; Murad, 2006) (Fig. 1.4). NOS proteins use complex cosubstrates, including molecular oxygen, NADPH, FAD, FMN and heme iron groups, and require the cofactors calmodulin and BH_4 to bind the enzyme as a homodimer (Murad, 2006). This reaction has to occur at adequate oxygen and cofactor levels (BH_4, NADPH), or the uncoupling of the electron will form superoxide instead of NO (Moncada and Higgs, 1993; Murad, 2006). The enzyme activity can be inhibited by substituted arginine analogues such as LNMMA and LNAME. NO can also be generated non-enzymatically from nitrite in the

Figure 1.4 Generation of NO from NOS.

NO is formed by NOS enzymes through oxidizing a guanidino nitrogen of L-arginine to NO and L-citrulline. This reaction usually occurs at adequate oxygen and cofactor levels (BH4, NADPH). The enzyme activity can be inhibited by substituted arginine analogues such as L-NAME, and NO can also be generated pharmacologically by compounds *in vitro* such as organic nitrates, sodium nitroprusside or nitroglycerin. There are three NOS proteins, nNOS, iNOS and eNOS. nNOS and eNOS activities are regulated by intracellular calcium and calmodulin. Cytokines can induce iNOS to produce NO at a high level for a prolong time period.


LNAME
L-Arginine +O2
L-Citrulline +
NO
NADPH
NOS
NADP+
nNOS
eNOS
iNOS
NO donors
CaM
Ca2+
iNOS gene
Cytokines

acid environment of the stomach (van't Hof and Ralston, 2001) and pharmacologically by compounds such as organic nitrates, sodium nitroprusside or nitroglycerin, which have been used clinically for treatment of angina pectoris for over two centuries (Murad, 1999).

Three different NOS proteins have been identified so far. The neuronal form of NOS (nNOS or NOS1) was first identified in brain (Moncada and Higgs, 1993), then found to be expressed in many cell types. The endothelial form of NOS (eNOS or NOS3) was first discovered in endothelial cells (Ignarro, 1999), but most cell types express eNOS. The inducible form of NOS (iNOS or NOS2) can be induced by LPS or many inflammatory cytokines in almost every cell type and plays an important role in inflammatory diseases (Furchgott, 1999) (Tab. 1.1). These proteins are encoded by three different genes on different chromosomes and have about 50-60% homology with each other and the cytochrome P450 enzymes (Murad, 2006).

Both eNOS and nNOS (collectively called cNOS) constitutively produce NO at low levels (picomolar range) in many cell types. Their activity is mainly regulated by changes in intracellular Ca^{2+} concentration and activities of calmodulin (Ignarro, 1999; Moncada et al., 1991; Scher et al., 2007). Expression of eNOS has been shown to be activated by phosphorylation at serine 1177 by many signaling pathways, such as heat-shock protein (HSP90), Akt or acetylcholine (Ach) (Feelisch, 2008; Forstermann and Kleinert, 1995). Additionally, eNOS gene promoter activity is enhanced by shear stress and changes in estrogen levels (Armour et al., 2001a; Klein-Nulend et al., 1998; van't Hof and Ralston, 2001). Consistent with this, eNOS mRNA level has been found to be increased in endothelial cells after exposure to physiological concentration of estrogen

(Armour et al., 2001a) and in response to shear stress (Klein-Nulend et al., 1998). nNOS protein is found in the synaptic endplates, scarcoplasic reticulum, and mitochondria (Feelisch, 2008) and is upregulated upon activation of synaptic endplates in the central nervous system (Moncada et al., 1991). The regulation of nNOS expression seems to be very complex as reflected by at least eight different promoters transcribing eight different exon sequences in different cell types (Forstermann and Kleinert, 1995).

In contrast, iNOS is capable of generating large quantities of NO (nanomolar range) over a prolonged time period. However, because iNOS activity depends on transcription, response to external stimuli is not as rapid as that of cNOS. Properties of these three NOS are summarized in Table 1. (Tab. 1.1). Activation of the transcription factor NF-κB seems to be an essential step for iNOS induction in most cells (Feelisch, 2008). iNOS can be activated by the endotoxin LPS and by pro-inflammatory cytokines, such as interleukin 1 (IL-1), tumor necrosis factor alpha (TNFα) and interferon γ (INFγ) through NF-κB pathways, whereas glucocorticoids and the anti-inflammatory cytokines IL-4, IL-10 and TGFβ are inhibitory (van't Hof and Ralston, 2001). It is now clear that the iNOS promoter is markedly upregulated by several cytokines and endotoxins (Forstermann and Kleinert, 1995). Interestingly, different combinations of cytokines differ in their abilities to produce NO in different cell types. For example, human chondrocytes can be induced by single cytokines such as IL-1β or TNFα to produce NO, whereas human primary osteoblasts require two or three cytokines for significant induction of NO production (Ralston, 1997).

Table 1.1 Sumary table of Nitric Oxide Synthases (NOS)

	Isoforms	Examples of source	Calcium/ Calmodulin	Characters	Examples of Function
Constitutive NOS	nNOS, NOS1	Brain	Dependent	Picomole NO fast release; Short lasting; Signaling molecule; Stimulated by Ach	Learning
	eNOS, NOS3	Macrophage	Dependent		Vasodilation
Inducible NOS	iNOS, NOS2	Endothelium	Independent	Nanomole NO slow release; Long lasting; Induced by cytokines	Cytotoxicity

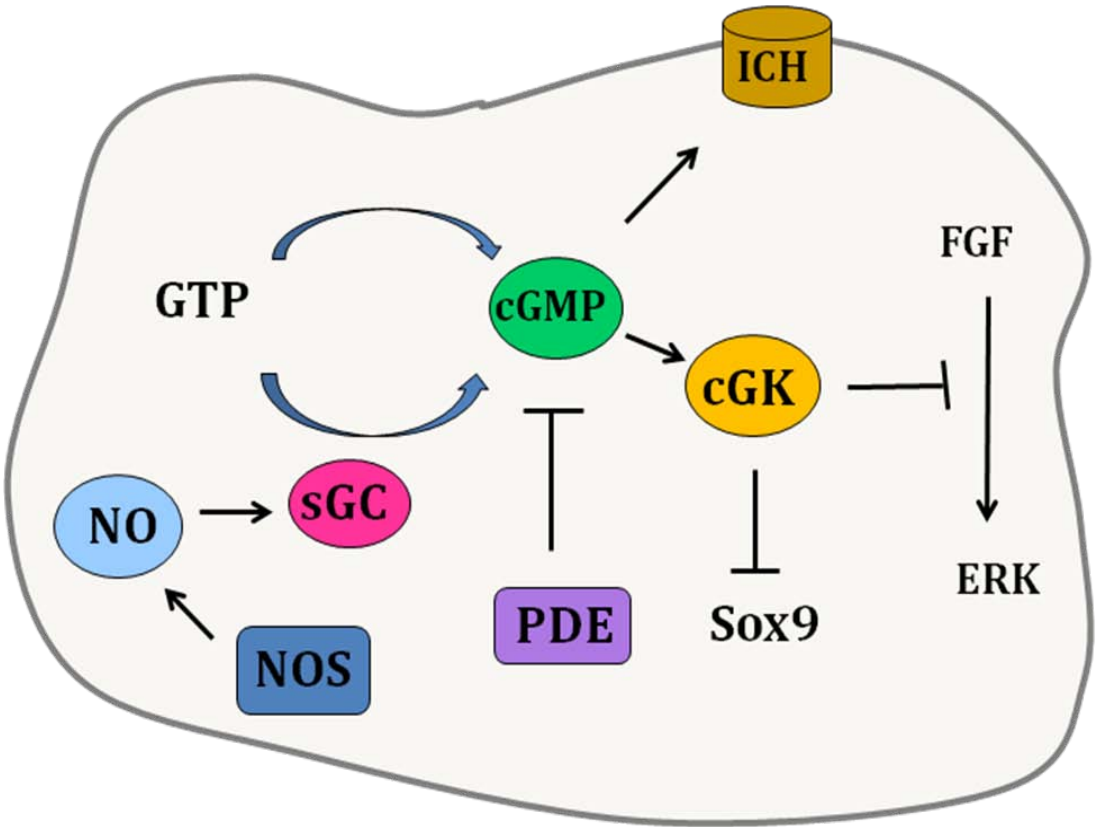
Additionally, high levels of NO produced by iNOS actually inhibit NF-κB activity itself, thus limiting iNOS and NO production in a negative feed-back loop (Moncada et al., 1991). Recently, cNOS were also found to be inducible by cytokines while iNOS was found constitutively expressed in many cell types (Feelisch, 2008) including chondrocytes (Rosa et al., 2008).

1.4.4 Molecular pathways of NO action

NO is a gaseous uncharged free radical with an unshared electron, making it very reactive. Because it is uncharged, it can freely diffuse to surrounding cells once it's formed (Murad, 1999). In most processes, NO from cNOS serves as a messenger molecular and its physiological functions are mediated by its direct activation of the heme-containing moiety of sGC in target cells (Collin-Osdoby et al., 1995). Activation of sGC causes an increase in the intracellular cGMP levels (Moncada and Higgs, 1993; Moncada et al., 1991). cGMP is a very important second messenger in many cell types and interacts with specific binding sites in target proteins, including cGMP-dependent protein kinases (PKG), cGMP-binding proteins (e.g. phosphodiesterases, PDEs) and cGMP-dependent ion channels (e.g. ICH) (Feelisch, 2008; Murad, 2006) (Fig. 1.5). Activation of PKG triggers a cascade of phosphorylation events that culminate in the alteration of various cellular processes, e.g. cell proliferation, smooth muscle relaxation, cytotoxicity and neurotransmission (Collin-Osdoby et al., 1995).

Figure 1.5 Molecular pathways of NO in cartilage.

Once NO is produced, it can freely diffuse to surrounding cells and activates sGC, which causes an increase in the intracellular secondary messenger cGMP levels. cGMP is a very important second messenger in many cell types and interacts with specific binding sites in target proteins, including cGMP-dependent protein kinases (PKG), cGMP-binding proteins (e.g. phosphodiesterases, PDEs) and cGMP-dependent ion channels (e.g. ICH). An important role of cGMP-dependent protein kinase II (PKGII) in bone formation has been identified. Mice lacking PKGII or its upstream activator C-type natriuretic peptide (CNP) demonstrated severe skeletal phenotypes and dwarfism. Possible downstream pathways of cGKII include the inhibition of nuclear translocation of transcription factor SOX9, suppression of FGF signaling, and activation of MAPK (p38 and ERK). (Adapted from Teixeira et al., 2008)


ICH
GTP
cGMP
cGK
FGF
NO
sGC
PDE
Sox9
ERK
NOS

In contrast, high concentrations of NO, for example from iNOS, can bind to tyrosine or cysteine residues of many thiol containing proteins and lead to nitration and nitrosylation of the proteins (Lundberg et al., 2008; Marshall and Stamler, 2001; Stamler et al., 2001). For example, S-nitrosylation of glutathione, leads to inhibition of glutathione and other redox-signaling regulation pathways (Stamler et al., 2001).

1.4.5 Mechanisms of nitrite, nitrate, peroxynitrite

The half life of NO is only a few seconds because it converts to nitrite and nitrate shortly after it is formed (Furchgott, 1999). These products have been used as markers of NO formation for a long time. It is clear now that these derivatives of nitrogen are responsible for many biological effects of NO. For example, nitrite has been shown to protect tissues against ischemia/reperfusion-related injuries in several organs (Feelisch, 2008). This is particularly important for cartilage, because cartilage is an avascular tissue, and the oxygen level is only about 1-6% in deeper articular chondrocytes (Hirao et al., 2006; Schipani et al., 2001). NO formation by NOS requires abundant oxygen levels, while nitrite formation is independent of oxygen. Therefore, when oxygen levels fall below a critical threshold, nitrite and nitrate react to mediate indirect effects of NO, and nitrite reduction to NO may serve to prevent a drop in NO concentration (Feelisch, 2008).

At the same time, NO can interact with superoxide to form the very reactive peroxynitrite and hydroxyl radicals that are responsible for tissue damage during inflammatory process by inducing lipid peroxidation (Feelisch, 2008). Peroxynitrite becomes a marker for inflammation and formation of NO in many cell types (Stamler et al., 2001).

1.4.6 Expression of NOS proteins in cartilage

All three NOS proteins are expressed in chicken, and NO derivatives accumulate in the calcified region of the chicken cartilage (Teixeira et al., 2005), suggesting a role of NOS genes in cartilage development. iNOS was found spontaneously expressed in normal human articular cartilage, but at a higher level in chondrocytes from osteoarthritis (OA) (Rosa et al., 2008). This implies an important role of iNOS in articular cartilage, and indeed iNOS is speculated to participate in almost every aspect of OA pathophysiology (Abramson, 2008; Abramson et al., 2001; Amin and Abramson, 1998; Feelisch, 2008; van den Berg, 2001).

Over years NO has been known to regulate bone cell metabolism and bone remodeling (Abramson, 2008; Teixeira et al., 2008; van't Hof and Ralston, 2001). Recently, with the reported roles of cGKII and CNP as regulators of endochondral bone formation, the biological role of NO in bone formation has been investigated further.

1.4.7 NO in cartilage development and endochondral bone formation

In vitro studies suggest that NO signaling is required for chondrocyte maturation (Teixeira et al., 2005). In chicken cartilage, NO synthesis is required for development of the mature chondrocyte phenotype by upregulating alkaline phosphatase (ALP) and type X collagen expression (Teixeira et al., 2005). Inhibition of NOS, sGC, or cGK blocked retinoic-acid induced chondrocyte maturation *in vitro* (Kirimoto et al., 2005). High levels of NO, whether from nitric oxide donors or endogenously induced by cytokines, has been shown to induce chondrocyte apoptosis, and recently this apoptosis has been linked to generation of reactive oxygen species in chondrocyte (Lotz et al., 1999). Interestingly, a study showed that low levels of NO protect chondrocytes from cell death through

upregulation of heme oxygenase 1 (HO-1) and NF-κB, and concomitant downregulation of both extracellular signaling regulated protein kinases ERK 1/2 and p38 activation (Kim et al., 2005).

In recent years, a study of genetically altered mice discovered that *eNOS* gene knockout mice display reduced bone volume and bone formation at 6 weeks of age, but a normal bone phenotype was restored at 12-18 weeks (Aguirre et al., 2001). Another study showed that *eNOS* knockout mice present limb deficiencies with short digits, reduced weight and delayed growth (Hefler et al., 2001). It was proposed that these abnormities were caused by insufficient blood flow to the limbs and hemorrhage. However, the exact mechanism and role of eNOS in earlier stages of skeletal development is not well understood. Other groups demonstrated that *nNOS* knockout mice present higher bone mineral density in 10-week old female mice, particularly in trabecular bone (van't Hof et al., 2004). Moreover, administration of NOS inhibitors to drinking water of pregnant rats induced fetal growth retardation and hindlimb disruptions in pups (Diket et al., 1994). However, because of potential compensatory mechanism between the three NOS proteins and the possibility of non-specific actions of NOS inhibitors, the *in vivo* role of NO and NOS genes in endochondral ossification still waits to be elucidated.

Interestingly, double knockout mice for two NOS genes do not display significant growth defects as also shown for triply knockout mice of all three *NOS* genes (Sabanai et al., 2008; Teixeira et al., 2008). However, the double and triple knockout mice present high frequency of embryonic lethality and very low survival rates postnatally (15% in triple knockout mice) (Sabanai et al., 2008; Teixeira et al., 2008). This suggests occurrence of major abnormalities during development in most embryos. Postnatal death

of triple knockout mice is mostly due to spontaneous myocardial infarction accompanied by severe coronary arteriosclerotic lesions (Nakata et al., 2008), but the surviving knockout mice surprisingly live a normal life. This suggests that other effects may compensate for loss of all three *NOS* genes. One study of triple knockout mice showed increased bone mineral density in 12-20 week-old mice, but these parameters were normal in younger (4 week-old) mice (Sabanai et al., 2008). This study also demonstrated increases in bone formation rate, mineral apposition rate, and serum alkaline phosphatase concentration in triple knockout mice (Sabanai et al., 2008). These results further confirm the role of NOS in bone homeostasis. More generally, analyses of mice with inactivation of two or all three *NOS* genes suggest that the phenotypes of these mice are variable, ranging from very mild phenotypes to lethality (Teixeira et al., 2008). More detailed studies on the knockout mice and other compensatory mechanisms need to be completed.

1.4.8 Effects of NO on bone remodeling, osteoblasts and osteoclasts

NO appears to have biphasic effects on osteoblasts and osteoclasts. The constitutive production of NO at low concentrations promotes the proliferation and differentiation of osteoblasts and modulates osteoblast function. Some investigators have shown that slow release of NO donors stimulates osteoblast proliferation and differentiation *in vitro,* while NOS inhibitors had little effects on them (MacPherson et al., 1999). eNOS signaling was found to be important for the osteoblast lineage (Grassi et al., 2006). Several studies showed reduced bone formation in *eNOS* knockout mice accompanied by reduced osteoblast number and mineralization (alkaine phosphatase, ALP and mineral deposition) *in vivo* and *in vitro* (Aguirre et al., 2001). Additionally, eNOS signaling was shown to be essential for estrogen and mechanical loading induced bone formation (Armour et al.,

2001a; Furchgott, 1999). Another study revealed that eNOS may act to mitigate an exaggerated state of estrogen-deficiency-induced bone remodeling (Aguirre et al., 2001). *nNOS* knockout mice present reduced bone remodeling with reduced remodeling surface and a reduction in osteoblast numbers *in vivo* (van't Hof et al., 2004). In contrast, high concentrations of NO are also inhibitory for the osteoblast lineage, and NO production induced by IL-1β, TNF-α, and IFN-γ appears to be partly responsible for the inhibitory effects on osteoblast proliferation (Evans and Ralston, 1996) and on the promotion of osteoblast differentiation and apoptosis (Lotz et al., 1999). As a result, high levels of NO and iNOS regulate osteoblast function and inhibit bone formation with important implications for the pathogenesis of inflammation-mediated osteoporosis (Armour et al., 2001b).

These results also support the idea that moderate induction of NO potentiates bone resorption. NO therefore appears to be an important regulatory molecule for both osteoblast and osteoclast lineages and represents one of the molecules produced by osteoblasts which directly regulate osteoclastic activity (Evans and Ralston, 1996).

It is now well-established that a high concentration of NO is a potent inhibitor of osteoclast-mediated bone resorption. When combined with other cytokines, IFN-γ markedly induces NO production, which suppresses osteoclast formation and activity of mature osteoclasts (Brandi et al., 1995). This high concentration of NO is largely responsible for the selective inhibitory effect of IFN-γ on cytokine-induced bone resorption. It is suggested that the inhibitory effects of NO on bone resorption are partly explained by induction of apoptosis in osteoclast progenitors rather than osteoclast formation (Ralston, 1997). Therefore, it is of interest to study the selectivity of NO-

induced bone cell apoptosis and the communication between bone cells, and it is of clinical relevance that modulating NO concentrations may have therapeutical potential.

There is no evidence to support a role of eNOS for normal osteoclast formation and activity under physiological conditions in knockout mice (van't Hof and Ralston, 2001). *nNOS* knockout mice present reduced bone remodeling and a reduction in osteoclast numbers *in vivo*, suggesting a role of nNOS in stimulation of bone turnover (van't Hof et al., 2004).

1.4.9 Downstream targets of NO in bone

The cellular pathways downstream of NO in skeletal cells are complex and poorly understood. As discussed above, an important role of cGMP-dependent protein kinase II (PKGII) in bone formation has been identified (Chikuda et al., 2004; Pfeifer et al., 1996). Mice lacking PKGII or its upstream activator C-type natriuretic peptide (CNP) demonstrated severe skeletal phenotypes and dwarfism (Chusho et al., 2001; Miyazawa et al., 2002; Pejchalova et al., 2007; Pfeifer et al., 1996). Because both NO and CNP stimulate the production of cGMP via soluble or particulate guanylyl cyclases (GC) (Chusho et al., 2001), the cGMP/cGKII pathway would be a likely mediator of NO signaling in cartilage. Identification of the downstream targets of cGKII in bone formation is under investigation by several groups. Some studies of CNP signaling suggested that possible downstream pathways of cGKII include the inhibition of nuclear translocation of transcription factor SOX9 (Miyazawa et al., 2002), suppression of FGF signaling, and activation of MAPK (p38 and ERK) (Agoston et al., 2007; Teixeira et al., 2008; Yasoda et al., 2004) (Fig. 1.5). In agreement with these models, IL-1 caused a release of FGF in rabbit articular chondrocyte and stimulated cell proliferation, which

was blocked by NOS inhibition (Jang and Murrell, 1998). However, cGKII is not the only downstream target of NO (Feelisch, 2008; Kim et al., 2005). The role of cGKI and the indirect effects of the NO derivatives nitrite, nitrate, and peroxynitrite in individual bone cells need to be studied further.

1.5 *Overall objectives and hypotheses*

1.5.1 Rational

Endochondral ossification is controlled by a process of chondrogenesis, chondrocyte proliferation, differentiation and eventually hypertrophy. During postnatal development, proliferation and hypertrophy occur in a controlled fashion in the cartilage growth plate, regulating the full length of adult bone. As we discussed above, NO promotes chondrocyte maturation and apoptosis *in vitro,* and *NOS* genes play an important role in bone formation and remodeling. However, little is known about the role of these genes in the cartilage growth plate and in early endochondral bone formation. The recent identification of CNP/cGMP/cGKII as important regulators of endochondral bone formation provides further motivation to study the role of NO/NOS in growth plate development and endochondral ossification. The overall objective of this study was to identify the effects of the NO/NOS pathways on specific events during the endochondral ossification process, in particular chondrocyte proliferation and hypertrophic differentiation.

1.5.2 Overall hypothesis

NOS/NO controls endochondral ossification by regulating chondrocyte proliferation and differentiation.

1.5.3 Research plan and specific aims

1.5.3.1 Aim 1: Analyze the roles of iNOS/NO in growth plate physiology and its interaction with the *Rac1* gene *in vivo*

Objective: We had shown recently that chondrocyte-specific deletion of the *Rac1* gene in mice leads to severe dwarfism due to reduced chondrocyte proliferation, but the molecular pathways involved remained unclear. Our study examined the iNOS/NO pathway as a novel mediator of mitogenic Rac1 signaling and implicated it as a target for therapies for growth disorders.

Hypothesis: iNOS/NO signaling mediates mitogenic signaling of *Rac1* gene in cartilage

Main findings:

1. *Rac1*-deficient chondrocytes display severely reduced levels of iNOS protein and NO production. NO donors reversed the proliferative effects induced by *Rac1* deficiency, whereas inhibition of NO production mimicks the effects of Rac1 loss of function.
2. Examination of the growth plate of *iNOS*-deficient mice revealed reduced chondrocyte proliferation and lower expression of cyclin D1, a key regulator of chondrocyte proliferation.

3. Rac1-NO signaling inhibits the expression of ATF3, a known suppressor of cyclin D1 expression in chondrocytes, identifying ATF3 as a link between Rac1/iNOS and the chondrocyte cell cycle.

1.5.3.2 Aim 2: Characterize the roles of eNOS/NO pathway in growth plate physiology *in vivo*

Objective: NO has been implicated both in the bone remodeling and pathogenesis of osteoarthritis, but the role of specific NOS enzymes in chondrocyte physiology is unclear. The objective of this study was to examine the effects of inactivation of *eNOS* on cartilage development using genetically modified mice.

Hypothesis: eNOS/NO signaling promotes endochdondral bone growth through direct effects in growth plate chondrocytes.

Main findings:

1. *eNOS*-deficient mice show increased lethality and reduced bone growth, with hypocellular growth plates and a marked reduction in the number of proliferating chondrocytes.
2. *In vitro* studies demonstrated lower chondrocyte numbers and reduced endochondral bone growth in mutants, suggesting that the role of eNOS signaling in chondrocyte proliferation is cell-autonomous.
3. Reduced chondrocyte numbers appear to be caused by decreased cyclin D1 and increased p57 expression in mutants, resulting in slower cell cycle progression and earlier cell cycle exit.

4. The prehypertrophic markers P57, RORα and Hif-1α were expressed prematurely in mutant mice.

1.5.3.3 Aim 3: Determine the roles of nNOS/NO pathway in cartilage growth plate *in vivo*

Objective: We recently described the effects of *eNOS* deficiency on cartilage development. Interestingly we found an upregulation of nNOS transcription levels in *eNOS*-deficient cartilage. Therefore, now we extended these studies to mice deficient for *nNOS*.

Hypothesis: nNOS/NO signaling promotes chondrocyte proliferation and inhibits differentiation in the cartilage growth plate *in vivo*.

Main findings:

1. *nNOS*-null mice show transient growth retardation and shorter long bones. *nNOS*-deficient growth plates display a marked reduction in replicating proliferating chondrocytes.
2. Histological sections also demonstrated thinner cortical bone and fewer trabeculae in mutant mice.
3. Reduced chondrocyte numbers may in part be due to premature cell-cycle exit because of decreased cyclin D1 and increased p57 expression in mutants.
4. Increased chondrocyte apoptosis was shown by increased cleaved-caspase 3 staning in mutants.
5. Expressions of prehypertrophic markers p57, RORα, and c-Fos were increased, while the expression of Hif1α was not changed in mutants.

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CHAPTER TWO

iNOS/NITRIC OXIDE SIGNALING MEDIATES THE MITOGENIC ACTIVITY OF RAC1 DURING ENDOCHONDRAL BONE GROWTH*

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Wang, G.*, Yan, Q. *, Woods, A., Feng, Q. and Beier, F. iNOS/nitric oxide signaling mediates the mitogenic activity of Rac1 during endochondral bone growth. (*contributed equally). We are currently revising this manuscript for *J. of Cell Science.*

2.1 *Chapter summary*

Coordinated proliferation and differentiation of growth plate chondrocytes controls endochondral bone growth and final height in humans, and disruption of this process results in diseases of the growing and adult skeleton such as chondrodysplasias or osteoarthritis. We had shown recently that chondrocyte-specific deletion of the *Rac1* gene in mice leads to severe dwarfism due to reduced chondrocyte proliferation, but the molecular pathways involved remained unclear. Here, we demonstrate that *Rac1*-deficient chondrocytes display severely reduced levels of iNOS protein and NO production. NO donors reversed the proliferative effects induced by *Rac1* deficiency, whereas inhibition of NO production mimicks the effects of Rac1 loss of function. Examination of the growth plate of *iNOS*-deficient mice revealed reduced chondrocyte proliferation and expression of cyclin D1, a key regulator of chondrocyte proliferation. Finally, we demonstrate that Rac1-NO signaling inhibits the expression of ATF3, a known suppressor of cyclin D1 expression in chondrocytes, identifying ATF3 as a link between Rac1/iNOS and the chondrocyte cell cycle. In conclusion, our studies identify the iNOS/NO pathway as a novel mediator of mitogenic Rac1 signaling and implicate it as a target for therapies for growth disorders.

2.2 *Introduction*

Growth of endochondral bones is regulated by the coordinated proliferation and differentiation of growth plate chondrocytes (Kronenberg, 2003; Olsen et al., 2000). Cell volume increase during hypertrophy provides one driving force for bone growth, while ordered proliferation is required for generation of sufficient cell numbers for subsequent hypertrophy (Beier, 2005). Thus, inhibition of either proliferation or hypertrophy can both result in dwarfism. Numerous diseases are caused by disruption of these processes, for example by gene mutations, endocrine imbalances and mechanical or nutritional influences. Many growth factors and hormones have been shown to regulate chondrocyte proliferation, including fibroblast growth factors, parathyroid hormone-related peptide, and Indian hedgehog (Ballock, 2003; van der Eerden et al., 2003). However, the intracellular mechanisms controlling chondrocyte proliferation and hypertrophy are much less understood.

We have previously reported that chondrocyte-specific inactivation of the *Rac1* gene, encoding a Rho family GTPase with a central role in cellular signaling pathways, causes dwarfism and numerous skeletal abnormalities (Wang et al., 2007). A similar phenotype of reduced long bone growth was observed upon limb-specific inactivation of the *Rac1* gene (Morita et al., 2007). Rho family GTPases act as molecular switches that become activated in response to specific extracellular stimuli through the activity of guanine nucleotide exchange factors (GEFs) that load GTP onto the GTPase (Heasman and Ridley, 2008; Vega and Ridley, 2007; Vega and Ridley, 2008). Activated Rho proteins then interact with a large variety of downstream effectors, including cytoskeletal

regulators and various kinases, to control a number of cellular events such as cell migration, cell cycle progression and gene expression.

The defects we observed in cartilage-specific *Rac1* KO mice appear to be due, at least to a large part, to reduced chondrocyte proliferation (Wang et al., 2007), but the downstream mechanisms connecting Rac1 to chondrocyte proliferation are unknown. Rac1 has been shown to control many cellular processes, including actin dynamics, gene expression and generation of reactive oxygen species (ROS) (Bustelo et al., 2007; Hordijk, 2006). There are many interactions between ROS and NO (Thomas et al., 2008); however, NO or the enzymes involved in its synthesis have not been identified as major mediators of Rac1 signaling. Three different NOS have been identified, eNOS, iNOS and nNOS (endothelial, inducible and neuronal NOS, respectively) (Bryan et al., 2009). Here we identify iNOS as a novel target of Rac1 that mediates the mitogenic activities of this pathway during endochondral bone growth.

2.3 *Materials and methods*

2.3.1 Animals and materials

Timed-pregnant CD1 mice were purchased from Charles River Laboratories at embryonic day 15.5 mice (E15.5). S-nitroso-N-acetylpenicillamine (SNAP), 2,2'-(Hydroxynitrosohydrazino) bis-ethanamine (NOC-18), and the Rac1 inhibitor (NSC23766) were obtained from Calbiochem. N (G)-nitro-L-arginine methyl ester (L-NAME), 3-morpholinosydnonimine (SIN-1) and sodium nitroprusside (SNP) were obtained from Sigma and reconstituted in Dimethyl sulfoxide (DMSO, vehicle) according to the manufacturer's instructions. Cell culture materials and general chemicals were obtained from Invitrogen, Sigma or VWR unless otherwise stated.

The following antibodies were employed in this study: iNOS #ab15323-500, and eNOS #ab3868-100 (abcam); Rac1 #sc-217, Nitrotyrosine #sc-32757, ATF3 #sc-188, Goat-anti-mouse #sc-2005, and Goat-anti-rabbit #sc-2004 (all from Santa Cruz Biotechnology); Cyclin D1 (SP4) #9104-S1 (NeoMarkers Inc.); BrdU #03-3900 (Zymed Laboratories).

2.3.2 Mouse breeding and genotyping

Mice with a deletion of the *iNOS* gene (*NOS2*^{tm1Lau,} stock# 002609) (Laubach et al., 1995) and control mice with C57/BL6 background were obtained from Jackson Laboratory (Bar Harbor, ME), and exposed to a 12-h light–dark cycle and fed tap water and regular chow at libitum. All procedures involving animals were approved by the University of Western Ontario Animal Care and Use Committee. For PCR genotyping, tail snips were used to prepare DNA for PCR analysis. PCR genotyping was performed by simultaneous amplification of the wild-type and null *iNOS* alleles as described

(Tranguch and Huet-Hudson, 2003). PCR fragments were analyzed by agarose gel electrophoresis.

2.3.3 Primary cell culture and treatments

Primary chondrocytes were isolated essentially as described (James et al., 2006; Woods et al., 2009c). Long bones from limbs were isolated from E15.5 mouse embryos and placed in α-MEM media (Invitrogen) containing 0.2% Bovine Serum Albumin (BSA), 1 mM β-glycerophosphate, 0.05 mg/ml ascorbic acid and penicillin/streptomycin. Then the medium was removed and the bones placed in 4 ml of 0.25% trypsin-EDTA (Invitrogen) for 15 min at 37°C. Trypsin was subsequently replaced with 1 mg/ml collagenase P (Roche) in DMEM/10% fetal bovine serum (Invitrogen), and cells were incubated at 37°C with rotation at 100 rpm for 90 min. Following digestion, the cell suspension was centrifuged for 5 min at 1000 rpm, and the collagenase containing supernatant was decanted. Chondrocytes were resuspended in medium containing 2:3 DMEM:F12, 10% fetal bovine serum, 0.5 mM L-glutamine, and penicillin/streptomycin (25 units/ml). Cells were seeded in 6-well NUNC plates at a density of 5×10^4 cells per ml and incubated overnight. Primary monolayer chondrocytes were treated with 10^{-3} M L-NAME, 10^{-5} M SNAP or NOC-18 or the DMSO control (vehicle) diluted in fresh media supplemented with 0.25 mM ascorbic acid (Sigma) and 1 mM β-glycerophosphate (Sigma) and incubated for up to 5 days with replacement of the medium and treatments every other day.

2.3.4 Adenoviral infections

Adenovirus expressing Cre Recombinase or GFP (control) was purified using the Adeno-XTM virus mini-purification kit (Clontech) and incubated with primary chondrocytes from *Rac1fl/fl* mice at a multiplicity of infection (MOI) of 50. Four hours after culturing, medium was changed and cells were cultured as described above.

2.3.5 ROS and NO measurements

Intracellular ROS and NO were measured fluorometrically. The nonfluorescent dye 2′,7′-dichlorofluorescein diacetate (DCF-DA) permeated cells easily and hydrolyzed to fluorescent 2′,7′-dichlorofluorescein (DCF) upon interaction with intracellular ROS. Cultured cells were washed twice with PBS. Cells were then incubated with 20 μM of DCF-DA for 30 min and washed with PBS. The DCF fluorescence was analyzed using a fluorometer (SAFIRE microplate reader, TECAN, Australia) at an excitation of 485 nm and an emission of 530 nm. Nitric oxide production in chondrocytes was analyzed using DAF-FM DA (4-amino-5-methylamino-2',7'-difluorofluorescein diacetate), a fluorescence probe specific for NO, that was added (10 μM) to cells. The probe was diluted in culture medium. Cells were incubated with DAF-FM for 30 min at 37°C and and then washed with PBS. Measurements were made using the SAFIRE microplate reader (TECAN, Australia; excitation, 495 nm; emission, 515 nm).

2.3.6 Cell proliferation assay

Cell proliferation assays were performed using CyQUANT NF Cell Proliferation Assay Kit (Invitrogen) according to manufacturer’s protocol, as described (Yan et al., 2010). Chondrocytes were isolated as described above and seeded in 96-well NUNC plates at a density of 1000 cells/100μl/well. After treatment, the medium was discarded

and replaced with CyQUANT NF dye reagent diluted in HBSS buffer and incubated for 30 min in 37^{o}C incubator. Measurement of the fluorescence intensity of each sample was done using SAFIRE microplate reader (TECAN, Australia) with excitation at 480 nm and emission detection at 520 nm with a minimum of 6 individual wells per treatment in duplicate and three independent cell isolations. Images of primary chondrocyte were taken after treatments of 1 day in culture using a Nikon SMZ1500 microscope with Photometric CoolSNAP colour digital camera (Nikon Canada) and PTI Image Master 5 program.

2.3.7 Tibia organ culture

Tibiae were isolated from E15.5 embryos from CD1 timed-pregnant mice (Charles River Canada) using the Stemi DV4 Stereomicroscope (Zeiss) as described (Agoston et al., 2007; Wang et al., 2007). Dissection day was considered to be day 0 and tibiae were allowed to recover from dissection overnight in serum-free α-MEM media containing 0.2% BSA, 0.5 mM L-glutamine, 40 units penicillin/mL and 40 μg streptomycin/mL as described (Agoston et al., 2007). The following morning, bones in 24-well Falcon plates were measured using an eyepiece in the Stemi DV4 Stereomicroscope and treated with 10^{-3} M L-NAME, 10^{-5} M SNAP or NOC-18 or 5 X 10^{-5} M Rac1 inhibitor (NSC23766) with or without 10^{-5} M SNP, and DMSO as the vehicle. Medium was changed every 48 hrs beginning on day 1, and bones measured on days 1, 3 and 6. Results are expressed as change in length relative to day 1. Experiments were repeated at least three times, with 4–6 bones per treatment for each trial.

2.3.8 Histology, immunohistochemistry and BrdU labeling

Tibia samples from organ cultures or newborn mice were rinsed with PBS and fixed in 4% paraformaldehyde overnight. Bones were then placed in 10% formalin solution, and sent for embedding and sectioning in the Pathology lab at the Molecular Pathology Core Facility at the Robarts Research Institute (London, Ontario, Canada). Following sectioning, bones were stained with hematoxylin and eosin or safranin O/fast green using standard protocols (Solomon et al., 2009; Wang et al., 2007; Yan et al., 2010). For immunohistochemistry, sections were incubated with primary anti-Cyclin D1 antibody (1:50 dilution, NeoMarkers), anti-ATF3 antibody (1:200 dilution, Santa Cruz), anti-nitrotyrosine antibody (1:200 dilution, Santa Cruz) or anti-iNOS antibody (1:1000 dilution, abcam) overnight at 4°C. For BrdU labeling, newborn mice were injected 1h before sacrifice intraperiteally with BrdU (Roche) at a dose of 0.01 ml/g body weight. BrdU was detected in paraffin sections using anti-BrdU antibody (1:100 dilution, Zymed Laboratories). Bound antibody was visualized using the UltraVision LPValue detection system (Lab Vision) with AEC chromogen substrate (Lab Vision).

2.3.9 RNA isolation, and real-time RT-PCR

Total RNA was isolated from epiphyseal cartilage of long bone from newborn mice or primary cells using TRIzol (Invitrogen), according to the manufacturer's recommendations. Taqman real-time PCR was performed as described (James et al., 2006; Woods et al., 2005) with primers and probe sets from Applied Biosystems (*ATF3* Mm00476032_m1; *Gapdh* Mm99999915_g1). Data were normalized to *Gapdh* mRNA levels and represent averages and SEM from direct comparison of mutant and control littermates from three different crosses.

2.3.10 Statistical analysis

Statistical significance was determined by Student's T-Test or by one-way ANOVA with Bonferroni post test using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. In figures where values are shown as percentages, data were first log-transformed before applying ANOVA.

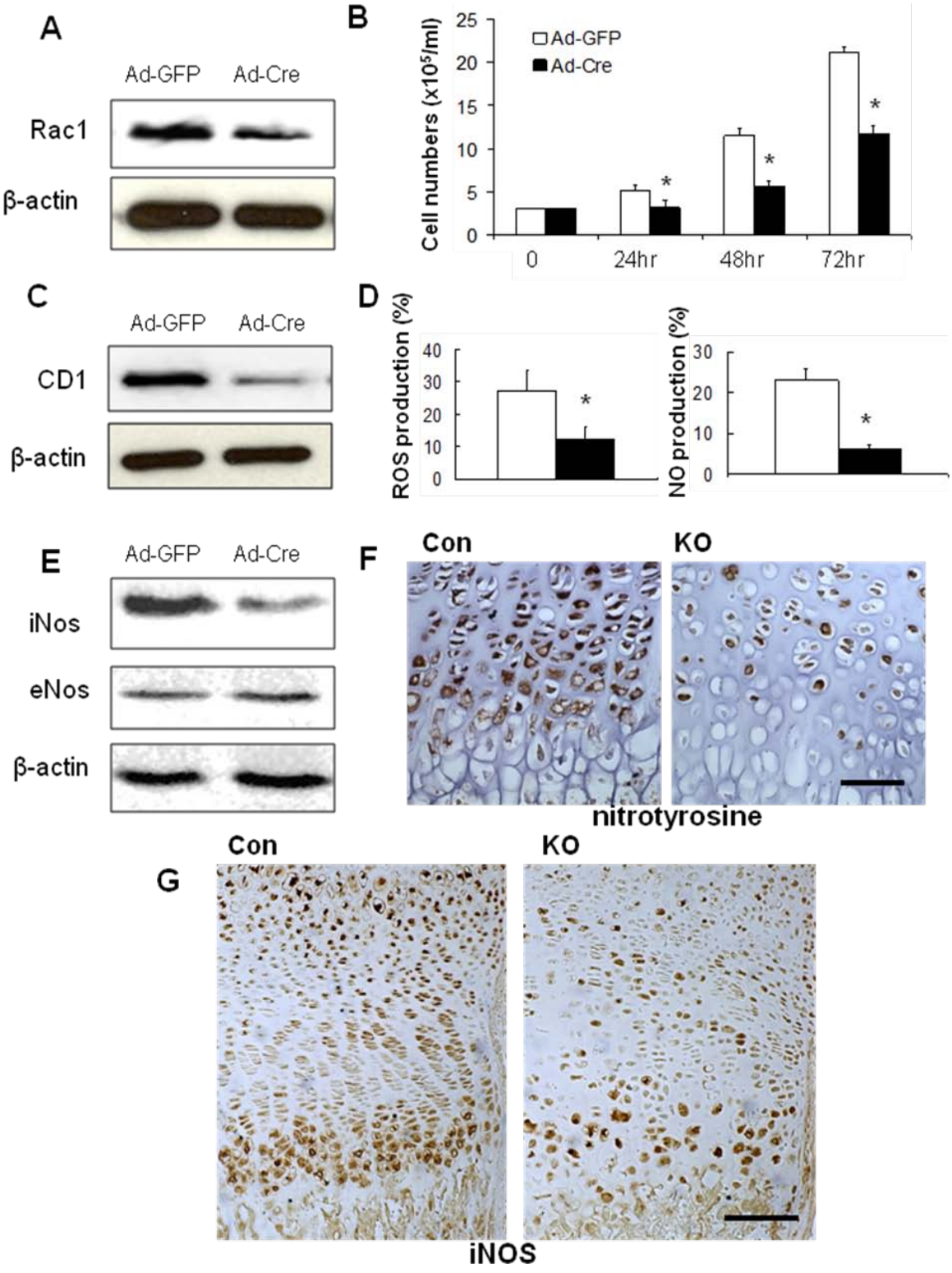
2.4 *Results*

2.4.1 Rac1 deficiency results in reduced iNOS expression and NO production

We have previously shown hypocellularity and reduced proliferation in *Rac1*-deficient growth plates (Wang et al., 2007). We first examined whether we could recapitulate this phenotype *in vitro* to facilitate biochemical studies on the underlying mechanisms. Primary chondrocytes were isolated from $Rac1^{fl/fl}$ mice, carrying two alleles of a conditional (floxed) *Rac1* allele. Cells in monolayer culture were infected with adenoviruses expressing green fluorescent protein (GFP, control) or Cre recombinase. Infection with Ad-Cre resulted in a marked reduction of Rac1 protein (Fig. 2.1A); densitometry determined that Rac1 proteins levels were reduced by 70 % in Ad-Cre-infected cultures (data not shown). Control cells increased their numbers steadily over the three-day time course of the experiment, whereas cells infected with Ad-Cre multiplied at a much slower rate (Fig. 2.1B). This was accompanied by reduced expression of cyclin D1 (Fig. 2.1C), as we had observed *in vivo*. These data documented that our *in vitro* culture

Figure 2.1 iNOS expression and NO generation are reduced in *Rac1*-deficient chondrocytes.

Primary chondrocytes were isolated from $Rac1^{fl/fl}$ mice and infected with adenovirus expressing green fluorescent protein (GFP, control) or Cre recombinase. Infection with Ad-Cre resulted in approximately 70 % reduction of Rac1 protein (A). Cell counts demonstrated that control cells increased their numbers steadily over the three-day time course of the experiment, whereas cells infected with Ad-Cre multiplied at a much slower rate (B). This was accompanied by reduced expression of cyclin D1 in mutant cells, compared to control (C). Rac1 deletion reduced ROS levels, but caused a more pronounced reduction in NO production (D) in Cre recombinase-infected cells. Western blotting demonstrated markedly reduced expression of iNOS protein in Cre-expressing chondrocytes, whereas eNOS expression was similar to control cells (E). By immunohistochemistry, iNOS staining was notably reduced in growth plates from cartilage-specific *Rac1* KO mice compared to control mice (F). Scalebar: 50µm. A similar decrease in staining in mutant mice was observed for nitrotyrosine, an indicator of cellular NO levels (G). Scalebar: 100µm. Figures are representative of 3 independent trials.


A
Ad-GFP
Ad-Cre
Rac1
β-actin
B
Cell numbers (x10⁵/ml)
Ad-GFP
Ad-Cre
0
24hr
48hr
72hr
C
Ad-GFP
Ad-Cre
CD1
β-actin
D
ROS production (%)
NO production (%)
E
Ad-GFP
Ad-Cre
iNos
eNos
β-actin
F
Con
KO
nitrotyrosine
G
Con
KO
iNOS

system provides a good model to study the effects of Rac1 deletion on chondrocyte proliferation.

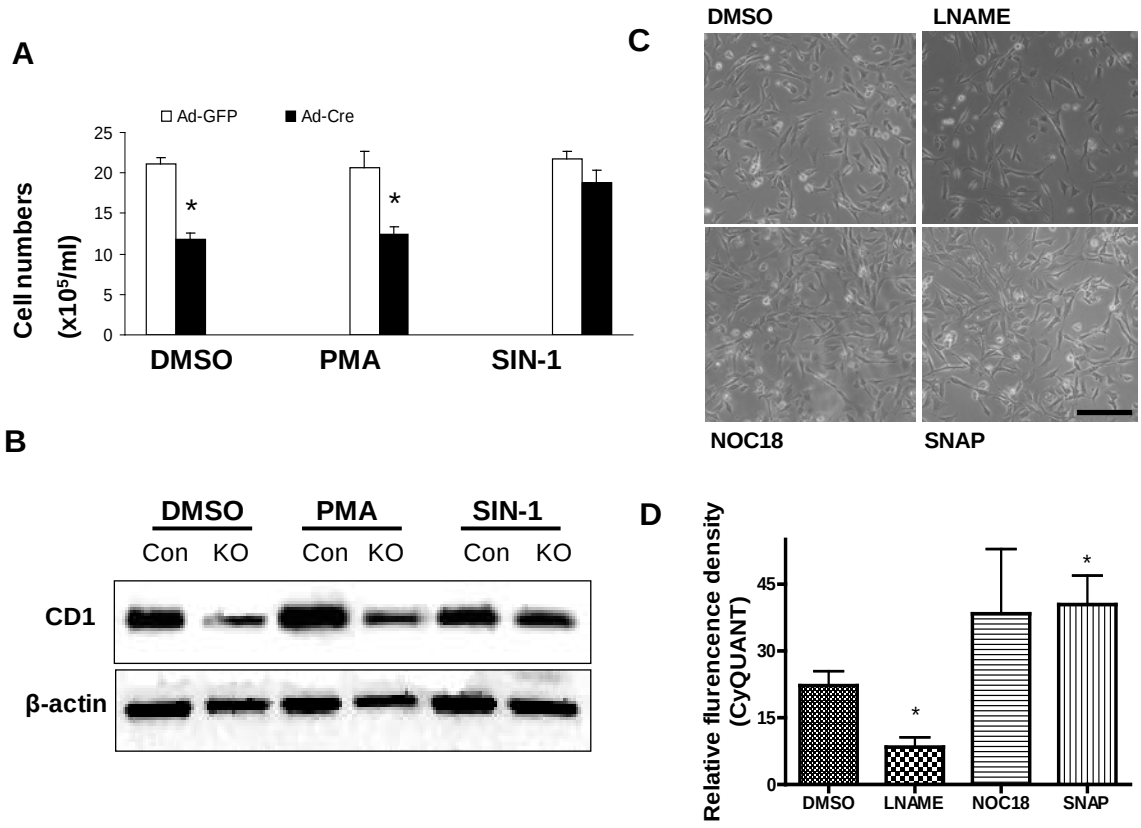
Since Rac1 is able to stimulate ROS generation, we measured levels of ROS and NO. Rac1 deletion caused an approximately 50 % reduction in ROS levels, but NO production was inhibited to a much larger degree (Fig. 2.1D). We therefore examined the molecular cause of reduced NO production and showed that expression of iNOS protein was markedly reduced in *Rac1*-deficient chondrocytes, whereas eNOS expression was similar to control cells (Fig. 2.1E). To analyze effects of *Rac1* deficiency on iNOS expression in *vivo*, we performed immunohistochemistry of paraffin sections from control and cartilage-specific *Rac1* KO mice. In control mice, iNOS protein was found mostly in late proliferating and prehypertrophic chondrocytes (Fig. 2.1F). iNOS staining was notably reduced in growth plates from mutant mice that also showed disorganization of the proliferative zone, as we described earlier (Wang et al., 2007). A similar decrease in staining in mutant mice was observed using an antibody for nitrotyrosine, an indicator of cellular NO levels (Fig. 2.1G). Collectively, these data suggest that iNOS expression and NO generation are severely reduced in *Rac1*-deficient chondrocytes.

2.4.2 NO promotes chondrocyte proliferation in cell and organ culture

We next examined whether addition of exogenous NO could overcome the effects of Rac1 deficiency. Addition of SIN-1, which induces both ROS and NO generation, rescued the numbers of *Rac1*-deficient chondrocytes in monolayer culture (Fig. 2.2A). In parallel, we treated cells with the phorbol ester PMA (an inducer of ROS only that did not change NO levels; data not shown) and observed no effects on chondrocyte numbers,

Figure 2.2 NO can rescue the effects of *Rac1* deficiency.

Primary chondrocytes were isolated from $Rac1^{fl/fl}$ mice and infected with adenoviruses expressing green fluorescent protein (GFP, control) or Cre recombinase. Addition of SIN-1, but not PMA, in this culture system increased numbers of Rac1-deficient chondrocytes (A). Western blotting showed that cyclin D1 expression in primary chondrocytes was restored by SIN-1, but not PMA (B). Incubation of chondrocytes with the NOS inhibitor L-NAME reduced chondrocyte cell numbers, while the NO donors SNAP and NOC18 caused an increase in chondrocyte cell numbers (C and D). Scale bar for C: 50μm. Figures are representative of 3 independent trials.


A
Ad-GFP
Ad-Cre
Cell numbers (x10⁵/ml)
25
20
15
10
5
0
*
*
DMSO
PMA
SIN-1
B
DMSO
PMA
SIN-1
Con KO Con KO Con KO
CD1
β-actin
C
DMSO
LNAME
NOC18
SNAP
D
Relative flurencence density (CyQUANT)
45
30
15
0
*
*
DMSO
LNAME
NOC18
SNAP

suggesting that the mitogenic defects in *Rac1*-deficient chondrocytes are not due to lower ROS production. Similarly, Western blotting demonstrated that cyclin D1 expression in *Rac1*-deficient chondrocytes is restored by SIN-1, but not PMA (Fig. 2.2B). These data suggested that NO production is required for Rac1-induced chondrocyte proliferation and that inhibition of NO synthesis by other means would result in similar phenotypes as deletion of Rac1, while NO donors should increase chondrocyte proliferation. To test this prediction, we incubated chondrocytes with the NOS inhibitor L-NAME and the NO donors SNAP and NOC18. L-NAME reduced chondrocyte cell numbers, while both donors increased cell numbers (although only SNAP reached statistical significance; Fig. 2.2C and D). Collectively, these data suggest a model where Rac1 promotes chondrocyte proliferation through iNOS/NO-mediated induction of cyclin D1 expression.

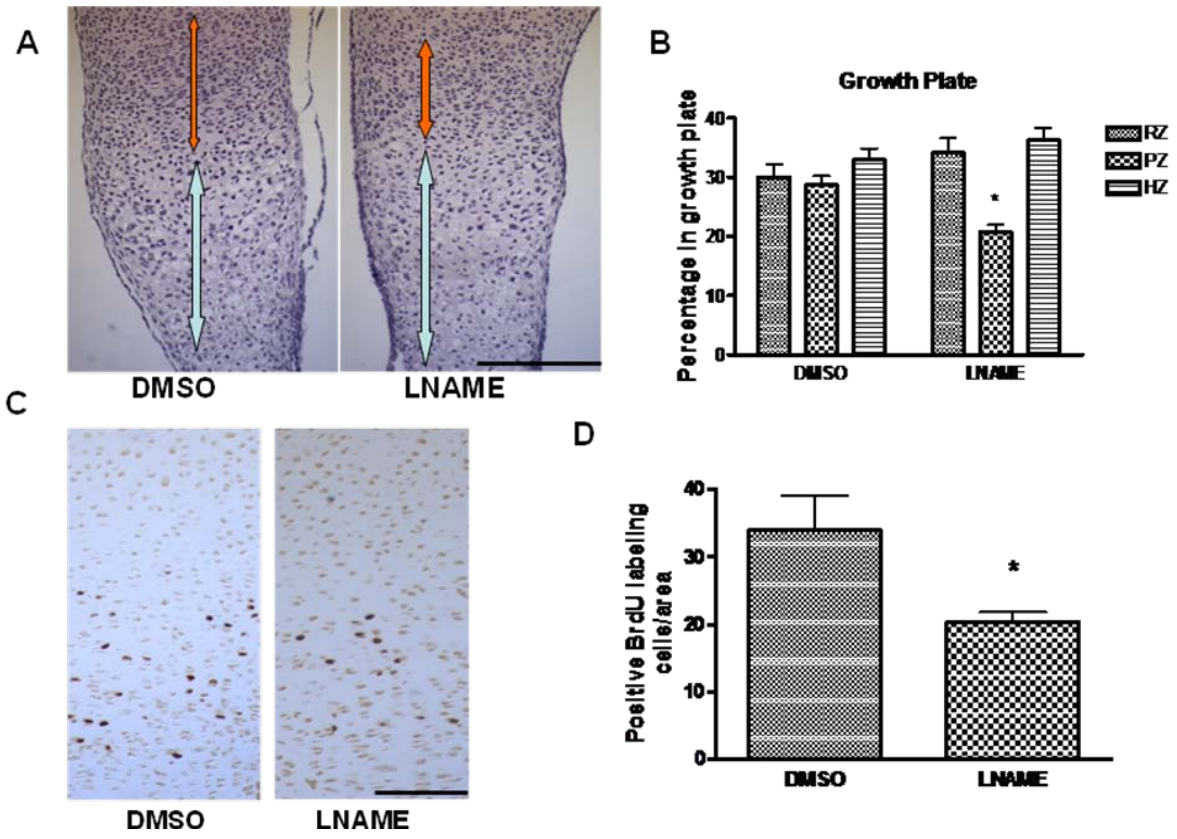
We next examined how NO regulates chondrocyte proliferation and tissue architecture in intact growth plates using an organ culture system of embryonic mouse tibiae (Agoston et al., 2007). Tibiae were grown with DMSO (control) and L-NAME for six days. Measurement of growth plate zones in H&E-stained tissue sections demonstrated that L-NAME treatment resulted in a shortened proliferative zone, while the resting and hypertrophic zones of the growth plate were not affected (Fig. 2.3A and B). This was accompanied by reduced numbers of BrdU-incorporating cells (Fig. 2.3C and D).

2.4.3 NO donors rescue the effects of Rac1 inhibition on bone growth

To investigate whether NO donors can rescue effects of Rac1 inhibition in intact growth plates, tibiae were incubated with the pharmacological Rac1 inhibitor NSC23766 (Gao et al., 2004). Our earlier studies had demonstrated that this compound has similar

Figure 2.3 Inhibition of NO production results in reduced proliferation in tibia organ cultures.

Tibiae isolated from E15.5 mice were incubated in organ culture with DMSO or the NOS inhibitor L-NAME (1mM). After six days, tibiae were fixed, embedded and sectioned, and sections were stained with hematoxylin and eosin (H&E). Staining and measurement of zone length demonstrated a shorter proliferative zone in L-NAME treated tibiae (A; red arrow: proliferating cell zone; blue arrow: hypertrophic cell zone A, B; RZ; resting zone, PZ: proliferative zone, HZ; hypertrophic zone. Scale bar 250 μm). This was accompanied by reduced numbers of BrdU-incorporating (C and D; dark brown nuclei; Scale bar 100 μm). Immunohistochemistry figures are representative of 3 independent trials.


A
DMSO
LNAME
B
Growth Plate
Percentage in growth plate
40
30
20
10
0
RZ
PZ
HZ
*
DMSO
LNAME
C
DMSO
LNAME
D
Positive BrdU labeling cells/area
40
30
20
10
0
*
DMSO
LNAME

effects on early chondrogenesis as genetic inactivation of *Rac1*, providing evidence for the specificity of the inhibitor (Woods et al., 2007a). NSC23766 treatment for six days resulted in drastic reduction of bone growth in our organ culture system (Fig. 2.4A and B), accompanied by shortening of the proliferative zone (Fig. 2.4C). The NO donor SNP did not affect bone growth on its own, but completely rescued the effects of Rac inhibition on overall tibia growth and the length of the proliferative zone (Fig. 2.4A-C). These data demonstrate that restoration of NO signaling overcomes the effects of Rac1 deficiency in the authentic three-dimensional context of the growth plate.

2.4.4 Reduced chondrocyte proliferation in *iNOS*-deficient mice

Since our data suggested a role for endogenous Rac1-iNOS signaling in growth plate chondrocyte proliferation, we next analyzed the cartilage phenotype of *iNOS*-deficient mice (Laubach et al., 1995). Growth plates from these mice showed normal zonal and columnar architecture, but appeared slightly hypocellular in their centers (Fig. 2.5A), similar to but more subtle than those of mice with cartilage-specific deletion of the *Rac1* gene (Wang et al., 2007). Cell counts confirmed hypocellularity in the proliferative zone of mutant mice (Fig. 2.5B). Immunohistochemistry and counts of positive cells demonstrated lower numbers of chondrocytes incorporating BrdU (Fig. 2.5C and D) and expressing cyclin D1 (Fig. 2.5E and F) in *iNOS*-deficient growth plates. Both of these features had also been observed in cartilage-specific *Rac1* KO mice (Wang et al., 2007).

Figure 2.4 The NO donor SNP rescues the effects of Rac inhibition in organ culture. E15.5 tibiae were incubated with control medium, the Rac inhibitor NSC23766 (50 μM), the NO donor SNP (10 μM) or both for six days. Rac inhibition resulted in drastic reduction of bone growth in our tibia organ culture system (A and B), accompanied by shortening of the proliferative zone (C). The NO donor SNP (10 μM) did not affect bone growth on its own, but completely rescued the effects of Rac inhibition on growth and the length of the proliferative zone (A-C). Scale bar 100 μm. Images are representative of 3 independent trials.

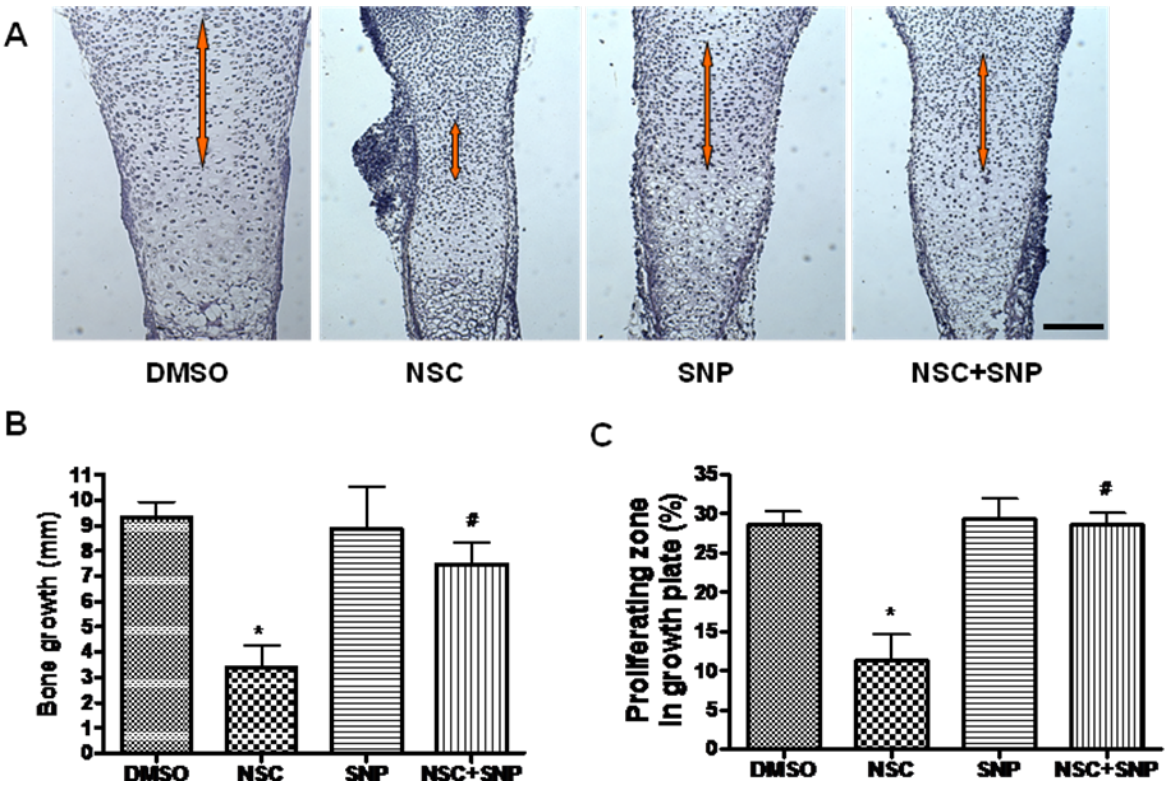
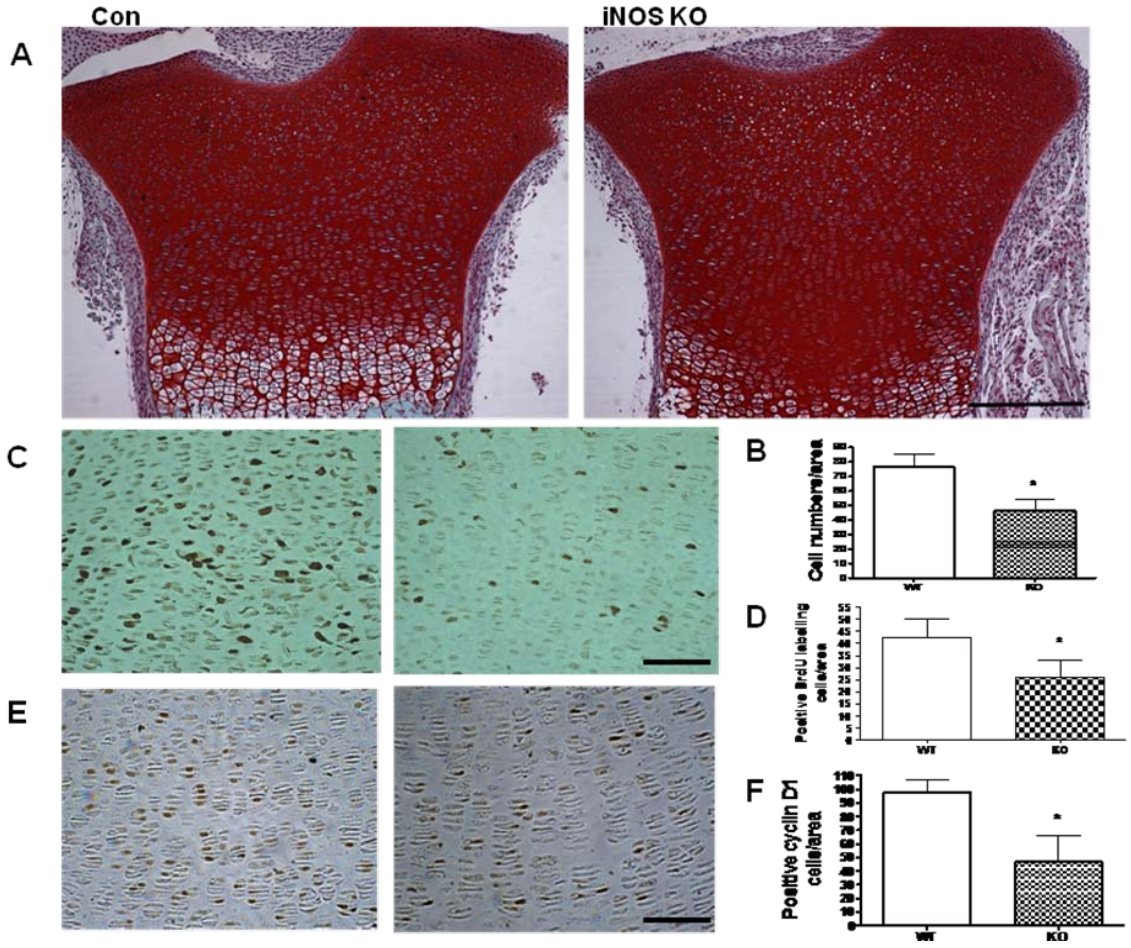

A
DMSO
NSC
SNP
NSC+SNP
B
Bone growth (mm)
0
1
2
3
4
5
6
7
8
9
10
11
*
#
DMSO
NSC
SNP
NSC+SNP
C
Proliferating zone
In growth plate (%)
0
5
10
15
20
25
30
35
*
#
DMSO
NSC
SNP
NSC+SNP

Figure 2.5 Reduced chondrocyte proliferation in *iNOS* KO mice.

Growth plates from iNOS mutant mice were hypocellular (A), as shown by Safranine O/Fast Green staining. Cell counts confirmed hypocellularity in the proliferative zone of mutant mice (B). Immunohistochemistry and counts of positive cells demonstrated lower numbers of chondrocytes incorporating BrdU (C and D) and expressing cyclin D1 (E and F) in *iNOS*-deficient growth plates. Scalebar for A 250 μm, C and E 100 μm. Images are representative of 3 independent trials.

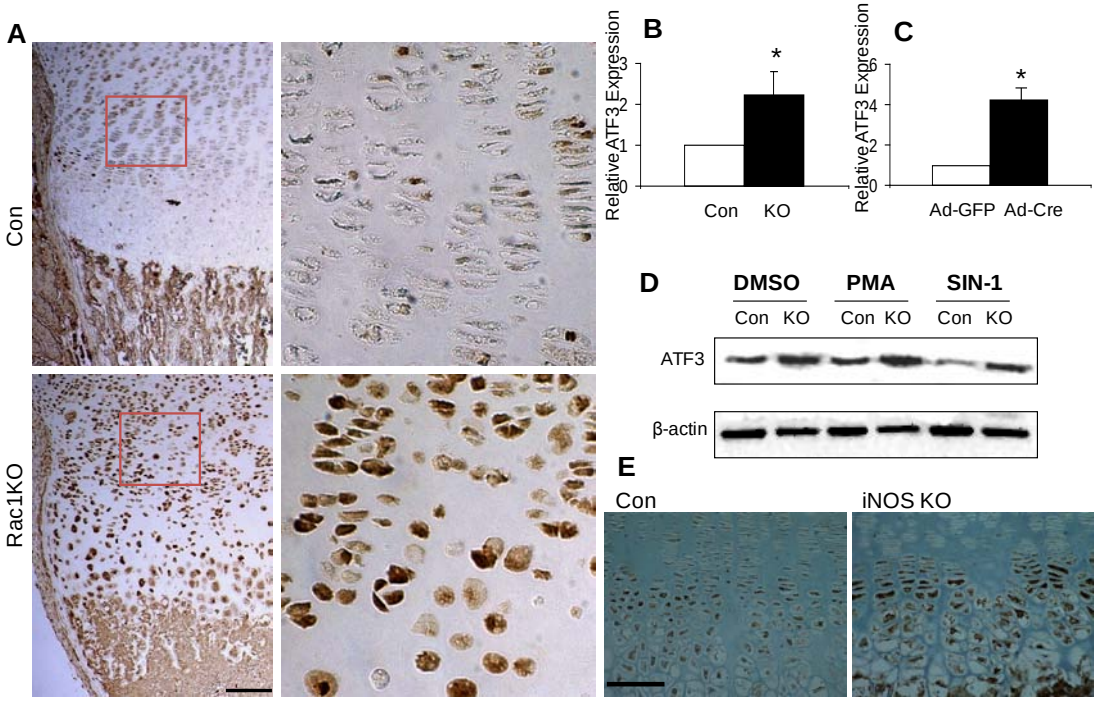

Con
iNOS KO
A
C
E
B
D
F
Cell number/area
Positive BrdU labelling cells/area
Positive cyclin D1 cells/area
WT
KO
*

2.4.5 The transcriptional repressor ATF3 is upregulated upon loss of *Rac1* or *iNOS*

We had shown earlier that cyclin D1 expression in chondrocytes is positively regulated by the transcription factors ATF-2 and CREB and repressed by ATF3 (Beier et al., 2001; Beier et al., 1999a; James et al., 2006). Immunohistochemistry demonstrated limited expression of ATF3 protein in chondrocytes of control mice, but markedly increased numbers of stained cells upon loss of *Rac1* (Fig. 2.6A). In agreement with these data, ATF3 mRNA levels were increased in *Rac1*-deficient cartilage *in vivo* (Fig. 2.6B) and in primary $Rac1^{fl/fl}$ chondrocytes infected with Ad-Cre (Fig. 2.6C). This suggests premature induction of ATF3 which in turn can repress cyclin D1 transcription and result in earlier cell cycle withdrawal. SIN-1 treatment reduced ATF3 protein levels both in control chondrocytes and in *Rac1*-depleted chondrocytes in cell culture (Fig. 2.6D), suggesting that NO represses ATF3 expression. Finally, the number of ATF3-expressing chondrocytes is increased and its expression domain is expanded in *iNOS* KO growth plates, similar to *Rac1* mutants (Fig. 2.6E). These data suggest that the Rac1-iNOS-NO pathway acts to suppress ATF3 expression in order to maintain cyclin D1 transcription and chondrocyte proliferation.

Figure 2.6 ATF3 suppresses cyclin D1 expression and inhibit chondrocyte proliferation.

Immunohistochemistry demonstrated relatively little expression of ATF3 protein in control mice (see also in magnification in right panel), with markedly increased expression upon loss of *Rac1* (A). Scale bar 50 µm. Real-time RT-PCR demonstrated that ATF3 mRNA levels are increased in Rac1-deficient cartilage in vivo (B) and in primary $Rac1^{fl/fl}$ chondrocytes infected with Ad-Cre (C). SIN-1 treatment reduced expression of ATF3 protein in primary wild type and *Rac1*-deficient chondrocytes, while PMA did not affect either (D). The number of ATF3-expressing chondrocytes was increased in *iNOS* KO growth plates, similar to Rac1 mutants (E). Scale bar 100 µm. Figures are representative of 3 independent trials.

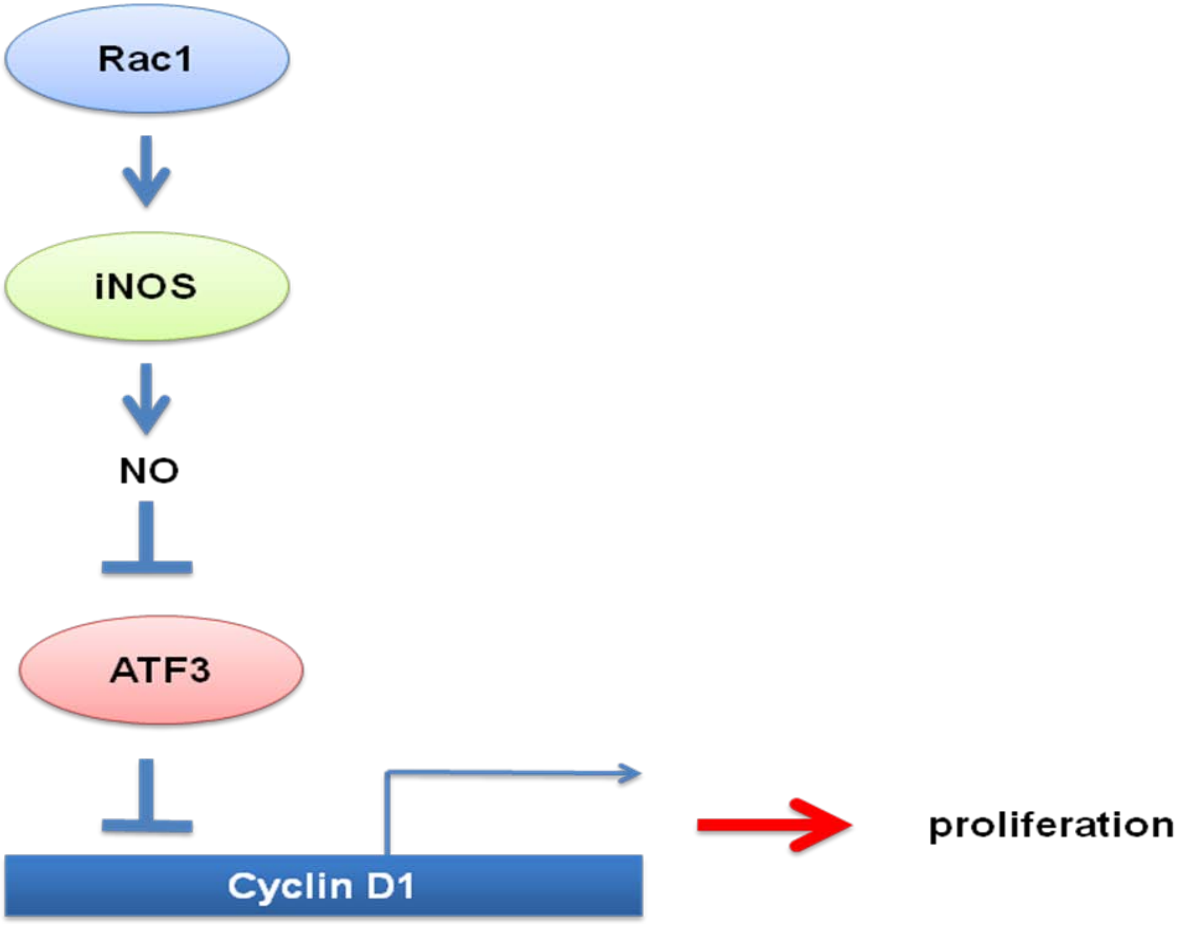

A
Con
Rac1KO
B
Relative ATF3 Expression
Con
KO
C
Relative ATF3 Expression
Ad-GFP
Ad-Cre
D
DMSO
PMA
SIN-1
Con KO Con KO Con KO
ATF3
β-actin
E
Con
iNOS KO

2.5 *Discussion*

Chondrocyte proliferation is one of the main determinants of endochondral bone growth and thus final height. While many extracellular signals that control these processes are known, the intracellular signaling pathways connecting cell surface receptors to specific cell cycle genes are less well understood. We had shown that the small GTPase Rac1 is essential for normal chondrocyte proliferation and cyclin D1 expression, but the molecular mechanisms involved were not known. Here we demonstrate that Rac1 controls chondrocyte proliferation by stimulation of iNOS expression and NO production, which in turn suppresses ATF3 expression and thus allows increased transcription of the cyclin D1 gene (Fig. 2.7).

While the regulation of ROS generation by Rac1 is well established, there has been much less attention on interactions between Rac proteins and NO signaling. The requirement for Rac1 for normal iNOS expression in our study is consistent with an earlier report showing reduced iNOS mRNA levels upon adenoviral delivery of dominant-negative Rac to rat livers (Harada et al., 2003). However, Rac1 activation or inhibition did not alter iNOS induction by interleukin 1β in smooth muscle cells (Finder et al., 2001), suggesting that the connections between both pathways are stimulus- and/or cell type-specific. Interestingly, *Rac1* deficiency in endothelial cells leads to reduced expression and activity of eNOS (Sawada et al., 2008), while eNOS protein levels were not altered in our system. These data suggest that the connections between Rac1 (and possibly other Rho GTPases) and components of the NO signaling pathway are connected in a cell type-specific manner. In addition, it appears likely that

Figure 2.7 Model of Rac1 –iNOS/NO – ATF3 – cyclin D1 signaling in chondrocytes. Our data show that Rac1 is required for normal levels of iNOS expression and NO production in chondrocytes. NO suppresses expression of ATF3, which acts as transcriptional repressor of cyclin D1. In *Rac1*- or *iNOS*-deficient chondrocytes, ATF3 levels are increased, resulting in reduced activity of the cyclin D1 promoter which in turn slows chondrocyte proliferation and induces premature cell cycle exit.


Rac1
iNOS
NO
ATF3
Cyclin D1
proliferation

the Rac1-iNOS interaction demonstrated by us is not exclusive; for example, Rac1 probably acts through additional effectors including ROS and the actin cytoskeleton, and iNOS activity and expression in chondrocytes is likely controlled by additional upstream regulators.

NO and *NOS* genes have been studied extensively in the context of bone remodeling and arthritis (Scher et al., 2007; van't Hof and Ralston, 2001), but much less is known about the roles of this pathway in endochondral bone development (Teixeira et al., 2008). Teixeira and colleagues had shown that excess NO promotes hypertrophic differentiation and apoptosis of chondrocytes in chicken (Teixeira et al., 2005; Teixeira et al., 2001), while our data suggest that basal levels of NO are required for chondrocyte proliferation. Studies from other tissues suggest that the role of iNOS in cellular proliferation is cell-type and/or context-specific; for example, iNOS is required for hepatocyte proliferation during liver regeneration (Yasoda et al., 2009) but suppresses proliferation of smooth muscle cells (Kibbe et al., 2000). However, to our knowledge neither ATF3 nor cyclin D1 have been implicated in the mitogenic or anti-mitogenic effects of iNOS. It should be noted that since *in vitro* data from our and other laboratories suggest Rac1 functions during chondrocyte hypertrophy (Kerr et al., 2008; Wang and Beier, 2005), it is quite possible that iNOS or other NOS enzymes also contribute to this role of Rac1.

Interestingly, we recently demonstrated a similar phenotype as described here (e.g. reduced proliferation and cyclin D1 expression, increased levels of ATF3 and premature cell cycle exit) in mice deficient for the *eNOS* gene (Yan et al., 2010). Together, these studies suggest a dose-dependent effect of nitric oxide in the growth plate where low

levels are required for proliferation, but high levels promote terminal differentiation and ultimately apoptosis. This model is in agreement with suggested dose-dependent effects of NO (Thomas et al., 2008). The overlap in phenotypes in this and our earlier study (Yan et al., 2010) also imply partial overlap of iNOS and eNOS function in chondrocytes. NO generated from either source inhibits the onset of ATF3 expression, allowing continued expression of cyclin D1 and thus delaying cell cycle exit. However, eNOS expression was not affected by loss of Rac1, suggesting that the two genes are regulated by different upstream pathways and mediate mitogenic responses to different external stimuli.

In conclusion, this study identified the iNOS/NO pathway as a central mediator of the mitogenic activity of Rac1 in growing endochondral bones. Further research into this will provide us with novel approaches towards the treatment of various skeletal diseases such as chondrodysplasias.

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CHAPTER THREE

ENDOTHELIAL NITRIC OXIDE SYNTHASE DEFICIENCY RESULTS IN REDUCED CHONDROCYTE PROLIFERATION AND ENDOCHONDRAL BONE FORMATION*

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3.1 *Chapter summary*

Nitric oxide (NO) and aberrant chondrocyte differentiation have both been implicated in the pathogenesis of osteoarthritis, but whether these processes are connected is unknown, and the role of specific nitric oxide synthase (NOS) enzymes in chondrocyte physiology is unclear. The objective of this study was to examine the effects of inactivation of endothelial nitric oxide synthase (*eNOS*) on cartilage development in mice. Skeletal growth and development of mice carrying a null mutation in the *eNOS* gene was studied in comparison to control littermates. In situ analyses were complemented by experiments with primary chondrocytes and tibial explants from these mice. *eNOS*-deficient mice show increased lethality and reduced bone growth, with hypocellular growth plates and a marked reduction in the number of proliferating chondrocytes. In vitro studies demonstrated lower chondrocyte numbers and reduced endochondral bone growth in mutants, suggesting that the role of *eNOS* signaling in chondrocyte proliferation is cell-autonomous. Reduced chondrocyte numbers appear to be caused by decreased cyclin D1 and increased p57 expressions in mutants, resulting in slower cell cycle progression and earlier cell cycle exit. In addition, expression of early chondrocyte markers such as Sox9 was reduced and prehypertrophic markers were expressed prematurely in mutant mice. Our data identify a novel and important role of *eNOS* in chondrocyte proliferation and endochondral bone growth and demonstrate that loss of *eNOS* results in premature cell-cycle exit and prehypertrophic chondrocyte differentiation during cartilage development.

3.2 *Introduction*

Nitric Oxide (NO) plays an important role in the progression of osteoarthritis (OA), in part by influencing the physiology of chondrocytes (Abramson, 2008). NO is synthesized through L-arginine by nitric oxid synthases (NOS) in many cell types. Three different types of NOS have been identified. The neuronal (nNOS or NOS1) and endothelial (eNOS or NOS3) forms are constitutively expressed, and their activity is regulated by intracellular signaling and the calcium binding protein calmodulin (van't Hof and Ralston, 2001). The inducible form (iNOS or NOS2) is stimulated at the level of expression by factors including lipopolysaccharide and cytokines, such as IL-1, TNFα and IFNα, and leads to a sustained and high levels of NO, mainly in inflammatory disease (Abramson, 2008; Mungrue et al., 2003).

Changes in the articular chondrocyte phenotype, such as initiation of hypertrophic differentiation, are thought to play an important role in the pathogenesis of OA (Appleton et al., 2007; Drissi et al., 2005; Gauci et al., 2008). Physiologically, chondrocyte hypertrophy occurs in the process of endochondral ossification that gives rise to most bones in the vertebrate skeleton, such as the long bones of the limbs (Karsenty and Wagner, 2002; Zelzer and Olsen, 2003). Endochondral ossification involves the formation of a highly controlled and precisely shaped cartilage template that is subsequently replaced by bone tissue and bone marrow (Wagner and Karsenty, 2001). Once mesenchymal cells commit to the chondrogenic lineage, the subsequent events of endochondral bone formation occur through the epiphyseal growth plate (Olsen, 1999), which consists of zones of resting, proliferating, and hypertrophic chondrocytes organized in distinguishable columnar arrays (Wagner and Karsenty, 2001). Within the

growth plate, proliferation of chondrocytes occurs in a unidirectional manner, resulting in longitudinal bone growth (Goldring et al., 2006; Kronenberg, 2003). After exiting the cell cycle, chondrocytes start to differentiate into prehypertrophic and eventually hypertrophic chondrocytes (Hoffmann and Gross, 2001; Mackie et al., 2008; Stanton et al., 2003). Hypertrophic chondrocytes direct mineralization of the surrounding extracellular matrix, attract blood vessel invasion, and ultimately undergo apoptosis while being replaced by bone and bone marrow (Kronenberg, 2003; Olsen et al., 2000). Proliferation and hypertrophy of growth plate chondrocytes and extracellular matrix production drive the longitudinal growth of endochondral bones and eventually determine body length in mammals (Kronenberg, 2003; Okazaki and Iwamoto, 2006).

Since both NO signaling and altered chondrocyte differentiation contribute to OA, it is important to examine whether both processes are connected. Studies in the chicken suggest that NO signaling promotes chondrocyte hypertrophy (Teixeira et al., 2008; Teixeira et al., 2005), but the roles of NO and in particular the individual *NOS* genes in mammalian cartilage development are not well understood. In this study, we used a genetic approach to address the role of *eNOS* in chondrocyte differentiation and endochondral ossification *in vivo*. Inactivation of the *eNOS* gene resulted in a number of phenotypes, including reduced proliferation and earlier cell cycle exit of chondrocytes. Overall, these data provide novel information regarding the importance of eNOS/NO signalling in chondrocyte development and identify downstream regulators of endochondral ossification.

3.3 *Materials and methods*

3.3.1 Animals and materials

Timed-pregnant CD1 mice were purchased from Charles River Laboratories. Cell culture materials and general chemicals were obtained from Invitrogen, Sigma or VWR unless otherwise stated. The following antibodies were employed in this study: Kip2/p57 #sc8298 (rabbit antibody against human protein), RORα #sc28612 (rabbit antibody against human protein), ATF3 #sc188 (rabbit antibody against human protein), Goat-anti-mouse #sc2005, Goat-anti-rabbit #sc2004 (all from Santa Cruz Biotechnology); Cyclin D1 (SP4) #9104-S1 (rabbit antibody against human, mouse and rat proteins; NeoMarkers Inc.); PCNA #2586, (mouse antibody against human, mouse and rat proteins; Cell Signaling Inc.); Hif1α #07-628 (rabbit antibody against human protein; Upstate Inc.); BrdU #03-3900 (mouse antibody, species-independent; Zymed Laboratories).

3.3.2 Mouse breeding and genotyping

Mice with a deletion of the *eNOS* gene (Stock #2684) (Shesely et al., 1996) and control mice in the C57/BL6 background were obtained from Jackson Laboratory (Bar Harbor, ME), and exposed to a 12-hour light-dark cycle and fed tap water and regular chow at libitum. All procedures involving animals were approved by the University of Western Ontario Animal Care and Use Committee. All experiments were done using crosses of heterozygote mice to compare KO animals to wild type controls within litters. For PCR genotyping, tail snips were used to prepare DNA for PCR analysis. PCR genotyping was performed by simultaneous amplification of the *eNOS* wild type and null alleles as described (Hefler et al., 2001). PCR fragments were analyzed by agarose gel electrophoresis.

3.3.3 Skeletal staining and histology

Animals were skinned, eviscerated and dehydrated in 95% ethanol overnight and then in acetone overnight. Skeletons were stained with 0.015% alcian blue, 0.05% alizarin red and 5% acetic acid in 70% ethanol for several days. Skeletons were then cleared in 1% KOH, passed through a decreasing KOH series and stored in glycerol/ethanol (1:1) (Wang et al., 2007). After dissection of mice, bones were rinsed with PBS, fixed in 4% Paraformaldehyde (PFA) overnight, placed in 10% formalin solution and sent for embedding and sectioning into 4μm sections at the Molecular Pathology Core Facility at the Robarts Research Institute (London, Ontario, Canada). Following sectioning, bones were stained with hematoxylin and eosin or safranin O/fast green using standard protocols (Solomon et al., 2009; Wang et al., 2007) or used for immunohistochemistry as described below.

3.3.4 Immunohistochemistry and BrdU labeling

For immunohistochemistry, sections unstained prior to use were incubated in 3% H_2O_2 for 15 min at room temperature, following by boiling for 20 min in 10 mM sodium citrate (pH 6.0) and blocking with 5% goat serum at room temperature for 30 min. Sections were incubated with primary antibody overnight at 4°C and secondary antibodies according to the manufacturers' recommendations. After washing, the sections were incubated for 1-10 min with DAB substrate solution (Dako North America, Inc.), washed and mounted. All images were taken at room temperature with a Retiga EX camera connected to a Leica DMRA2 microscope using OpenLab 4.0.4 software. For cell counts in sections, all cells and cells positive for staining with primary antibody labeling were counted from three different areas of one section. Sections from at least three mice

per genotype, and at least three sections from every mouse, were analyzed, and averages and standard deviation from all counts/genotype are shown.

For BrdU labeling, newborn mice were injected intraperitoneally with BrdU (Roche) at a dose of 0.03 mg/g body weight one hour before sacrifice. BrdU was detected in paraffin sections using anti-BrdU antibody (Zymed Laboratories) as described above. For all antibodies, negative controls were conducted following the same protocol in the absence of the primary antibody.

3.3.5 Primary cell culture and MTT assays

Tibiae, femuri and humeri were isolated from embryonic day 15.5 (E15.5) KO and control mouse using a Stemi DV4 Stereomicroscope (Zeiss). 20 bones from the same genotype were placed in 2ml α-MEM media (Invitrogen)/well containing 0.2% bovine serum albumin (BSA), 1 mM β-glycerophosphate, 0.05 mg/ml ascorbic acid and penicillin/streptomycin in a 6-well culture dish (Falcon), as described (James et al., 2005). Then medium was removed and every 10-12 bones from each group placed in 1 ml of 0.25% trypsin-EDTA (Invitrogen) for 15 min at 37°C. Trypsin was subsequently replaced with 1 mg/ml collagenase P (Roche) in DMEM/10% fetal bovine serum (Invitrogen), and cells were incubated at 37°C with rotation at 100 rpm for 90 min. Following digestion, the cell suspension was centrifuged for 5 min at 1000 rpm, and the collagenase containing supernatant was decanted. Chondrocytes were resuspended in medium containing 2:3 DMEM:F12, 10% fetal bovine serum, 0.5 mM L-glutamine, and penicillin/streptomycin (25 units/ml) supplemented with 0.25 mM ascorbic acid (Sigma) and 1 mM β-glycerophosphate (Sigma). Chondrocytes (10000 cell/ml) were seeded in 96-well culture dish (Falcon), containing 100 µl of medium per well and cultured in the

presence of 5 % CO_2. Cell numbers were determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay as described (Halawani et al., 2004; Wang et al., 2004).

3.3.6 Tibia organ culture

Tibiae were isolated from E15.5 embryos from wild type and KO mice as described above (Agoston et al., 2007; Wang et al., 2007). Dissection day was considered to be day 0 and tibiae were allowed to recover from dissection overnight in serum-free α-MEM medium 1 ml/well, 1 tibia/well containing 0.2% Bovine Serum Albumin (BSA), 0.5 mM L-glutamine, 40 units penicillin/mL and 40 μg streptomycin/mL in 24-well Falcon plates as described (Agoston et al., 2007; Wang et al., 2007). All culture dishes were maintained in an incubator containing 5% CO_2. The following morning, bone length was measured using an eyepiece in the Stemi DV4 Stereomicroscope. Medium was changed every 48 hrs beginning on day 1, and bones measured on days 2, 4 and 6. Results are expressed as growth in length relative to day 1. Experiments were repeated at least three times, with 4–6 bones per treatment for each trial.

3.3.7 Western blotting

Fresh cartilage from limbs of newborn mice was dissected in cold Puck's solution A (PSA; 80 g/l NaCl, 4.0 g/l KCl, 0.6 g/l KH_2PO_4, 0.9 g/l $Na_2HPO_4 \cdot 7H_2O$, and 10 g/l glucose, pH 7.4). Modified Radioimmunoprecipitation (RIPA) Buffer extracts of cartilage were used directly for Western blotting (Appleton et al., 2007; Wang et al., 2007). Blocking, incubation with antibodies and washing were carried out in accordance with instructions of the supplier of the primary antibody. Immunoblots were developed

using the ECL detection system (Amersham). Representative blots from three independent littermate comparisons are shown.

3.3.8 RNA isolation, and real-time RT-PCR

Total RNA was isolated from epiphyseal cartilage of long bone from newborn mice using TRIzol (Invitrogen), according to the manufacturer's recommendations. Taqman real-time PCR was performed as described (James et al., 2005; Woods et al., 2005) with primers and probe sets from Applied Biosystems (*Sox9* Mm00448840_m1; *Col2a1* Mm00491889_m1; *Col10a1* Mm00487041_m1; *Rora* Mm00443103_m1; *Hif1a* Mm00468869_m1; *Nos1* Mm00435175_m1; *Nos2* Mm00440485_m1; *Nos3* Mm00435204_m1; *Gapdh* Mm99999915_g1). Data were normalized to *Gapdh* mRNA levels and represent averages and SEM from direct comparison of mutant and control littermates from three different crosses. Statistical significance of real-time PCR results was determined by one way ANOVA with Bonferroni post-test using GraphPad Prism 4.00 for Windows.

3.3.9 Statistical analysis

All experiments were performed with at least three independent litters. Statistical significance was determined by a one-way ANOVA with Bonferroni post test using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA.

3.4 *Results*

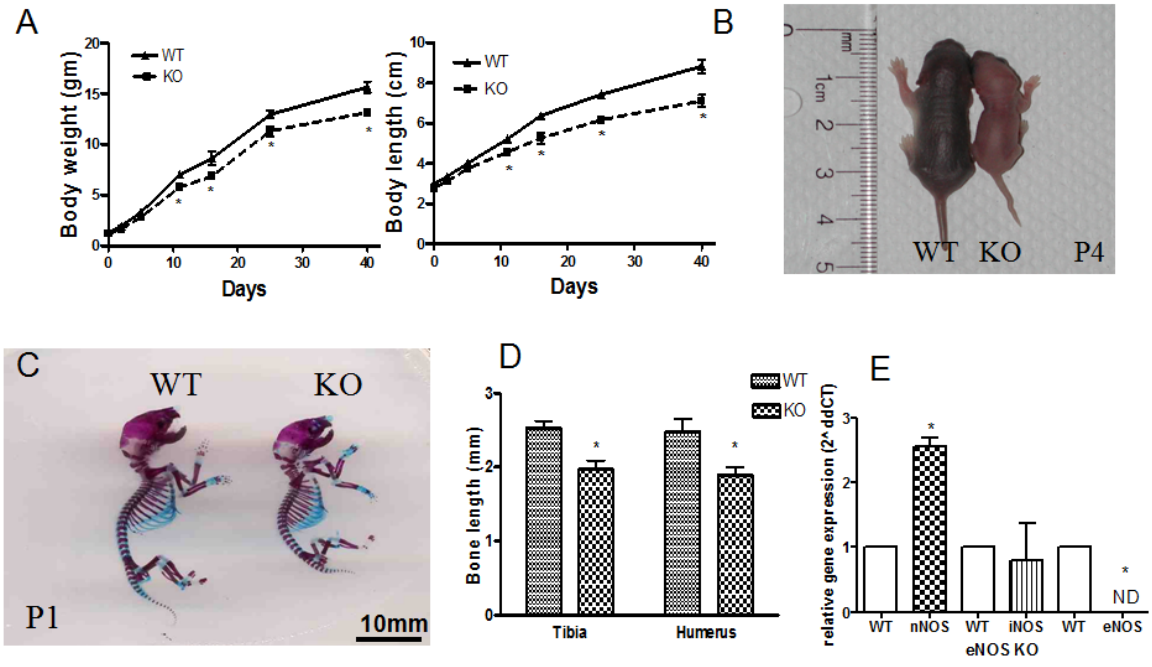
3.4.1 Inactivation of *eNOS* gene results in reduced viability and growth

We used *eNOS*-deficient mice to address the role of this gene in cartilage development *in vivo*. Based on our breeding protocol from heterozygote mice, Mendelian ratio would predict that 50% of newborn mice are heterozygote and 25% are homozygote *eNOS* null mice. However, examination of genotype distribution of newborn mice revealed that only 14% of pups are homozygous for the mutant *eNOS* allele, and 40% are heterozygous. Analyses of genotypes at different embryonic stages showed the expected Mendelian distribution (data not shown). These data suggest that loss of *eNOS* gene results in increased lethality at the late prenatal or the perinatal stage. Additionally, newborn $eNOS^{-/-}$ mice showed 44% neonatal death occurring shortly after birth. Slightly decreased body weight (86%) and length (92%) were found in $eNOS^{-/-}$ mice at birth. Analysis of both parameters over the first 42 days of life demonstrated that growth retardation became more pronounced in surviving $eNOS^{-/-}$ mice as the animals aged (Fig. 3.1A). Growth retardation was similar in female and male mice (data not shown). However, some $eNOS^{-/-}$ mice demonstrated more severe delays in growth and development, and some were born with a kinky tail (6 out of 20 KO mice from 9 litters) (Fig. 3.1B).

We next examined the skeletal phenotype of *eNOS*-deficient mice. Whole skeletal staining with alcian blue and alizarin red confirmed that mutant mice have a smaller skeleton than control littermates at postnatal day 1 (Fig. 3.1C). The majority of mutant mice showed no obvious morphological changes in the skeleton. Detailed observation of

Figure 3.1 Loss of eNOS results in reduced growth.

Body length and body weight of mice were determined during the first 42 days of their life. *eNOS* null mice show slower increases in both parameters compared to wild type littermates (A). Mean and standard deviation from at least 8 mice per genotype are shown for each data point. (*P<0.05) (WT: wild type; KO: knockout). Comparison of an *eNOS* knockout (KO) mouse and a wild type (WT) littermate at postnatal day 4 demonstrates delay in growth and development and a kinky tail in the mutant (B). Whole skeletal staining with alcian blue and alizarin red at postnatal day1 shows no morphological difference besides reduced size in mutants (C). Measurements of selected individual bones confirm reduced bone length in humeri and tibiae in neonatal mutant mice (D). Real-time RT-PCR using RNA extracted directly from neonate cartilage from *eNOS* KO mice demonstrated upregulated nNOS transcript levels; iNOS mRNA levels did not change; and eNOS transcripts could not be detected in KO mice (E). (ND: not detectable; N = 4, *P<0.05).


A
Body weight (gm)
WT
KO
Days
Body length (cm)
WT
KO
Days
B
WT KO P4
C
WT
KO
P1
10mm
D
Bone length (mm)
WT
KO
Tibia
Humerus
E
relative gene expression (2^ddCT)
ND
WT nNOS WT iNOS WT eNOS
eNOS KO

the whole skeleton of mutant mice demonstrated a smaller rib cage and skull and a shorter appendicular skeleton. Measurement of selected individual bones confirmed reduced bone length of humeri (76%) and tibiae (80%) in neonatal mutant mice (Fig. 3.1D). Real-time RT-PCR using RNA extracted directly from mutant neonate cartilage demonstrated upregulated *nNOS* transcription levels compared with control; *iNOS* mRNA levels were unchanged; and *eNOS* transcripts could not be detected in mutant mice, as expected (Fig. 3.1E).

3.4.2 *eNOS* deficiency reduces chondrocyte proliferation in the growth plate

To elucidate the cellular basis for the reduced skeletal growth, we analyzed growth plate organization. Growth plates from newborn mutant mice displayed a similar chondrocyte arrangement in proliferative and hypertrophic zones as control animals (Fig. 3.2A). Measurement of the width of the various growth plate zones showed that the proliferative zone is slightly smaller and the hypertrophic zone is slightly larger in KO growth plates, but these effects were not statistically significant (Fig. 3.2B). *eNOS*$^{-/-}$ growth plates were hypocellular (Fig. 3.2A). Counts of cell numbers in the proliferative zone of the growth plate revealed fewer cells per area in mutants (71% of control cell numbers), while cell counts in the epiphyseal cartilage did not show a significant difference between genotypes (Fig. 3.2C).

We next asked whether changes in cell proliferation are the cause of hypocellularity in *eNOS*-deficient growth plates. BrdU incorporation assays showed a marked reduction in replicating cells in mutant mice that was confirmed by cell counts (Fig. 3.3A). Control experiments in these and all subsequent immunohistochemistry studies were done without primary antibody and showed no staining (data not shown).

Figure 3.2 *eNOS* deficiency results in reduced proliferating chondrocyte numbers in growth plate.

Histological analyses (safranin O/Fast Green staining) demonstrate that growth plates from newborn mutant mice displayed a similar chondrocyte arrangement into proliferating and hypertrophic zones as control mice (A). Measurement of the width of the resting zone (RZ), proliferative zone (PZ) and hypertrophic zone (HZ) in the growth plates did not show significant changes in the mutant (B). However, cell numbers per area in the proliferative zone of the mutant growth plate are lower (71%) (C). (EC: epiphyseal cartilage; PZ: proliferative zone) (Scale bar=500μm) Mean and standard deviation from at least 8 mice per genotype are shown (*$P<0.05$).

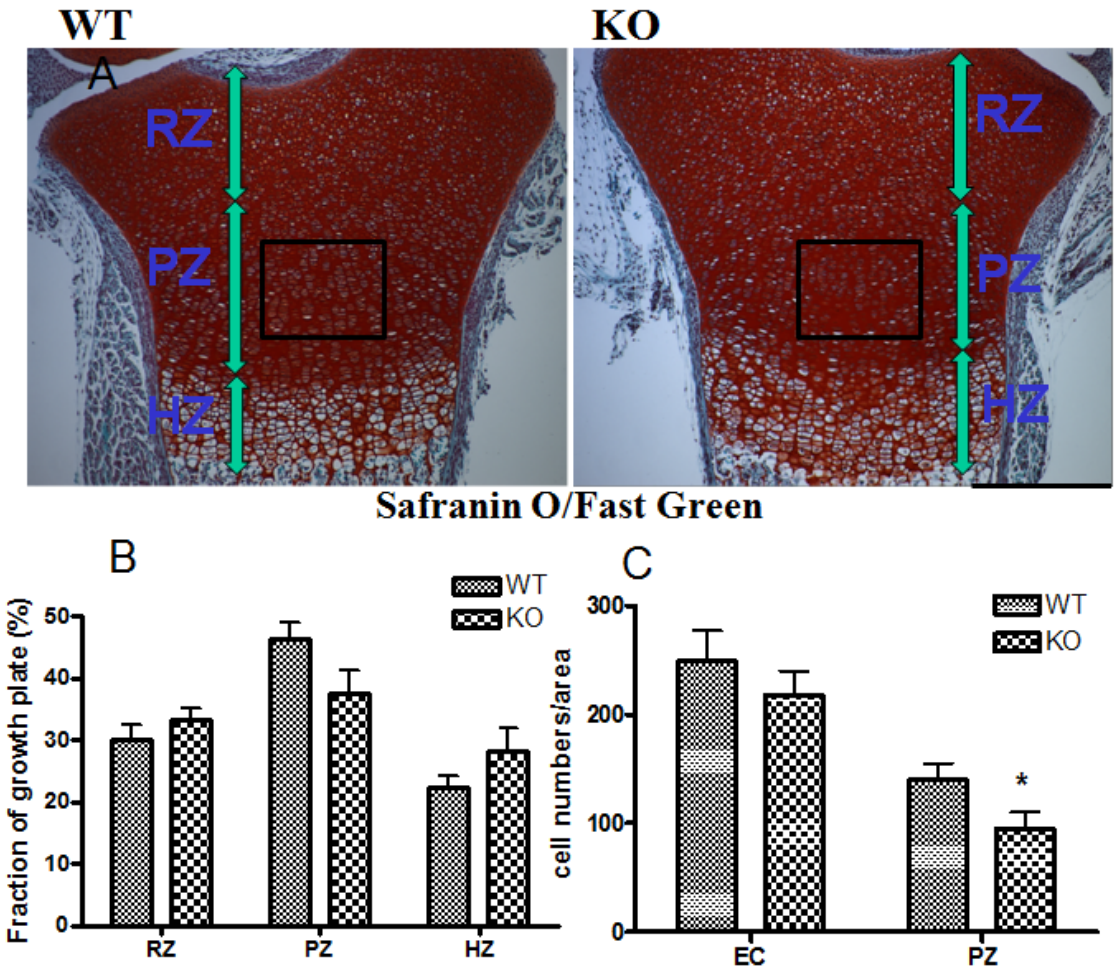
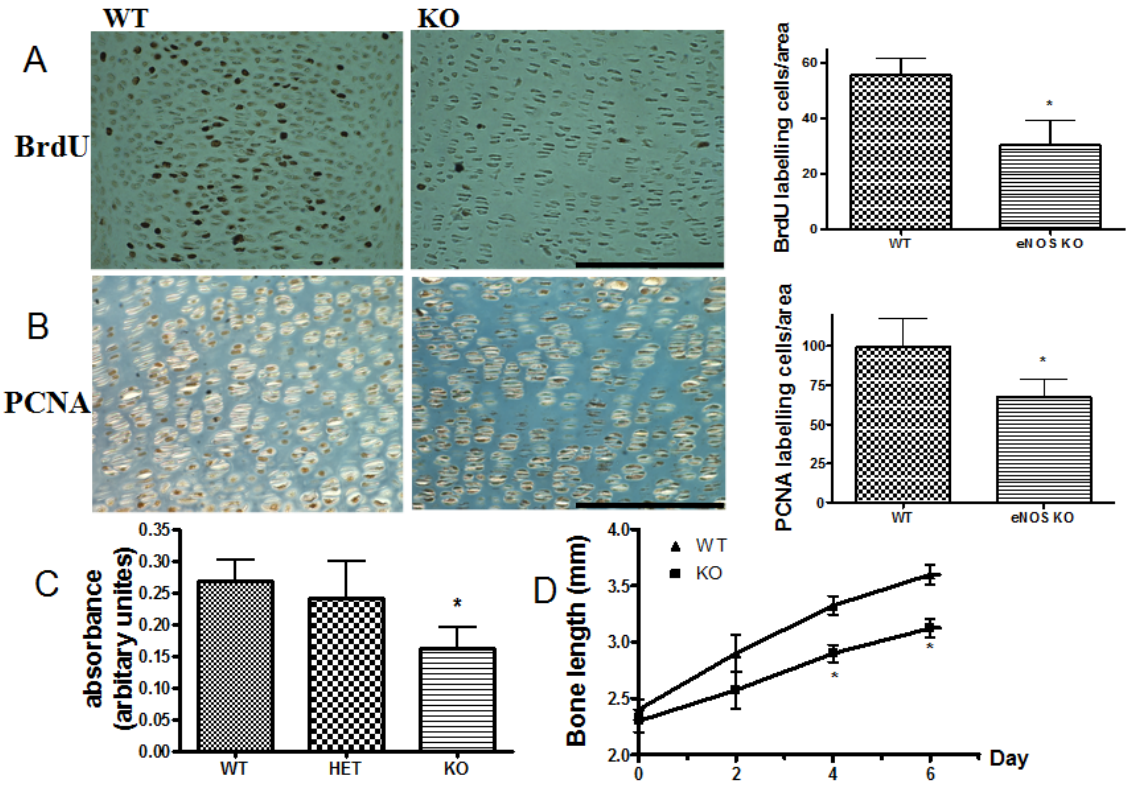

WT
KO
A
RZ
PZ
HZ
RZ
PZ
HZ
Safranin O/Fast Green
B
WT
KO
Fraction of growth plate (%)
50
40
30
20
10
0
RZ
PZ
HZ
C
WT
KO
cell numbers/area
300
200
100
0
*
EC
PZ

Figure 3.3 *eNOS* deficiency reduces chondrocyte proliferation and endochondral bone growth *in vitro*.

BrdU incorporation assays show a remarked reduction in replicating cells in eNOS mutant mice (A), which is confirmed by counting of labeled cells. Similarly, immunohistological staining of proliferating cell nuclear antigen (PCNA) showed reduced positive cell numbers per area in eNOS mutant mice (B). MTT assays demonstrate a slower increase in cell numbers of eNOS-/- primary chondrocytes than observed for cells from wild type or heterozygote littermates (C). In organ culture, mutant tibiae showed reduced bone growth compared with those from control animals at day 4 and day 6 of culture (D). (Scale bar=200μm) Mean and standard deviation from at least 4 independent experiments per genotype are shown (*$P<0.05$).


WT
KO
A
BrdU
B
PCNA
BrdU labelling cells/area
PCNA labelling cells/area
WT
eNOS KO
C
absorbance (arbitary unites)
WT
HET
KO
D
WT
KO
Bone length (mm)
Day

Immunohistochemical staining for proliferating cell nuclear antigen (PCNA) demonstrated a similar reduction in mutant mice (Fig. 3.3B). These data demonstrated reduced proliferation of chondrocytes upon loss of *eNOS*.

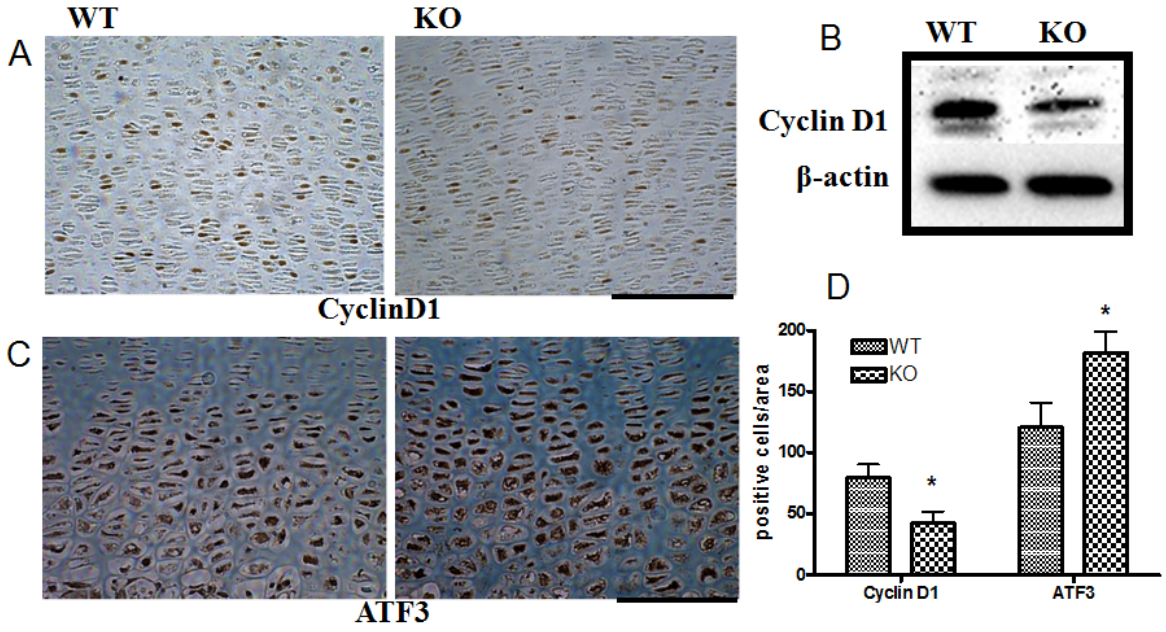
Because our mutant mice are *eNOS*-deficient in all cells of the body, it was possible that reduced bone growth and chondrocyte proliferation are secondary to loss of *eNOS* in other cell types. We therefore compared proliferation of primary chondrocytes from control and *eNOS*$^{-/-}$ mice in culture. MTT assays demonstrated that mutant cells increased their numbers more slowly than those form control littermates, while chondrocytes from heterozygote mice did not show any differences to wild type cells (Fig. 3.3C). In addition, we conducted organ culture experiments by growing tibiae from E15.5 *eNOS*$^{-/-}$ and wild type mice for 6 days in culture. While bones from both genotypes were of similar length at the beginning of the culture, mutant bones showed significantly reduced growth compared with those from control animals at day 4 and day 6 (Fig. 3.3D). These data suggest that *eNOS* is required for normal chondrocyte proliferation and endochondral bone growth in a cell-autonomous fashion.

3.4.3 *eNOS* deficiency results in reduced cyclin D1 expression

To elucidate the reason for reduced cell numbers in *eNOS*$^{-/-}$ mice, we examined cell cycle protein expression in growth plates. Immunohistochemical staining of cyclin D1, which is required for normal chondrocyte proliferation (Beier, 2005; Beier et al., 2001), demonstrated reduced numbers of positive cells in mutant mice (Fig. 3.4A). Reduced cyclin D1 protein expression in *eNOS*-deficient cartilage was confirmed by western blot (Fig. 3.4B). We recently described that the transcription factor ATF3 is upregulated

Figure 3.4 *eNOS* deficiency causes reduced cyclin D1 expression.

Immunohistochemical staining for cyclin D1 showed reduced numbers of positive cells in *eNOS* null mice (A). Reduced cyclin D1 expression in mutants was confirmed by Western blot (B). In addition, activating transcript factor 3 (ATF3) was found upregulated in *eNOS* null mice by immunohistochemistry (C). Counting of the positive cells per area confirmed these changes in the mutant (D). (Scale bar=200μm) Representative pictures from at least 4 independent experiments are shown.


A
WT
KO
CyclinD1
B
WT
KO
Cyclin D1
β-actin
C
ATF3
D
WT
KO
positive cells/area
200
150
100
50
0
*
*
Cyclin D1
ATF3

during chondrocyte hypertrophy and represses activity of the cyclin D1 promoter in chondrocyte (James et al., 2006). Immunohistochemistry demonstrated increased ATF3 expression in *eNOS*-deficient growth plates (Fig. 3.4C), suggesting that premature induction of ATF3 expression in mutant mice leads to repression of cyclin D1 transcription and chondrocyte proliferation.

3.4.4 Premature differentiation of *eNOS*-deficient chondrocytes

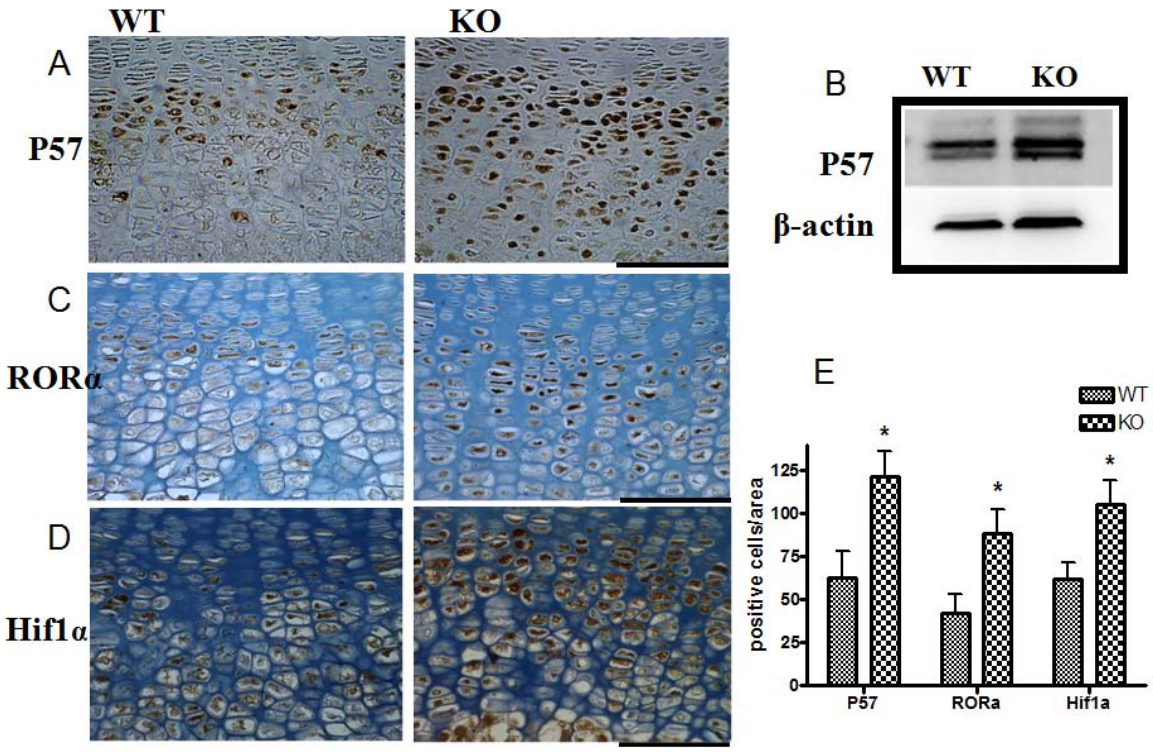
Since reduced chondrocyte proliferation is often associated with premature cell cycle exit and maturation (Beier, 2005), we examined the expression of additional markers of prehypertrophic chondrocytes. Expression of the cell-cycle inhibitor p57, which promotes cell cycle exit and is required for normal chondrocyte differentiation, was found to be increased by immunohistochemistry (Fig. 3.5A) and Western blot (Fig. 3.5B) in mutant mice. In wild type growth plates, p57 protein is restricted to a small band of prehypertrophic chondrocytes, while in KOs there is both earlier staining in late proliferative cells and sustained staining in hypertrophic chondrocytes. The nuclear receptor RORα is another marker for prehypertrophic and hypertrophic chondrocytes (Woods et al., 2009b) that showed a similar expansion in expression as p57 in mutant mice (Fig. 3.5C). Similar upregulation was observed for Hif-1α (Fig. 3.5D), a sensor for low oxygen tension (Hwang et al., 1988; Koshiji and Huang, 2004) that has been implicated in cartilage development (Schipani et al., 2001).

3.4.5 *eNOS* deficiency changes cartilage-specific gene expression

We sought to complement our immunohistochemistry results by quantitative analyses of gene expression, using real-time RT-PCR of RNA directly extracted from

Figure 3.5 Premature differentiation of *eNOS*-deficient chondrocytes.

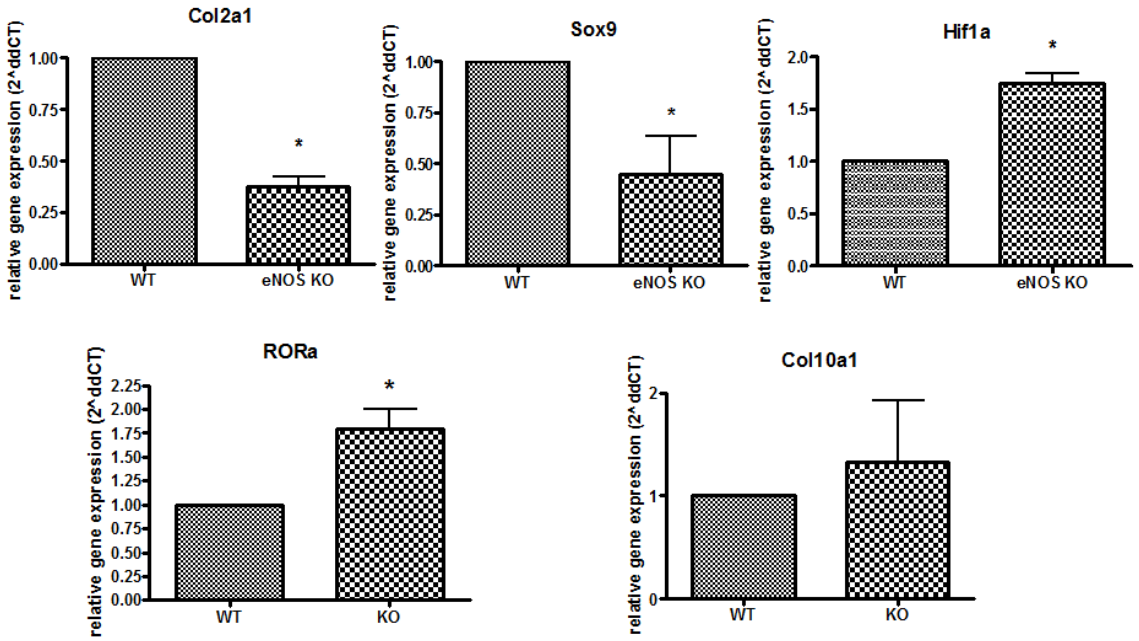
Immunohistochemistry demonstrates increased expression of the cell-cycle inhibitor protein p57 in mutant mice (A), which is confirmed by Western blot (B). The expression of prehypertrophic and hypertrophic markers retinoic acid related orphan receptor alpha (RORα) and hypoxia inducible factor 1 alpha (Hif-1α) is also upregulated in mutant growth plates, as shown by immunohistochemistry (C and D). Counting of the positive cells per area confirmed these changes in the mutant (E). (Scale bar=200μm) Representative pictures from at least 4 independent experiments are shown.


WT
KO
A
P57
B
WT
KO
P57
β-actin
C
RORα
D
Hif1α
E
WT
KO
positive cells/area
125
100
75
50
25
0
*
*
*
P57
RORa
Hif1a

cartilage. Our results revealed decreased expression of early chondrocyte markers, *Col2a1* and *Sox9* and increased expression of the prehypertrophic markers *RORa* and *Hif1a* (Fig. 3.6). In contrast, transcript levels for *Col10a1*, a marker of fully hypertrophic chondrocytes, were only elevated slightly (non-significantly) in mutant cells. These data suggest that the major phenotype in *eNOS*-deficient growth plates is the premature exit from the cell cycle and earlier prehypertrophic differentiation, with fewer effects on terminal differentiation.

Figure 3.6 *eNOS* deficiency promotes prehypertrophic gene expression.

Real-time RT-PCR analyses with RNA extracted directly from neonate cartilage reveals decreased expression of the early chondrocytes markers Col2a1 and Sox9, and increased expression of prehypertrophic markers RORα and Hif1a in *eNOS*-deficient cartilage, while expression of the hypertrophic marker Col10a1 was not significantly changed. Mean and standard deviation from at least 4 independent experiments per genotype are shown (*$P<0.05$).


Col2a1
relative gene expression (2^ddCT)
1.00
0.75
0.50
0.25
0.00
*
WT
eNOS KO
Sox9
relative gene expression (2^ddCT)
1.00
0.75
0.50
0.25
0.00
*
WT
eNOS KO
Hif1a
relative gene expression (2^ddCT)
2.0
1.5
1.0
0.5
0.0
*
WT
eNOS KO
RORa
relative gene expression (2^ddCT)
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
WT
KO
Col10a1
relative gene expression (2^ddCT)
2
1
0
WT
KO

3.5 *Discussion*

While NO has been shown to play an important role in controlling chondrocyte physiology in OA, the roles of the three NOS enzymes in cartilage development are less clear. In this study, we provide evidence for an important function of *eNOS* in the regulation of chondrocyte proliferation and differentiation. Our data show that genetic ablation of the *eNOS* gene *in vivo* results in reduced chondrocyte proliferation and endochondral bone growth. Analyses of the underlying molecular mechanisms suggest that these effects are likely due to altered expression of several cell-cycle proteins and the modulation of intracellular pathways with known roles in chondrocyte differentiation, including *Sox 9* and *Hif-1α.*

Although there were no gross morphological changes in the axial and appendicular skeleton of mutant mice, staining confirmed smaller rib cages, skull, spine and shorter long bones. Some KO mice showed more pronounced growth retardation and other defects such as kinky tails. The reasons for the variability in the phenotype are not known, but are consistent with earlier reports (Hefler et al., 2001). *eNOS*-deficient mice share some similarities to those observed in mice that lack C–type natriuretic peptide (CNP) (Chusho et al., 2001), especially dwarfism and a reduction in chondrocyte proliferation. Because both NO and CNP stimulate the production of cGMP via soluble or particulate guanylyl cyclases (GC) (Teixeira et al., 2008), these similarities are not surprising and provide further evidence for the importance of cGMP signaling in endochondral bone formation. However, the phenotype of *eNOS* null mice does not exactly resemble that of mice lacking CNP or the main effector of cGMP in cartilage, cGMP-dependent kinase II (cGKII) (Pfeifer et al., 1996). For example, mice lacking CNP

have strikingly narrow growth plates and shorter proliferating and hypertrophic zones (Chusho et al., 2001), which we did not see in our mutant mice. Because NO is produced through different NOS isoforms, deletion of *eNOS* might be compensated by the other two NOS enzymes. Indeed, we found a 2-fold upregulation of *nNOS* transcript levels in *eNOS* deficient mice, suggesting that these two constitutive forms have partially redundant roles in cartilage. Additionally, cGMP is not the only downstream target of NO; for example, NO can alter the activity of several proteins though tyrosine nitrosylation (Foster et al., 2009; Hess et al., 2005), which could account for some of the observed differences to *CNP*- and *cGKII*-deficient mice.

A requirement for *eNOS* in proliferation has been described for a number of cells, including endothelial cells and osteoblasts (Aguirre et al., 2001; Beier et al., 1999b). On the other hand, Teixeira and colleagues had reported that NO signaling stimulates chondrocyte hypertrophy in chicken chondrocytes (Teixeira et al., 2005), raising the question whether loss of *eNOS* would promote proliferation at the expense of differentiation or result in the opposite phenotype, e.g. reduced proliferation and earlier onset of maturation. Our results clearly support the latter model. While species-specific effects or differences in approaches (e.g. *in vivo* versus *in vitro*) could explain some of the differences between our study and Teixeira's (Teixeira et al., 2005), the more likely explanation is that NO effects in chondrocytes are differentiation stage- and concentration-dependent. For example, it is plausible to speculate that loss of only one NOS protein has different effects as pharmacological inhibition of all three.

eNOS inactivation results in reduced chondrocyte proliferation *in vivo* and *in vitro*. Considering that *eNOS* was deleted in all cells of the body in our mice, it was unclear

whether the reduced chondrocyte proliferation is because of loss of *eNOS* in cartilage or secondary to defects in other tissues (e.g. in endocrine tissues or endothelial cells). Therefore, we conducted organ culture and primary cell culture *in vitro*, thus removing systemic and endocrine influences. Our organ culture data demonstrated reduced tibia growth upon loss of *eNOS*, and mutant chondrocytes showed reduced multiplication of cells in culture. While these culture systems contain perichondral and periosteal cells that might be responsible for some of the observed changes, these results are consistent with the *in vivo* phenotype of *eNOS* KO mice and a cell-autonomous role of *eNOS* in chondrocyte proliferation.

The progression of the eukaryotic cell cycle is controlled by cyclins and cyclin-dependent kinases (Beier, 2005). For example, cyclin D1 has been shown to regulate the G1 phase of the cell cycle, is expressed specifically in the proliferating zone of growth plates and required for maximal chondrocyte proliferation *in vivo* and *in vitro* (Beier, 2005; Beier et al., 2001). Our data showed a decline in cyclin D1 and an increase in p57 expression, presumably resulting in slower cell-cycle progression and early cell-cycle exit upon loss of *eNOS*. The earlier cell cycle exit and resulting acceleration in the onset of prehypertrophic differentiation is also supported by increased expression of prehypertrophic markers such as RORα and Hif-1α, and by reduced expression of markers for proliferating chondrocytes, such as collagen II and Sox9. Of note, growth plate zone measurements and analyses of type X collagen expression suggest that *eNOS* deficiency accelerates prehypertrophic differentiation without accompanying acceleration of terminal hypertrophic differentiation.

In summary, our data demonstrate a role for *eNOS* in chondrocyte proliferation and endochondral bone growth. Loss of *eNOS* affects numerous aspects of cartilage physiology, including chondrocyte proliferation and gene expression. Further investigations into the specific mechanisms involved will result in a better understanding of physiological and pathological ossification and the role of *eNOS* in arthritic diseases.

3.6 *References*

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CHAPTER FOUR

REDUCED CHONDROCYTE PROLIFERATION INCREASED APOPTOSIS AND PREMATURE DIFFERENTIATION IN NEURONAL NITRIC OXIDE SYNTHASE DEFICIENT MICE*

***This chapter has been reproduced from:**
Yan, Q., Feng, Q. and Beier, F. Reduced chondrocyte proliferation increased apoptosis and premature differentiation in neuronal nitric oxide synthase-deficient mice. Submitted to *Arthritis Res Ther*.

4.1 *Chapter summary*

Nitric oxide (NO) has been implicated in the local regulation of bone metabolism. However, the contribution made by specific nitric oxide synthase (NOS) enzymes to skeletal development is unclear. The objective of this study was to examine the effects of inactivation of neuronal nitric oxide synthase (*nNOS*) on cartilage development in mice. Mice carrying a null mutation in the *nNOS* gene were used in this study to address our objectives. Histological staining, immunohistochemistry and in situ analyses were employed along with real-time RT-PCR. *nNOS*-null mice show transient growth retardation and shorter long bones. *nNOS*-deficient growth plates are hypocellular, with a marked reduction in replicating cells. Reduced chondrocyte numbers may in part be due to premature cell-cycle exit because of decreased cyclin D1 and increased p57 expressions in mutants. In addition, apoptosis was increased as shown by increased cleaved-caspase 3 staining in terminal hypertrophic chondrocytes in mutants. Expression of early chondrocyte markers such as Sox genes was reduced in mutant mice, while expression of prehypertrophic markers Rorα and c-Fos was increased. Histological sections also demonstrated thinner cortical bone and fewer trabeculae in mutant mice. These data identify an important role of nNOS in chondrocyte proliferation and endochondral bone growth and demonstrate that nNOS coordinates cell-cycle exit and chondrocyte differentiation in cartilage development.

4.2 *Introduction*

NO is a small signaling molecule with important regulatory effects in many tissues. It can function as an intracellular messenger, an autacoid, a paracrine substance, or a neurotransmitter (Murad, 1999). The versatile functions of NO stem from the chemical properties of the compound. NO is a gaseous uncharged free radical with an unshared electron that makes it easy to react with many molecules and proteins to regulate biological processes (Moncada et al., 1991). Because it is uncharged, it can freely diffuse to the surrounding cells, making it ideal as a signaling molecule (Teixeira et al., 2008). In most processes, NO activates soluble guanylyl cyclase, resulting in increased levels of the intracellular secondary messenger cGMP (Moncada et al., 1991; Murad, 1999), but other modes of signaling such as protein nitrosylation also contribute to the biological effects of NO (Ignarro, 1999; Moncada et al., 1991). Different cells and tissues have different abilities to produce NO. In general, low levels of NO are produced from constitutive nitric oxide synthases (endothelial NOS/eNOS and neuronal NOS/nNOS) and high levels of NO stem from inducible nitric oxide synthase (iNOS). The ultimate effects of NO are determined by its concentration, its source, and molecules in the microenvironment that it can react with. Over years NO has been known to regulate bone cell metabolism, bone remodeling and chondrocyte physiology in osteoarthritis (OA) (Abramson, 2008; Teixeira et al., 2008; van't Hof and Ralston, 2001), but the contributions made by specific NO synthase (NOS) enzymes to bone growth and development are unclear (Collin-Osdoby et al., 1995; Teixeira et al., 2005). In light of the prevalent connections between chondrocyte differentiation and the pathogenesis of OA (Drissi et al., 2005), and the well-

known roles of NO in OA, the elucidation of the role of individual *NOS* genes in cartilage development is of great importance.

Formation of the skeleton is achieved through two independent mechanisms: intramembranous and endochondral ossification (Karsenty, 2003; Kronenberg, 2003). Endochondral bone formation is a precisely regulated process and responsible for the formation of most bones in the adult skeleton. It involves the steps of chondrogenesis, chondrocyte proliferation, differentiation, and hypertrophy and eventually replacement of cartilage tissue by bone tissue and bone marrow (Beier, 2005; Horton, 2003; Karsenty and Wagner, 2002; Kronenberg, 2003; Provot and Schipani, 2005; Teixeira et al., 2008). During postnatal development, proliferation and hypertrophy occur in a controlled fashion in the cartilage growth plate that determines the final length of the adult bone. Within the growth plate, chondrocytes are organized in distinguished zones (resting, proliferating, prehypertrophic and hypertrophic zones) based on their morphologic shapes and gene expression patterns (Beier, 2005). If any steps during this process are not properly regulated, skeletal diseases will result, such as different types of chondrodysplasias (Zelzer and Olsen, 2003).

We recently described cartilage development in *eNOS*-deficient mice that display reduced bone growth (Yan et al., 2010). This effect appears to be due to a chondrocyte-autonomous role of eNOS in chondrocyte proliferation. However, nNOS mRNA levels were upregulated in *eNOS*-null chondrocytes, suggesting that nNOS partially compensates for the loss of eNOS. *nNOS* KO mice have been found to be viable, have enlarged stomachs due to pyloric muscle hypertrophy, exhibit insulin resistance and resistance to neural damage as the result of stroke induced by middle cerebral artery

ligation (van't Hof et al., 2004). nNOS expression has been detected during skeletal development and fracture healing, and previous studies showed reduced bone remodeling with a significant reduction in osteoblast and osteoclast numbers in *nNOS*-deficient mice (van't Hof et al., 2004). However, the exact mechanisms and functions of nNOS in earlier stages of skeletal development have not been described. In this study, we investigated the effects of *nNOS* deficiency on cartilage development and endochondral bone formation.

4.3 *Materials and methods*

4.3.1 Antibodies and reagents

All reagent materials and general chemicals were obtained from Invitrogen, Sigma or VWR unless otherwise stated. The following antibodies were employed in this study: Kip2/p57 #sc8298 (rabbit antibody against human protein), RORα #sc28612 (rabbit antibody against human protein), ATF3 #sc188 (rabbit antibody against human protein), Sox9 #sc-17340 (goat antibody against human protein), c-Fos #sc-52 (rabbit antibody against human protein), Goat-anti-mouse #sc2005, Goat-anti-rabbit #sc2004 (all from Santa Cruz Biotechnology); Cyclin D1 (SP4) #9104-S1 (rabbit antibody against human, mouse and rat proteins; NeoMarkers Inc.); PCNA #2586, (mouse antibody against human, mouse and rat proteins; Cell Signaling Inc.).

4.3.2 Mouse breeding and genotyping

Mice with a deletion of the *nNOS* gene (Stock #2986) and control mice in the C57/BL6 background were obtained from Jackson Laboratory (Bar Harbor, ME), and exposed to a 12-hour light-dark cycle and fed tap water and regular chow at libitum (van't Hof et al., 2004). All procedures involving animals were approved by the University of Western Ontario Animal Care and Use Committee. For PCR genotyping, tail snips were used to prepare DNA for PCR analysis. PCR genotyping was performed by simultaneous amplification of the wild type and null nNOS alleles as described (Drissi et al., 2005; van't Hof et al., 2004). PCR fragments were analyzed by agarose gel electrophoresis.

4.3.3 Histology and Immunohistochemistry

After dissection of mice, bones were rinsed with PBS, fixed in 4% paraformaldehyde (PFA) overnight, placed in 10% formalin solution and sent for

embedding and sectioning into 4µm sections at the Molecular Pathology Core Facility at the Robarts Research Institute (London, Ontario, Canada). Following sectioning, bones were stained with hematoxylin and eosin or safranin O/fast green using standard protocols (Solomon et al., 2009; Wang et al., 2007) or used for immunohistochemistry as described below.

For immunohistochemistry, sections unstained prior to use were dewaxed and incubated in 3% H_2O_2 for 15 min at room temperature, followed by boiling for 20 min in 10 mM sodium citrate (pH 6.0) and blocking with 5% goat serum at room temperature for 30 min. Sections were incubated with primary antibody overnight at 4°C and secondary antibodies according to the manufacturers' recommendations. After washing, sections were incubated for 1-10 min with DAB (3,3′-diaminobenzidine tetrahydrachloride) substrate solution (Dako North America, Inc.), washed and mounted. All images were taken at room temperature with a Retiga EX camera connected to a Leica DMRA2 microscope using OpenLab 4.0.4 software. For cell counts in sections, all cells and cells positive for staining with primary antibody labeling were counted from three different areas of one section. Sections from at least three mice per genotype, and at least three sections from every mouse, were analyzed, and averages and standard deviation from all counts/genotype are shown.

4.3.4 RNA isolation and real-time RT-PCR

Total RNA was isolated from epiphyseal cartilage of long bone from newborn mice using TRIzol (Invitrogen), according to the manufacturer's recommendations. Taqman real-time PCR was performed as described (Agoston et al., 2007; Woods et al., 2005) with primers and probe sets from Applied Biosystems (*Sox5* Mm00488381_m1; *Sox6*

Mm00488393_m1; *Sox9* Mm00448840_m1; *P57* Mm00438170_m1; *MMP13* Mm00439495_m1; *Igf1* Mm00439560_m1; *Gapdh* Mm99999915_g1). Data were normalized to *Gapdh* mRNA levels and represent averages and SEM from direct comparison of mutant and control littermates from three different crosses.

4.3.5 Statistical analysis

All experiments were performed with at least three independent litters. Statistical significance was determined by a one-way ANOVA with Bonferroni post test using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA.

4.4 *Results*

4.4.1 Inactivation of *nNOS* gene results in reduced bone length

We used *nNOS*-deficient mice to address the role of this gene in cartilage development *in vivo*. The knockout mice were viable and fertile, and the general appearance of stature and gait were unremarkable, but male knockout mice appeared more aggressive. Body weight and length were not significantly changed in *nNOS*$^{-/-}$ mice at birth. Growth retardation developed in surviving *nNOS*$^{-/-}$ mice in the postnatal period at day 21 to 42, as shown by reduced body weight and length (Fig. 4.1A). Body weight showed significant reduction at postnatal day 11, before body length was reduced (Fig. 4.1A). However, analyses at 3 month and 1 year of age demonstrated similar body weight and body length between genotypes (data not shown), suggesting that KO mice catch up during ageing. Observation of alcian blue/alizarin red-stained skeletons showed no obvious morphological changes in the axial and appendicular skeleton of mutants (data not shown), but measurement of selected individual bones confirmed reduced bone length in tibiae and humerus in mutant mice at postnatal day 21 (Fig. 4.1B).

4.4.2 *nNOS* deficiency results in reduced chondrocyte proliferation in the growth plate

To elucidate the cellular basis for the skeletal phenotype, we analyzed growth plate organization at different developmental stages. Growth plates from newborn mutant mice displayed a similar chondrocyte arrangement in resting, proliferative and hypertrophic zones as control mice (Fig. 4.2A). Measurement of the lengths of resting, proliferating and hypertrophic zones did not show significant differences (data not shown) between genotypes, and cells in the resting and hypertrophic zones of nNOS null mice did not

Figure 4.1 Loss of *nNOS* results in reduced growth and bone length.

Analyses of body weight and length of wild type and nNOS homozygote null mice showed that growth retardation developed in nNOS null mice during the first 42 days of their life (A). Measurement of selected individual bones confirmed reduced bone length in humerus and tibiae in postnatal day 21 mutant mice (B). Mean and standard deviation from at least 8 mice per genotype are shown for each data point. (*$P<0.05$) (WT: wild type; KO: knockout).

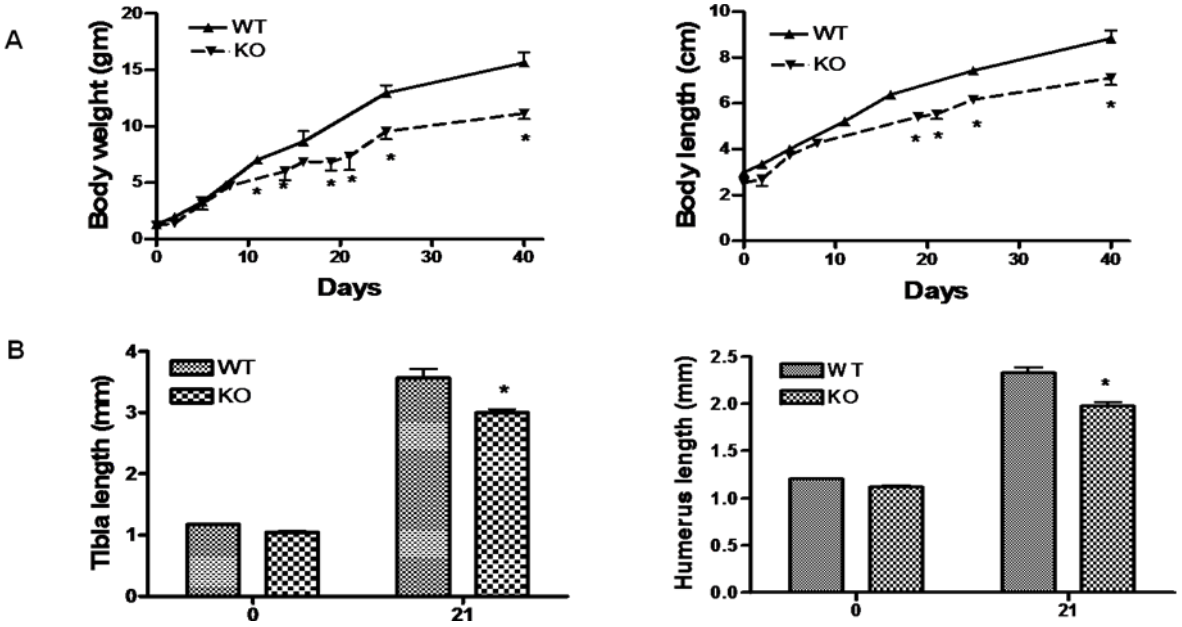
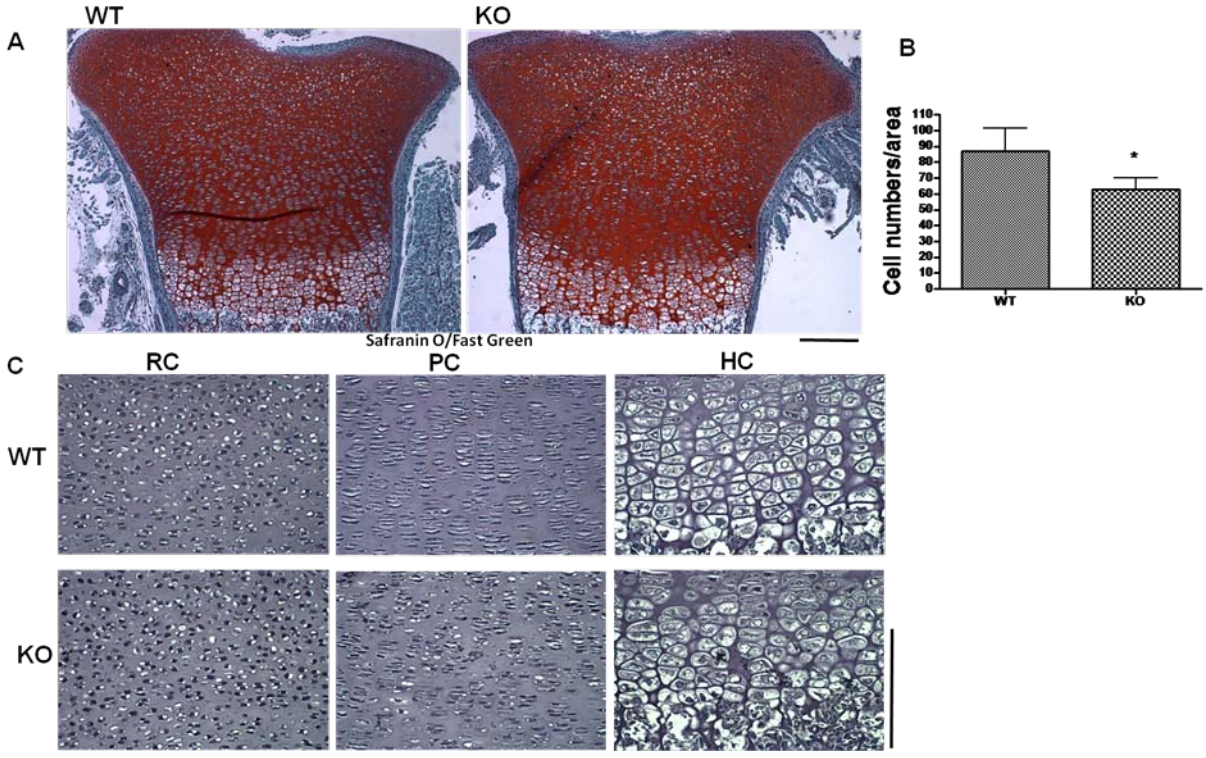

A
Body weight (gm)
WT
KO
Days
Body length (cm)
WT
KO
Days
B
Tibia length (mm)
WT
KO
0
21
Humerus length (mm)
WT
KO
0
21

Figure 4.2 Lack of *nNOS* causes reduced numbers of proliferating chondrocytes.

Safranin O/Fast Green staining of tibia growth plate sections from newborn mice demonstrated similar growth plate architecture in both genotypes, however, counting of cells revealed fewer cells in the proliferative zone of mutant growth plates (A and B). Detailed analyses of H&E stained cells in the resting, proliferative, and hypertrophic zones confirmed that the major differences occur in the proliferative zone (C). (RC: resting cells; PC: proliferating cells; HC: hypertrophic cells). (Scale bar=200μm) Mean and standard deviation from at least 6 mice per genotype are shown (*$P<0.05$).


A
WT
KO
Safranin O/Fast Green
B
Cell numbers/area
110
100
90
80
70
60
50
40
30
20
10
0
*
WT
KO
C
RC
PC
HC
WT
KO

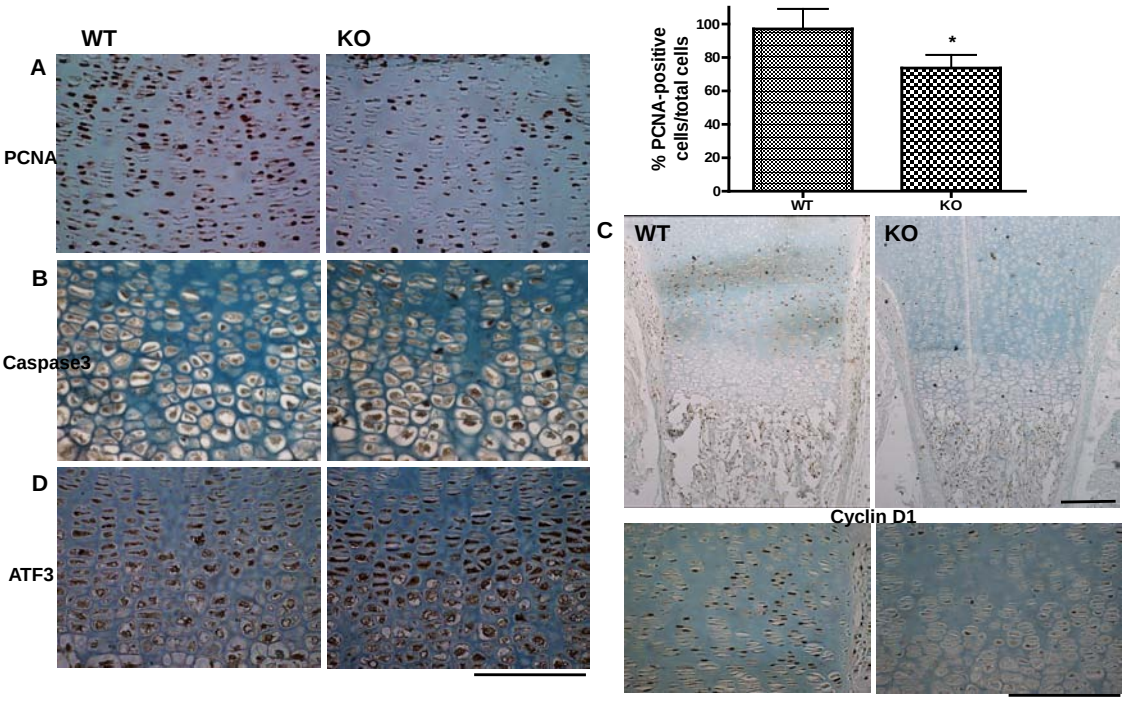
appear markedly different from controls. However, *nNOS*$^{-/-}$ growth plates were hypocellular, especially in the proliferative zone in the center of the growth plates. Some cells appear to be smaller in size, and the proliferative zone was disorganized with fewer and shorter columns (Fig. 4.2A,B and C). Counts of cell numbers in the proliferative zone of the growth plate revealed fewer cells in mutants (73% of control cell numbers per area) (Fig. 4.2B).

Since our mutant mice showed lower cell numbers in the proliferative zone of the growth plate, we next examined chondrocyte proliferation and apoptosis. Immunohistochemical staining for proliferating cell nuclear antigen (PCNA) demonstrated a reduction in positive cells in mutant mice (Fig. 4.3A). Reduced cell counts could also be due to increased cell apoptosis, which we examined by immunohistochemical staining for cleaved (activated) caspase 3. Staining for cleaved caspase 3 was increased in hypertrophic chondrocytes of mutant mice (Fig. 4.3B).

To elucidate the reason for reduced proliferation in *nNOS*$^{-/-}$ mice, we examined cell cycle protein expression in growth plates. Immunohistochemical staining for cyclin D1, which controls progression through the G1 phase of the cell cycle (Beier, 2005), demonstrated reduced number of positive cells in mutant mice (Fig. 4.3C). The transcription factor ATF3 acts as a repressor of cyclin D1 transcription in chondrocytes (James et al., 2006). Immunohistochemistry demonstrated a larger number of ATF3-expressing chondrocytes in *nNOS*-deficient growth plates (Fig. 4.3D), suggesting that premature induction of ATF3 expression in mutant mice leads to repression of cyclin D1 transcription and chondrocyte proliferation.

Figure 4.3 *nNOS* deficiency reduces chondrocyte proliferation and increases apoptosis.

Immunohistochemical staining of newborn tibia sections for proliferating cell nuclear antigen (PCNA; A) or cyclin D1 (C) showed a reduction in stained cells in mutant mice. Increased staining for cleaved caspase 3 was found in hypertrophic chondrocytes in mutant tibia sections (B). In addition, expression of ATF3 protein was increased in growth plates from newborn mutant mice (D). (Scale bar=200μm) Representative pictures from at least 3 independent experiments are shown. Mean and standard deviation from at least 6 mice per genotype are shown (*$P<0.05$).


WT
KO
A
PCNA
B
Caspase3
D
ATF3
% PCNA-positive cells/total cells
100
80
60
40
20
0
*
WT
KO
C
WT
KO
Cyclin D1

4.4.3 *nNOS*-deficient chondrocytes differentiate prematurely

Since reduced chondrocyte proliferation is often associated with premature cell cycle exit and maturation (Beier, 2005), we examined the expression of additional markers of prehypertrophic chondrocytes. Expression of the cell-cycle inhibitor p57, which promotes cell cycle exit and is required for normal chondrocyte differentiation, was found increased by immunohistochemistry in mutant mice (Fig. 4.4A). Immunohistochemistry also showed a similar increase in the number of cells expressing the transcription factors c-Fos (Fig. 4.4B) and RORα (Fig. 4.4C), both of which have been implicated in hypertrophic chondrocyte-specific gene expression (Riemer et al., 2002; Woods et al., 2009a). In contrast, Hif-1α protein expression was not changed in KO mice (Fig. 4.4D).

4.4.4 *nNOS* deficiency affects cartilage-specific gene expression

In parallel, we analyzed expression of several cartilage marker genes in the mutant mice by real-time RT-PCR, using RNA directly extracted from cartilage. Our results revealed decreased expression for early chondrocyte markers *Sox5 and Sox6* (Fig. 4.5A, B) while the reduction in mRNA levels for *Sox9* did not reveal statistical significance (Fig. 4.5C). Real-time RT-PCR also demonstrated increased expression of prehypertrophic and hypertrophic markers p57 and *Mmp13* (Fig. 4.5D,E). Transcript levels for *Igf1,* which plays an important role in regulating skeletal development and bone remodeling (Wang et al., 1992), were also strongly decreased in the mutant mice (Fig. 4.5F). Transcript levels for several other genes did not show significant responses to disruption of the *nNOS* gene in cartilage, including *Ihh, aggrecan, Col 10a1, Vegfa, Runx2,* and *RANKL* (data not shown).

Figure 4.4 Premature differentiation of *nNOS*-deficient chondrocyte

Expression of the cell-cycle inhibitor p57, which promotes cell cycle exit and is required for normal chondrocyte differentiation, was found increased by immunohistochemical analyses of newborn tibia sections in mutant mice (A). Immunohistochemistry also showed a similar increase in the number of cells expressing the transcription factors c-Fos (B) and RORα (C), while Hif1α expression did not change in the mutant mice (D) from newborn tibia sections. (Scale bar=200μm) Representative pictures from at least 3 independent experiments are shown.

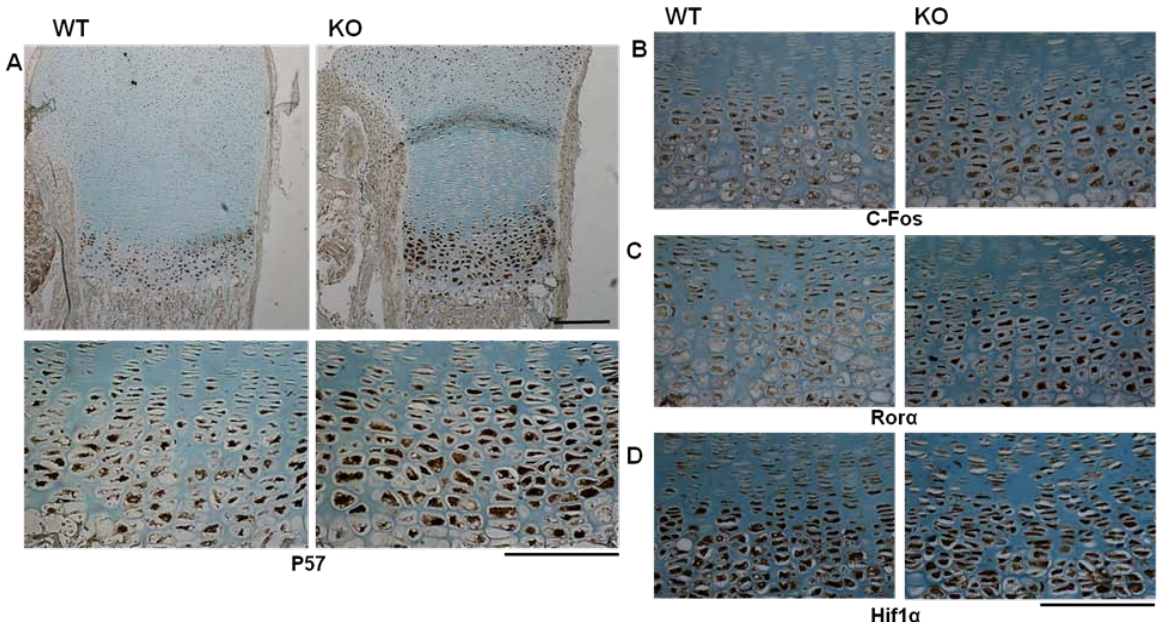
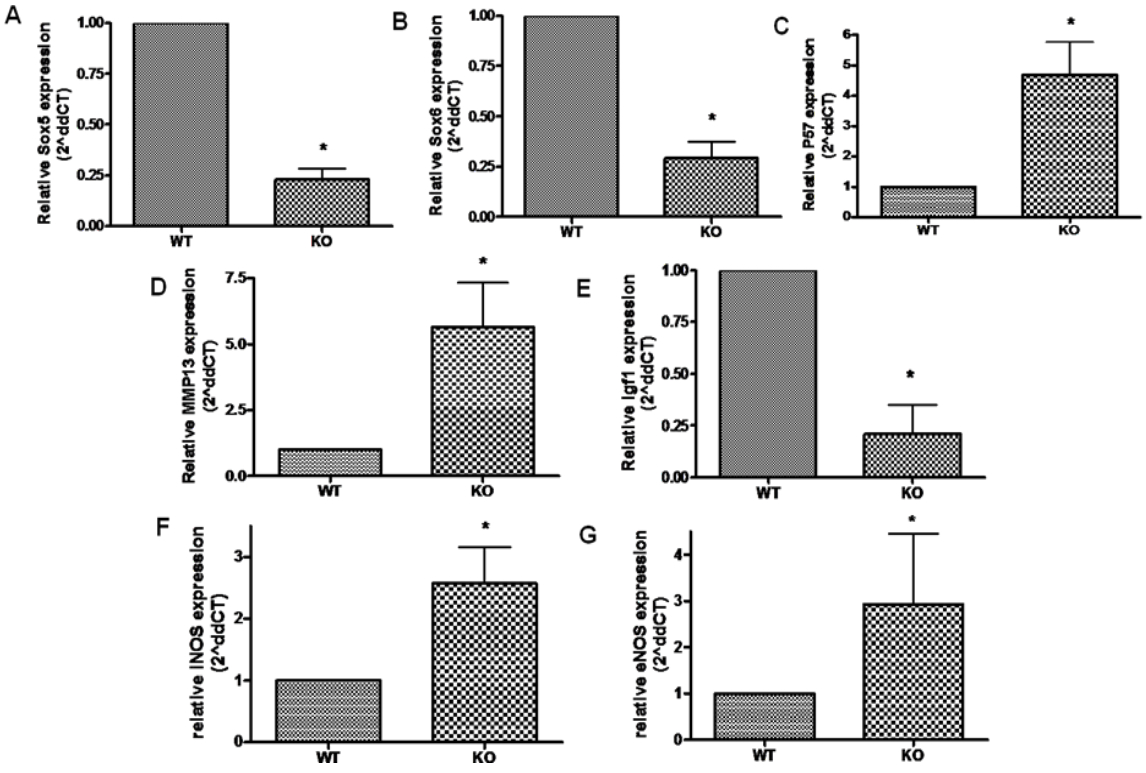

WT
KO
A
P57
WT
KO
B
C-Fos
C
Rorα
D
Hif1α

Figure 4.5 *nNOS* deficiency affects cartilage-specific gene expression.

Real-time RT-PCR revealed decreased expression for early chondrocyte markers Sox5 and Sox6 (A, B). And increased expression of the prehypertrophic and hypertrophic markers p57 and MMP13 were also demonstrated in cartilage of KO mice (C,D). Transcript level for Igf1 was significantly decreased in the mutant mice (E). mRNA levels of iNOS and eNOS were also found upregulated in *nNOS* KO cartilage (F,G). Mean and standard deviation from at least 4 independent experiments per genotype are shown (*$P<0.05$).


A
Relative Sox5 expression (2^ddCT)
1.00
0.75
0.50
0.25
0.00
*
WT
KO
B
Relative Sox6 expression (2^ddCT)
1.00
0.75
0.50
0.25
0.00
*
WT
KO
C
Relative P57 expression (2^ddCT)
6
5
4
3
2
1
0
*
WT
KO
D
Relative MMP13 expression (2^ddCT)
7.5
5.0
2.5
0.0
*
WT
KO
E
Relative Igf1 expression (2^ddCT)
1.00
0.75
0.50
0.25
0.00
*
WT
KO
F
relative INOS expression (2^ddCT)
3
2
1
0
*
WT
KO
G
relative eNOS expression (2^ddCT)
4
3
2
1
0
*
WT
KO

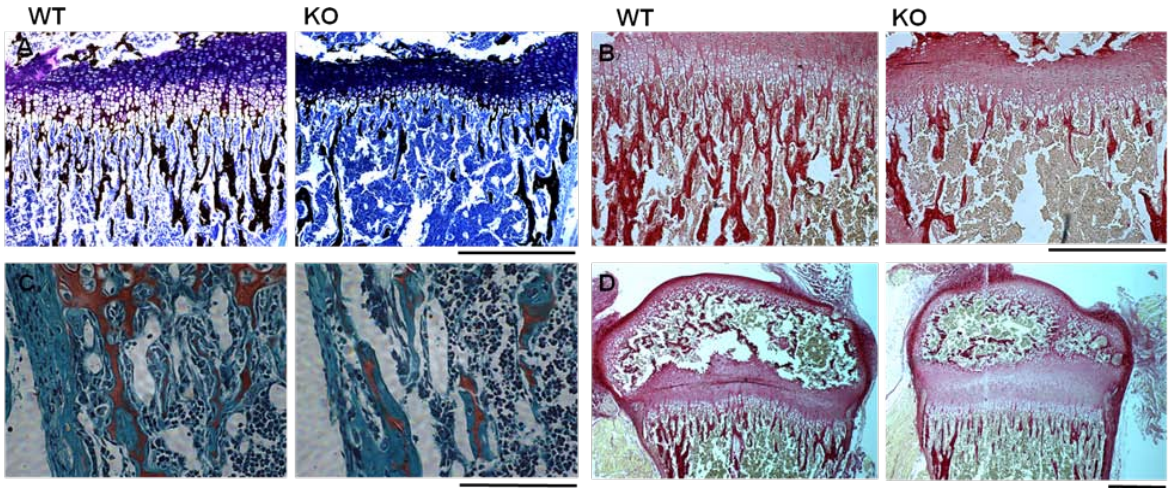
Interestingly, we also demonstrated upregulated eNOS and iNOS mRNA levels in the nNOS KO samples (Fig. 4.5G,H).

4.4.5 Loss of *nNOS* results in reduced trabecular bone and calcium deposition

To analyze whether *nNOS* deficiency had effects on endochondral ossification, we next examined trabecular bone formation and ossification patterns in mutant mice. Von Kossa staining demonstrated mineralization of trabecular and cortical bone in tibia sections from postnatal day 21 mice, which was clearly decreased in mutant mice (Fig. 4.6A). A lack of trabecular structures was seen by picrosirius red staining for fibrillar collagen (Fig. 4.6B), suggesting that these effects are not simply due to delayed mineralization, but to a delay or reduction in bone formation. Additionally, thinner cortical bone was also shown in mutant mice by Safranin O/Fast green staining from postnatal day 12 mice (Fig. 4.6C). Secondary ossification centers were advanced in control mice at postnatal day 12, but age-matched KO mice displayed underdeveloped ossification centers (Fig. 4.6D).

Figure 4.6 Deficient of *nNOS* leads to reduced trabecular bone and calcium deposition.

Mineralization of trabecular and cortical bone shown by VonKossa staining in tibia sections from postnatal day 21 mice was clearly decreased in mutant mice (A). A similar lack of trabecular structures was also seen by picrosirius red staining for fibrillar collagen (B). Additionally, thinner cortical bone was also showed in mutant mice by Safranin O/ Fast green staining (C) from postnatal day 12 mice tibiae. Secondary ossification centers were advanced in control mice at postnatal day 12, but displayed underdeveloped ossification centers in mutant mice (D). (Scale bar: 200µm for A,B,D; 50µm for C) Representative pictures from at least 3 independent experiments are shown.


WT
KO
WT
KO
A
B
C
D

4.5 *Discussion*

The signaling pathways that control growth plate chondrocyte proliferation and differentiation during endochondral ossification are incompletely understood. In this study, we provide evidence for an important role of the *nNOS* gene in these processes. Our data show that genetic ablation of *nNOS* results in reduced chondrocyte proliferation and endochondral bone growth *in vivo*. Analyses of these changes in cellular and molecular levels suggest that these effects are likely due to altered expression of several cell-cycle proteins, such as cyclin D 1 and p57, as well as modulation of other genes with known roles in cartilage differentiation, including Sox genes, RORα and c-Fos.

Although there were no obvious morphological changes in the axial and appendicular skeleton, measurement of individual bones confirmed shorter tibiae and femurs in postnatal day 21 mutant mice. Detailed analyses of nNOS null tissue sections revealed several abnormalities, most notably reduced chondrocyte proliferation. *nNOS*-deficient growth plates resemble those we observed in mice lacking the *eNOS* gene (Yan et al., 2010) and also share similarities to those in mice deficient for C-type natriuretic peptide (CNP) (Chusho et al., 2001). Because both NO and CNP stimulate the production of cGMP via soluble or particulate guanylyl cyclases (GC) (Chusho et al., 2001), these similarities are not surprising and further document the importance of cGMP signaling in endochondral bone formation. However, it should be noted that a major phenotype of CNP null mice is a reduction in the length of the hypertrophic zone (Chusho et al., 2001) that we did not observe here. One potential explanation for this difference is that during hypertrophy, when all three NOS genes are expressed highly (Teixeira et al., 2005),

eNOS and/or iNOS (or potentially CNP) can compensate for the loss of nNOS, while they are not able to do so in the proliferating zone.

The growth plate phenotype of nNOS KO mice is strikingly similar to that of eNOS null mice (Yan et al., 2010). Given the similar functions of eNOS and nNOS, the similarities between the phenotypes of both KO lines are not surprising. The compensatory upregulation of transcripts for both enzymes in the *eNOS and nNOS* KO mice provides further evidence for overlapping function of the two genes. It is likely that simultaneous inactivation of both genes would have more severe outcomes, but we have not been able to obtain double KO mice despite extensive efforts. However, it should be noted that mice lacking all 3 NOS genes have been described (Sabanai et al., 2008). Even though those mice show a very high rate of lethality (85%), the surviving triple NOS knockout mice live a surprisingly normal life (Nakata et al., 2008), suggesting that additional compensatory mechanisms may exist.

nNOS inactivation, very similar to deletion of the *eNOS* gene (Yan et al., 2010), results in reduced chondrocyte proliferation and increased apoptosis. However, increased staining for active caspase 3 mainly located to terminally differentiated hypertrophic chondrocytes, suggesting that apoptosis plays no or only a minor role in the hypocellularity that we observed mainly in the proliferative zone.

One of the major reasons for the reduced proliferation appears to be the induction of the transcription factor ATF3, which is able to suppresses cyclin D1 transcription (James et al., 2006). Cyclin D1 expression is induced by many mitogenic stimuli and pathways in chondrocytes (Beier, 2005). Our studies suggest that in addition to positive regulation through transcription factors such as ATF2 and CREB (Beier et al., 2001;

Beier et al., 1999b; Beier and LuValle, 2002), cyclin D1 transcription is also under negative regulation by ATF3 (James et al., 2006), for example in response to reduced NO levels. The cyclin-dependent kinase inhibitor p57 is a second cell cycle protein showing altered expression in the absence of eNOS or nNOS. Immunohistochemsitry showed earlier and broader expression in mutant mice, along with other prehypertrophic markers such as RORα. At the moment, we don't know whether NO signaling regulates cyclin D1 and p57 expression through a common pathway or through different effectors. It is also possible that premature differentiation to prehypertrophic chondrocytes is due to the reduced expression of *Sox5, Sox6* and potentially Sox*9* in *nNOS* KO cartilage, but the relationship between these transcription factors and cyclin D1 expression has not been addressed. Finally, reduced expression of *Igf1* could also contribute to the dwarf phenotype of *nNOS* KO mice, although the reduced growth in *Igf1*-deficient mice is largely due to smaller size of hypertrophic chondrocytes, as opposed to the primary effect on cell proliferation in our mice (Wang et al., 2006).

Loss of *nNOS* results in reduced von Kossa staining of cortical and trabecular bone. Picrosirius red staining suggests that this effect is due to paucity of trabecular structures per se, not just a delay in mineralization. At the moment, it is unclear whether this phenotype is secondary to defects in cartilage development or due to intrinsic functions of nNOS in osteoblasts or osteoclasts. As has been shown before (van't Hof et al., 2004), the similar phenotypes between trabecular and cortical bone (which does not develop from a cartilage intermediate) suggest the latter, but cell type-specific KO models will be required to resolve this question.

In summary, our data demonstrate a role for nNOS in chondrocyte proliferation apoptosis and maturation. Loss of nNOS affects numerous aspects of cartilage physiology, including chondrocyte proliferation and gene expression, as well as mineralization of bone. It appears likely that the overall phenotype of *nNOS* deficient mice is a combination of alternations in all these aspects. Further investigations into the specific mechanisms involved will result in a better understanding of physiological and pathological skeletal development and associated diseases, such as osteoarthritis and osteoporosis.

4.6 *References*

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CHAPTER FIVE

GENERAL DISCUSSION AND CONCLUSIONS

5.1 *Overview of the research project*

The focus of this thesis was to investigate the role of NOS/NO signaling in endochondral bone formation. The roles of NO and NOS genes have been studied extensively in bone remodeling and arthritis (Abramson, 2008; Scher et al., 2007; van't Hof and Ralston, 2001). This pathway has also been implicated in chondrocyte physiology during development. Teixeira and colleagues had shown that excess NO promotes hypertrophic differentiation and apoptosis of chondrocytes in chicken (Teixeira et al., 2005; Teixeira et al., 2001). However, much less is known about the specific roles of individual NOS genes in cartilage development and endochondral ossification (Teixeira et al., 2008).

Endochondral bone formation is a precisely regulated process, involving the steps of chondrogenesis, chondrocyte proliferation, differentiation, hypertrophy and eventually replacement of cartilage tissue by bone tissue and bone marrow (Beier, 2005; Horton, 2003; Karsenty and Wagner, 2002; Kronenberg, 2003; Provot and Schipani, 2005; Teixeira et al., 2008). If any steps during this process are not properly regulated, skeletal diseases will result, such as different types of chondrodysplasias (Zelzer and Olsen, 2003). Therefore, it is important to investigate the cellular mechanisms controlling growth plate regulation, for a better understanding of pathophysiological processes resulting in skeletal diseases.

The intracellular signaling pathways that control growth plate chondrocyte proliferation and differentiation during endochondral ossification are incompletely understood. Over the last several years, our studies in the laboratory have already identified *Rac1* gene as an important regulator of chondrocyte differentiation *in vitro* and

in vivo (Wang et al., 2007; Woods et al., 2007b). In particular, mice with cartilage-specific deletion of the *Rac1* gene display high mortality, profound dwarfism and numerous skeletal defects (Wang et al., 2007). The severe dwarfism we observed upon cartilage-specific deletion of *Rac1* gene is mostly due to the profound reduction in chondrocyte proliferation (Wang et al., 2007; Woods et al., 2007b). However, the downstream mechanisms connecting Rac1 to chondrocyte proliferation are unknown.

Rac1 has been shown to control many cellular processes, including actin dynamics, gene expression and generation of reactive oxygen species (ROS) (Bustelo et al., 2007; Hordijk, 2006). The first thought during this project was to indentify whether ROS signaling mediates Rac1 effects on chondrocyte proliferation. However, a recent study showed that elevated ROS levels inhibit chondrocyte proliferation and induce hypertrophy *in vivo* and *in vitro*, which did not support the notion of ROS mediating mitogenic signaling by Rac1 (Morita et al., 2007). Indeed, we only found a moderate reduction of ROS levels in Rac1-deficient chondrocytes. There are many interactions between ROS and nitric oxide (NO) (Thomas et al., 2008); for example, NO can react rapidly with superoxide and other oxygen species to form nitrite, nitrate, and perioxynitrite (collectively called reactive nitrogen species, RNS) (Asou et al., 2002; Feelisch, 2008; Ignarro, 1999; Moncada et al., 1991). However, NO or the enzymes involved in its synthesis have not been identified as major mediators of Rac1 signaling. Therefore we measured NO levels upon deletion of Rac1 in primary chondrocytes and indeed found a significant reduction in NO levels. Interestingly, *Rac1* deficiency in primary chondrocytes resulted in reduced iNOS protein expression but no changes in eNOS protein levels. Immunohistochemistry also showed reduced iNOS expression and

nitrotyrosine staining, which is an indicator of intracellular NO, in *Rac1* null cartilage. Therefore we decided to examine the role of iNOS, and subsequently the other two NOS genes, in cartilage development.

First we performed primary cell culture to investigate the effects of NOS inhibitors and NO donors on chondrocytes. We found that inhibition of NOS by LNAME results in reduced cell numbers, while NO donors increase chondrocyte numbers. After 6 days of tibia organ culture, NOS inhibition resulted in a shortened proliferating zone. In addition, we analyzed the rate of cell cycle progression by labeling growth plate chondrocytes in the S-phase of the cell cycle with bromo-deoxyuridine (BrdU), demonstrating reduced BrdU labeling upon NOS inhibition. Thus we demonstrated that NOS/NO signaling regulates chondrocyte proliferation *in vitro*. In the next step, we started to examine this signaling pathway in cartilage growth plate biology *in vivo*.

Using genetically modified mice, we examined the role of iNOS in growth plate physiology. The physical appearances of the *iNOS* KO mice were not distinguishable from WT mice. There were no obvious skeletal phenotypes in the KO mice. However, close examination of the growth plate showed hypocellularity, especially in the center of the growth plate. Counting of proliferating cells showed reduced cell numbers in the KO mice. Therefore, we next analyzed cell cycle progression by using BrdU incorporation assay which demonstrated reduced labeling in the *iNOS* KO mice, suggesting slower cell proliferation in the KO mice.

Although these data established a role of iNOS in regulating chondrocyte proliferation, we still did not know whether it mediated Rac1 effects on chondrocyte proliferation. We used primary chondrocyte and tibia organ culture models to examine

whether addition of exogenous NO could overcome the effects of Rac1 deficiency. Addition of SIN-1, a NO donor, rescued the numbers of Rac1-deficient chondrocytes in monolayer culture. In parallel, we treated cells with PMA, an inducer of ROS, and observed no effects on chondrocyte numbers. Rac1 inhibition resulted in drastic reduction of bone growth and shortening of the proliferative zone in the tibia organ culture system. The NO donor SNP did not affect bone growth on its own, but completely rescued the effects of Rac inhibition on overall tibia growth and the length of the proliferative zone. These data demonstrate that restoration of NO levels overcomes the effects of Rac1 deficiency in the intact growth plate.

To investigate this signaling pathway further, we examined the mechanism behind this regulation of chondrocyte proliferation by examining the expression of cell cycle proteins. Cyclin D1 was decreased in the *Rac1*-deficient chondrocyte culture, and treatment with SIN1 but not PMA restored cyclin D1 expression. In agreement with these data, expression of the cell-cycle progression protein cyclin D1 was decreased in the *Rac1* KO mice. We had shown in our laboratory that cyclin D1 expression in chondrocytes is repressed by ATF3 (Beier et al., 2001; Beier et al., 1999a; James et al., 2006). Therefore, we next analyzed the expression of ATF3 in the KO mice and in our chondrocyte monolayer culture. SIN-1 treatment reduced ATF3 protein levels both in control and *Rac1*-depleted chondrocytes in cell culture. Additionally, immunohistochemistry demonstrated increased numbers of ATF3-positive cells upon loss of *Rac1* as well as in *iNOS* KO mice. This suggests premature induction of ATF3 which can repress cyclin D1 transcription and result in earlier cell cycle withdrawal. We have shown for the first time here that Rac1 activates the iNOS/NO pathway that in turn

suppresses ATF3 expression in order to maintain cyclin D1 transcription and chondrocyte proliferation.

After demonstrating a role of iNOS in chondrocyte proliferation, we next aimed to examine whether other NOS genes regulate cartilage development and endochondral bone formation. I started by characterizing the phenotype of *eNOS*-null mice. These mice showed decreased viability and increased embryonic death. Examination of the genotype distribution of newborn mice revealed that only 14% of pups are homozygote for the mutant *eNOS* allele, and 40% are heterozygote, both numbers lower than the expected Mendelian ratios. These data suggest that loss of *eNOS* may lead to major abnormities during embryonic development and results in increased lethality at the prenatal or perinatal stages. Slightly decreased body weight and length were found in $eNOS^{-/-}$ mice at birth. Both of these parameters became more pronounced in surviving mutant mice as the animals aged (to 6 weeks). However, the phenotypes of the KO mice were variable. While the physical appearance of some KO mice was unremarkable in comparison to WT mice, some $eNOS^{-/-}$ mice demonstrated a more severe delay in growth and development and a significant reduction in size, and some were born with a kinky tail (6 out of 20 KO mice from 9 litters). Interestingly, when this phenotype was observed, it always affected all KO pups size in that litter.

We next examined the skeletal phenotypes of *eNOS*-deficient mice. There were no obvious morphological changes in the mutant skeleton, but neonatal mutant mice showed reduced bone length in humeri and tibiae. Appearance of primary and secondary ossification centers was delayed in the *eNOS* null mice compared with control mice and picrosirus-red staining showed fewer and shorter trabecular bones in mutant mice.

Because our mutant mice showed skeletal phenotypes, we next analyzed the cellular basis of this phenotype. Growth plates from newborn mutant mice displayed similar zonal organization as control animals. Measurement of the length of the growth plate zones did not show significant difference between genotypes. However, *eNOS*$^{-/-}$ growth plates were hypocellular, especially in the center of the growth plates (similar to *iNOS* null mice). Cell counts in the proliferative zone of the growth plate revealed fewer cells per area in mutants, while cell counts in the articular cartilage did not show a significant difference between genotypes. I next examined cell proliferation in *eNOS*-deficient growth plates. BrdU incorporation assay and immunohistochemical staining for PCNA showed a marked reduction in replicating cells in mutant mice.

Because our mutant mice are whole body KO mice, we did not know whether the skeletal phenotype was due to loss of *eNOS* gene in cartilage or secondary to defects in other organ systems. Therefore, I performed organ culture and primary cell culture *in vitro*. MTT assays demonstrated that mutant chondrocytes increased their numbers more slowly than those form control littermates. In addition, tibia organ culture experiments of E15.5 *eNOS*$^{-/-}$ and wild type mice showed that bones from KO mice grew significantly less compared to those from control animals. These data suggest that eNOS is required for normal chondrocyte proliferation in a cell-autonomous fashion.

Next I examined cell cycle protein expression in growth plates. Immunohistochemical staining of cyclin D1 demonstrated reduced numbers of positive cells in mutant mice, while ATF3 expression was increased in *eNOS*-deficient growth plates. Since reduced chondrocyte proliferation is often associated with premature cell cycle exit and maturation (Beier, 2005), I next examined the expression of markers of

prehypertrophic chondrocytes. Expression of the cell-cycle inhibitor p57 was found increased by immunohistochemistry and Western blot in mutant mice. Other prehypertrophic and hypertrophic markers, nuclear receptor RORα and Hif-1α, showed a similar upregulation in KO mice. Their expression expanded from prehypertrophic zone in control animals to the whole prehypertrophic and hypertrophic zone in the growth plates of mutant mice. This phenotype is very similar to that we observed in *iNOS* KO mice. This raises the questions whether there are common functions of the different NOS genes in cartilage. By using real-time RT-PCR, I found a 3-fold increase of nNOS mRNA level in *eNOS* KO cartilage, while iNOS transcript levels did not change. These data suggest that there are compensatory effects between the two constitutively expressed *NOS* genes, *nNOS* and *eNOS*.

Due to the compensatory upregulation of *nNOS* we found in *eNOS* KO mice, we next decided to investigate the role of nNOS in cartilage development and endochondral bone formation. *nNOS* knockout mice were viable and fertile, and the general appearance of stature and gait were unremarkable. The mutant mice showed transient growth retardation in young adulthood (2-6-week old). Other than that, there were no obvious skeletal phenotypes. A lack of trabecular structures was seen by picrosirius red staining and reduced mineralization of trabecular and cortical bone was demonstrated by Von Kossa staining at postnatal day 21. Analyses of the mutant growth plate showed a very similar phenotype to *iNOS* and *eNOS* null mice. Growth plates from *nNOS* KO mice showed reduced numbers of proliferating and PCNA-positive cells. The mechanisms behind this phenotype appeared to be premature induction of ATF3 expression, resulting in reduced cyclin D1 expression and chondrocyte proliferation. This suggests that ATF3

and cyclin D1 present a common pathway downstream of all three *NOS* genes in cartilage biology.

Immunohistochemistry also showed increased staining for cleaved (active) caspase-3 in cartilage of *nNOS* KO mice, suggesting increased apoptosis in the mutant mice. Expression of the cell-cycle inhibitor p57 and the transcription factors c-Fos and RORα was found significantly increased by immunohistochemistry in mutant mice. Real-time RT-PCR confirmed increased expression of the prehypertrophic and hypertrophic markers p57 and *Mmp13* in *nNOS*-deficient cartilage. Interestingly, transcript levels of *Igf1*, which plays an important role in regulating skeletal development and bone remodeling (Wang et al., 1992), were also significantly decreased in the mutant mice. Moreover, *eNOS* and *iNOS* transcript levels were upregulated in *nNOS* KO mice, further confirming the compensatory mechanisms among these NOS enzymes.

Collectively, these findings implicate the NOS/NO signaling pathway in regulating chondrocyte proliferation, differentiation and apoptosis during endochondral bone growth. Table 5.1 summarized the observed phenotypes for all three mutant mouse lines.

Table 5.1 Summary table of the phenotypes in the three *NOS* mutant mouse lines.

mice	iNOS KO	eNOS KO	nNOS KO
Bone phenotype	Reduced bone length	Reduced bone length; delayed ossification; fewer trabecular bone; kinky tail	Reduced bone length; fewer trabecular bone and reduced mineralization
Growth plate	Reduced proliferating cell numbers	Reduced proliferating cell numbers	Reduced proliferating cell numbers
BrdU	↓	↓	
PCNA		↓	↓
Cyclin D1	↓	↓	↓
ATF3	↑	↑	↑
Caspase-3		↑	↑
P57	↑	↑	↑
Rorα		↑	↑
C-Fos			↑
Hif1α		↑	
Sox9		↓	

5.2 *Contributions and significance*

5.2.1 Contributions to the field of cartilage biology

NO has been intensively studied in bone remodeling and arthritis. I showed for the first time that NOS/NO pathways regulate chondrocyte proliferation *in vivo*. Inactivation of any *NOS* gene leads to reduced chondrocyte proliferation, and premature prehypertrophic differentiation in growth plates. The significant reduction in bone length suggests that these pathways might play an important role in the complex regulation of growth plate physiology. NO has been implicated in controlling cell-cycle progression. My work identified decreased cycinD1 and increased p57 expression, which could explain the reduction in chondrocyte proliferation and bone length. We also identified, for the first time, Rac1 as an upstream regulator of iNOS/NO signaling in chondrocytes. Similarly, our finding that NOS genes suppress ATF3 expression in chondrocytes is novel. This NO→ ATF3 → Cyclin D1 pathway appears to be shared by all three *NOS* genes to regulate chondrocyte proliferation and differentiation (Fig. 5.1). On the other hand, NOS/NO signaling delays the onset of chondrocyte maturation. Premature hypertrophic differentiation is also a common feature of all three *NOS* KO mice and they may contribute to the phenotypes observed. Most likely NO effects in chondrocytes are differentiation stage- and concentration-dependent. For example, it is plausible to speculate that loss of only one NOS protein has different effects as pharmacological inhibition of all three.

NO signaling has been implied in transducing signals from various extracellular stimuli, such as cytokines, chemokines, growth factors and hormones, for the regulation of cell growth and metabolism. In addition to ATF3, Sox9 is another candidate

downstream target of No signaling. Sox9 is one of the major transcription factors regulating early chondrocyte differentiation (Asou et al., 2002; Karsenty and Wagner, 2002) and was decreased in *eNOS* and *nNOS* KO mice.

My data also implicate the NO pathway for the first time in the development of secondary ossification centers in the epiphyseal cartilage. The formation of SOC had been investigated in a number of studies, but not to the same extent as the development of primary spongiosa. Therefore, additional insight into this type of regulation will help us to understand the similarities and differences between these two components of the endochondral bone formation process. My data show that NOS/NO signaling is required for the normal onset and progression of secondary ossification. The most important findings of my thesis for our understanding of cartilage development are summarized in Table 5.1.

5.2.2 Contribution to the field of NO research

The role of NO has been intensively studied in inflammatory diseases, tissue injury, infection and shock (Mander and Brown, 2004). Although not all details are understood, the cytostatic action of NO, when attained at high concentrations from iNOS, has been found essential in these processes (Akarasereenont et al., 1994; Swierkosz et al., 1994; Tomlinson et al., 1994; Vane et al., 1994). However, the stimulatory action of lower quantities of NO on cell physiological function, such as cell proliferation and migration, has received less attention, although its potential physiological importance is already apparent, such as in multiple organ development (Amin et al., 1997; Mander and Brown, 2004; Peunova et al., 2001; Reif et al., 2004). Knowledge about the molecular

mechanisms and signalling events that are responsible for the enhancement of cell proliferation induced by NO is still very limited.

eNOS-deficient mice showed a significant reduction in neuronal progenitor cell proliferation in the dentate gyrus with no difference on apoptotic cell death, suggesting that eNOS selectively exerts its effects on the proliferation of progenitor cells. This might be mediated by a decrease in VEGF transcripts (Reif et al., 2004). The knockout of the *nNOS* gene results in decreased proliferation of neural precursors with the subsequent depletion of immature neurons in the olfactory epithelium of newborn mice, and altered organization of glomerular cell layers in the adult olfactory bulb, suggesting a positive action of NO on early postnatal development (Chen et al. 2004). NO also enhances cell proliferation in neuroepithelial cells of the developing neural tube (Traister et al. 2002; Plachta et al. 2003). It has been demonstrated that in lung epithelial and pleural mesothelial cells the depletion of endogenous NO by carboxy-PTIO, an NO scavenger compound, stops cell proliferation by arresting the cell cycle (Janssen et al. 1998).

In the cardiovascular system and heart development, *eNOS* KO mice are hypertensive and lack NO-mediated, endothelium-dependent vasodilation, have suppressed smooth muscle cell proliferation and display a negative inotropic effect (Sato et al., 2000). In a human bone marrow-derived mesenchymal stem cell culture (HBMSC), nitroglycerin (0.1–10 μM) led to a dose-dependent increase in cell proliferation and osteoblastic differentiation of HBMSC (Aguirre et al., 2001). Two NO donors, sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP), stimulated an increase in myoblast cell number over a range (1-10 pM) of donor concentrations (Ulibarri et al., 1999).

Controversy exists about the antiproliferative role of NO in smooth muscle cells, Drosophila and Xenopus development and a kidney cell line. One study showed that NO is involved in controlling the size of body structures as an antiproliferative agent during Drosophila development (Kuzin et al., 1996). And NO is an essential negative regulator of cell proliferation in the Xenopus brain (Peunova et al., 2001). Constitutive expression of nNOS plays an antiproliferative role in human airway smooth muscle cells (Amin et al., 1997). *eNOS* gene transfer inhibited cellular proliferation in a human embryonic kidney cell line (Maeda et al., 2000).

Collectively, the regulatory action of NO on cell proliferation is exerted in a biphasic mode, enhancing and inhibiting the progression of the proliferative process depending on the actual concentration of NO encountered by the cell and likely also the specific cell type. My data will contribute to elucidate the physiological role of NO in chondrocyte proliferation and also provide the mechanisms underlying this regulation.

The action of NO stimulating the proliferation of tumor cells has also become an important pathophysiological issue (Yoon et al., 2004). The studies concerning the action of NO on cancerous cells could be significant to better understand the molecular mechanisms underlying this pathological process and its possible therapeutic control. It is postulated that the NOS/NO pathway may be implicated in cellular proliferation and DNA or RNA synthesis of cancer cells, apart from promoting tumor angiogenesis (Shang et al., 2006) as well as its role in regulation in cell cycle progression in cancer cells (Holt, 2000).

5.2.3 Contribution to the understanding and development of therapeutic strategies in skeletal diseases

Achondroplasia, caused by activating mutations in the *FGFR3* gene, is the most common genetic form of dwarfism (Yasoda et al., 2004; Zelzer and Olsen, 2003). Currently, there is no treatment for achondroplasia other than distraction osteogenesis. In a mouse model of achondroplasia, systemic administration of CNP or cartilage-specific overexpression of CNP in transgenic mice was shown to be very effective in promoting bone growth (Yasoda et al., 2009; Yasoda et al., 2004). No side effects, including tumor growth, were observed after intravenous administration of synthetic form of CNP, CNP-22 (Yasoda et al., 2009). CNP is a promising candidate for treatment of achondroplasia and potentially other skeletal dysplasias, but as a peptide it is relatively expensive to generate and difficult to deliver to target cells in non-vascularized cartilage. NO and CNP converge on the secondary messenger cGMP and thereby activate cGKII in cartilage. As my data suggest, NOS/NO promote chondrocyte proliferation in cartilage. This may imply a potential therapeutic target for treatment of achondroplasia, and possibly other chondrodysplasias. Similarly such approaches might be useful in the treatment of other skeletal diseases including trauma and osteoarthritis.

More generally, skeletal development is a well controlled, complex process, and improved understanding of this process will help us comprehend how disturbances can compromise the function of the entire system and how we can counteract such disturbances.

5.3 *Limitations of research project and future directions*

5.3.1 Limitations of interpretation

Reduced chondrocyte numbers in our mutant mice could also be due, in part, to increased apoptosis (in addition to reduced proliferation). Although the observed apoptosis, shown by cleaved caspase-3, was mainly located in terminal differentiated hypertrophic chondrocytes, there is a chance that apoptosis or other forms of cell death also play a role to reduce chondrocyte numbers. Therefore the decreased chondrocyte cell numbers in KO mice could be a combination of reduced proliferation and increased apoptosis. It would be useful to examine growth plates at different developmental stages to elucidate this question. High concentrations of NO have long been linked to chondrocyte apoptosis (Moncada et al., 1991), while the low level of NO from cNOS (eNOS and nNOS) could be protective in chondrocyte survival. As we did not study this effect in *iNOS* and *eNOS* KO mice, it would be useful to examine chondrocyte apoptosis in these KO mice.

The regulation of Sox9 expression by the NOS/NO signaling pathway in chondrocytes is very interesting and potentially important, but I cannot conclude whether this is due to direct regulation by NOS/NO or secondary to other events. In addition to our results, Sox9 nuclear translocation has been found to be regulated by cGKII in rat chondrocytes (Chikuda et al., 2004), suggesting that regulation of Sox9 by NO/cGMP/cGKII is complex and occurs at multiple levels. Further analysis of these relationships is required for a more complete understanding of the downstream events of NO signaling during cartilage development.

We demonstrated in this project that NO suppresses the expression of ATF3 which we expect to be responsible for reduced chondrocyte proliferation. However, I cannot determine for sure whether suppression of ATF3 by NO is functionally important in this context. Ultimately, one will need to look at *ATF3* KO mice and potentially double KO with *NOS* to address this question. Although we identified the Rac1 as an upstream factor to stimulate iNOS expression and NO synthesis, I do not have data to examine whether they are under direct regulation or there are intermediate pathways connecting Rac1 to iNOS. Similar questions also exist regarding the inhibitory effects of NOS on ATF3 expression. The signaling pathways connecting NOS to ATF3 regulation need to be elucidated. Finally, the expression of cell cycle inhibitor p57 was clearly increased in all three *NOS* KO mice. But it is not clear whether upregulation of p57 and other prehypertrophic markers is secondary to premature cell cycle exit or independently regulated by NOS/NO. Addressing these questions will lead to a better understanding of cartilage biology.

5.3.2 Limitations of the experimental models

My results show that all three NOS share a common pathway in regulating chondrocyte proliferation and differentiation. However, phenotypes are most severe in *eNOS* KO mice, and minor differences between the three strains were seen (Table 5.1). Further investigation of any double or triple *NOS* KO mice are required to address redundancy and functional overlap between the three *NOS* genes. We have attempted such crossings, but were not successful in obtaining double KO mice. The use of conditional alleles that allow cartilage-specific deletion (and thus limit other phenotypes that could contribute to lethality) would be one approach to overcome this limitation.

Our mutant strains are conventional KO mice where the *NOS* genes have been deleted in each cell of the body. Therefore, some of the observed phenotypes could be due to primary defects in other cell types (e.g. the vasculature or endocrine organs). The similarity of our results in organ cultures and KO mice supports that the observed effects are due to direct effects of NO signaling in chondrocytes. However, ideally these results should be confirmed by in *vivo* experiments. The generation of conditional mutants using the Cre-lox system in combination with a cartilage-specific Cre driver line would be the best approach to address this question.

Another major concern of this project is the variable phenotype of the KO mice, especially in *eNOS* KO mice. Some KO mice (whole litters) showed a severe reduction in size compared to other KO mice. Based on this observation, we increased the sample size of the KO mice to at least eight litters to reach statistical difference. Growth of these KO mice is measured by body length and weight at different stages from newborn to six weeks to compare the difference in skeletal growth. It would be very useful to include prenatal stages and expand this study to a longer period of time. Moreover, some of the variance could be due to eNOS effects in other tissues; once again these could be overcome by the generation of cartilage-specific KO mice.

My data suggest that three *NOS* KO mice share a common signaling pathway to suppress cyclin D1 and inhibit chondrocyte proliferation. However, each individual NOS may respond to different upstream regulators in controlling chondrocyte fate. For example, we identified Rac 1 as activator of iNOS but not eNOS expression in cartilage. We do not know now what are the other upstream signaling to regulate eNOS and nNOS expression in cartilage. Interestingly, we found compensatory mechanisms among these

three *NOS* genes in cartilage; unregulated nNOS transcript levels in *eNOS*-null cartilage; and upregulated eNOS and iNOS mRNA in *nNOS* KO mice. This could explain the variability of the *NOS* KO phenotypes and the catch up growth of the KO mice after transient growth retardation at young age.

In this research project, we used primary chondrocyte monolayer culture and tibia explant culture. The monolayer culture system is an economical and technically simple model, allowing the culture of a high number of chondrocytes. Primary chondrocytes were isolated from long bone of E15.5 tibiae. At this stage mineralization and vascular invasion has just been initiated and a mixture of cell types is probably present (e.g. cells from primary spongiosa, and perichondrial cells could also be present in the monolayer cultures). However, cell types other than chondrocytes only represent a small percentage of the entire cell population. Nevertheless, because of the heterogeneous type of cell culture we cannot exclude that some of the observed NO effects are also due to the interference with other cell populations. Moreover, in primary monolayer culture, loss of chondrocyte phenotype was reported after a long period time of incubation. We limited the culture time to 48 hours to minimize the effects of this limitation and have not observed a changes in chondrocyte morphology. Nevertheless, three-dimensional cultures systems such as micromass cultures (Stanton et al., 2003; Woods et al., 2007b) would be one way to overcome this limitation.

Tibia explant culture was employed in this project for further validation the observed effects *in vitro*. The intact tibia is cultured in serum-free medium and pharmacological inhibitors can be used to alter signaling pathways in the correct three-dimensional context. Cell-cell and cell-matrix interactions are preserved in this system

and the effects of inhibitors or growth factors can be observed in a growth plate zone-specific manner. On the other hand, the organ culture system removes bone from other organ systems, such as endocrine and biomechanical environments, and allows us to study the effects of a single growth factor without influence of other organ systems. Using this system, I was able to observe specific effects in certain chondrocyte subpopulations, which would be impossible in a cell culture model.

5.3.3 Limitations of techniques and reagents

Using immunohistochemistry as a main technique in this project has certain disadvantages, such as the potential for nonspecific signals and difficulties in quantifying signals. Typically, sections of KO mice or organ culture from at least six independent trials, and representative images from each trials were analyzed to draw conclusions based on the immunostaining results (Stanton et al., 2007). Western blotting or RT-PCR was also conducted to further verify the results of protein or gene expression in chondrocytes in some key experiments. Controls included the omission of the primary antibody and, in some cases, use of tissues from KO mice for the protein of interest (e.g. the different *NOS* KO lines). Furthermore, expression patterns for proteins examined corresponded to those described in the literature. Nevertheless, we have to acknowledge the potential for lack of specificity of antibodies in IHC.

Another technical limitation in this project is that I cannot measure NO levels *in vivo*. NO is known to be formed in small amounts *in vivo* and is rapidly transformed by interaction with oxygen (Hashimoto et al., 1999), making measurement difficult. However, use of the fluorescent dye (DAF-FM) provides a sensitive and accurate method to analyze the relative quantification of NO production from chondrocytes in culture, but

it cannot give the absolute concentration of NO levels. For *in vivo* studies, we had to rely on the use of an antibody for nitrotyrosine as a substitute for measuring NO levels indirectly.

Another limitation to our study is the reliance on a pharmacological inhibitor, LNAME, to analyze the role of the NOS/NO in chondrocyte physiology. LNAME demonstrates potent inhibition of all three NOS proteins (Hashimoto et al., 2006); however, there are differences in its potency on the different enzymes, (IC_{50} of eNOS, iNOS and nNOS are 0.7, 3.9 and 0.65 μM respectively). By using a relatively high concentration, the inhibition was reported to be effective for all three NOS isoforms (Hashimoto et al., 2005). We used different concentrations of NOS inhibitors and measured NO production in parallel to test its effects and optimize the concentration under our culture conditions.

NO donors gave us the possibility to observe the effects of NO on chondrocyte without transfection. However, SNAP is a compound that is known to have peroxide effect to cause cell membrane lipid peroxidation (Kim et al., 2005). Therefore use of another NO donor helped us to validate the results. NOC-18 (DETA-NO) is a long-lasting diazeniumdiolate compound with a half-life of 27 hours (Chikanza et al., 2000). SIN1 is an inducer of NO and ROS. For the NO donors employed in this project, their effects on cell proliferation, NO and ROS levels were measured before we used them in these experiments. All donors had similar effects on the measured parameters.

5.4 *Conclusions*

In conclusion, this research project has shown that the NOS/NO signaling pathway plays important and physiological roles in multiple steps of endochondral bone formation, in particular in the control of chondrocyte proliferation. My thesis has identified several molecular mechanisms involved, such as NO suppression of ATF3 expression and subsequent effects on the chondrocyte cell cycle. These studies contribute to a better understanding of physiological bone growth and may ultimately result in improved therapeutic strategies for skeletal diseases.

5.5 *References*

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APPENDIX B

Western

07.01.08
*This is the 1st Renewal of this protocol
*A Full Protocol submission will be required in **2011**

Dear Dr. **Beier**

Your Animal Use Protocol form entitled:

Regulation of endochondral bone growth by hormones

has had its yearly renewal approved by the Animal Use Subcommittee.

This approval is valid from **07.01.08** to **06.30.09**

The protocol number for this project remains as **2007-045**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

c.c. Approved Protocol - F Beier, W Lagerwerf
Approval Letter - W Lagerwerf

***The* University *of* Western Ontario**
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University Education

University	Degree	Discipline	Year Received or Expected
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Awards & Scholarships

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- Western Graduate Research Scholarship, *The* University *of* Western Ontario, 2007-2010, $1,366 each year
- Western Graduate Research Scholarship, *The* University *of* Western Ontario, 2004-2007, $6,600 each year
- Academic Scholarships for Undergraduate Studies, Faculty of Pharmacy, *China Pharmaceutical University*, 1996-2000, $2000 each year

- Travel Award – Gordon Research Conference, Bone & Teeth, Maine, Massachusetts, 2009, $500
- Travel Award – Gordon Research Seminar, Bone & Teeth, Maine, Massachusetts 2009, $500
- Best Poster – Margaret Moffat Research Day, Schulich School of Medicine & Dentistry, *The* University *of* Western Ontario, 2008, $500

- Best Poster -- The International Conference on Chinese Medicinal Use, CHIR, Vancouver, Canada, 2005, $500

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- Graduate Teaching Assistant, Pharmacology & Toxicology 443, *The* University *of* Western Ontario, 2007-2008
- Graduate Teaching Assistant, Pharmcology & Toxicology 358, Principles of Drug Action, *The* University *of* Western Ontario, 2006-2007
- Graduate Teaching Assistant, BMSc 400 Medical Sciences Laboratory, *The* University *of* Western Ontario, 2004-2006

Academic Service

- Program Volunteer, Let's Talk Science Program, *The* University *of* Western Ontario, 2005-2006

Peer-Reviewed Publications

- **Yan Q.,** Feng Q., Beier F. (2010). Endothelial nitric oxide synthase deficiency in mice results in reduced chondrocyte proliferation and endochondral bone growth. *Arthritis & Rheum* 62(7):2013-2022.
- Wang, G.*, **Yan, Q.** *, Woods, A., Feng, Q. and Beier, F. iNOS/nitric oxide signaling mediates the mitogenic activity of Rac1 during endochondral bone growth. (*contributed equally). Revision will be submitted to *J. of Cell Science.*
- **Yan, Q.,** Feng, Q. and Beier, F. Reduced chondrocyte proliferation, increased apoptosis and premature differentiation in neuronal nitric oxide synthase-deficient mice. Submitted to *Arthritis Res Ther.*
- Liu, H., **Yan, Q.,** (2002) Protective Effects of *Baicalin* on Myocardial Injury induced by Ischemia and Reperfusion in Rats. *Journal of Railway Medical Science.* 18(2)198-208
- **Yan, Q.,** Li, Y., (2001) The Immune Effect of Chinese Drug Fengshikang-1. *Journal of Railway Medical Science.* 29(6).33-386
- Liu, H., Xu, D., **Yan, Q.**, et al. (1999) Pharmacokinetic study on Colonic -coated Oral Thymopeptidin 1&2. *Journal of Biochemistry.* 26(11). 369-375
- Liu, H., Wang, H., Pu, P., Pu, J., Xu, D., **Yan, Q.**, et al. (1998) Chemical and Pharmacological research of Chinese Drug Mohanlian. *Journal of Chinese Drugs.* 23(11) 680

Manuscript in Preparation:

- **Yan, Q.,** Beier, F. Nitric oxide and Bone. (Review, in preparation),
- **Yan, Q.,** E.M.K. Lui Protective effects of an aqueous extract of Salviae miltiorrhizae (Danshen) against homocysteine-induced loss of vascular reactivity in rats. *Life Science*. (In preparation)

Textbooks

- Zhao, L., **Yan, Q.,** (2000) Instruction of pharmacological experiments for continual education students. *Press of Medical School, Southeast University*.

Abstracts & Presentations

- Abstract: Yan, Q., Feng, Q., Beier, F. Reduced chondrocyte proliferation and endochondral bone formation in endothelial nitric oxide synthase deficient mice. *Osteoarthritis & Cartilage* (2009) S1.
- Abstract: Wang, G., Yan, Q. Rac1/iNOS/nitric oxide signalling is required for chondrocyte proliferation and endochondral bone growth. OR18. *Bone* 43(2008) S32
- Oral presentation: Endothelial nitric oxide synthase deficiency results in reduced chondrocyte proliferation and endochondral bone growth. 6th Canadian Nitric Oxide Society Conference. London, Canada. 2010.
- Oral presentation: Reduced chondrocyte proliferation and bone formation in endothelial nitric oxide synthase dificent mice. Gordon Research Seminar & Conference. Massachusetts, US. 2009.
- Oral presentation: Reduced chondrocyte proliferation and bone formation in endothelial nitric oxide synthase deficient mice. Great Lakes Mammalian Development Meeting, Toronto, Canada. 2009.
- Poster presentation; Endothelial nitric oxide synthase deficiency results in reduced chondrocyte proliferation and endochondral bone growth. Developmental biology Research Day, London, Canada, 2010.
- Poster presentation: Endothelial nitric oxide synthase deficiency results in reduced chondrocyte proliferation and endochondral bone growth. 16th Canadian Connective Tissue Conference, Toronto, Canada. 2010.
- Poster presentation: Endothelial nitric oxide synthase deficiency results in reduced chondrocyte proliferation and endochondral bone growth. Great Lakes Mammalian Development Meeting, Toronto, Canada. 2010.
- Poster presentation: Reduced chondrocyte proliferation and bone formation in endothelial nitric oxide synthase deficient mice. Gordon Research Conference. Massachusetts, US. 2009.
- Poster presentation: Reduced chondrocyte proliferation and bone formation in endothelial nitric oxide synthase deficient mice. Great Lakes Mammalian

Development Meeting, Toronto, Canada. 2009.

- Poster presentation: Reduced chondrocyte proliferation and bone formation in endothelial nitric oxide synthase deficient mice. Margaret Moffat Research Day, *The* University *of* Western Ontario, London, Canada. 2009.
- Poster presentation: Reduced chondrocyte proliferation and bone formation in endothelial nitric oxide synthase deficient mice. Department of Physiology & Pharmacology Research Day, *The* University *of* Western Ontario, London, Canada. 2009.
- Poster presentation: iNOS/nitric oxide signaling is required for chondrocyte proliferation during endochondral bone growth. 14th Canadian Connective Tissue Conference, Montreal, Canada. 2008.
- Poster presentation: Loss of endothelial nitric oxide synthase (eNos) leads to reduced chondrocyte proliferation and endochondral bone formation. Great Lakes Mammalian Development Meeting, Toronto, Canada. 2008.
- Poster presentation: NOS/NO signaling in endochondral bone formation. Margaret Moffat Research Day, *The* University *of* Western Ontario, London, Canada. 2008.
- Poster presentation: NOS/NO signaling in endochondral bone formation. Department of Physiology & Pharmacology Research Day, *The* University *of* Western Ontario, London, Canada. 2008.
- Poster presentation: Inhibition of NOS/NO pathway regulates differentiation of chondrocyte. 13th Canadian Connective Tissue Conference, Toronto, Canada, 2007.
- Poster presentation: Inhibition of NOS/NO signaling in chondrocyte. Margaret Moffat Research Day, *The* University *of* Western Ontario, London, Canada. 2007.

Academic Contracts

- Graduate Research Assistant, Department of Physiology and Pharmacology, University of Western Ontario, London, Canada, 2004-2010
- Research Assistant, Middlesex-London Health Unit, London, Canada, 2004-2006
- Assistant Professor, Clinical Pharmaceutical Lab, Medical School, Southeast University, Nanjing, China, 2000--2003