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Regulation and Function of Runx2 During Chondrogenic and Osteogenic Differentiation: a Dissertation

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A Dissertation Presented

By

Christopher Joachim Lengner

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Regulation and Function of Runx2 during Chondrogenic and Osteogenic Differentiation

A Dissertation Presented By
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Abstract

Members of the Runx family of transcription factors play essential roles in the differentiation and development of several organ systems. Here we address the contribution of the osteoblast-related Runx gene, Runx2, to the osteogenic and chondrogenic differentiation of mesenchymal stem cells. Using a transgenic mouse model, we observe Runx2 transcription through one of its two known promoters (designated P1 in pre-cartilaginous mesenchymal condensations as early as E9.5. Runx2 gene activity is later repressed at the onset of cartilage formation, both in vivo and in vitro, necessitating examination of the regulation and function of Runx2 in mesenchymal stem cells. We demonstrate that Runx2 gene activity is repressed by the direct interaction of the homeodomain transcription factor Nkx3.2 with the proximal Runx2 P1 promoter. This repression was found to be required for the progression of BMP-induced chondrogenesis, thereby identifying Runx2 as a modulator of BMP activity in the chondrogenic as well as osteogenic differentiation program. To further understand the regulation of the Runx2 P1 promoter and to determine the contribution of P1-derived gene product, Runx2 Type II, to the formation of mineralized tissue, we have generated a Runx2 Type II-LacZ gene replacement mouse model in which the initial coding sequences and splice donor sites of the Type II isoform are replaced with the LacZ reporter gene. Activity of the endogenous P1 promoter can therefore be monitored by β-galactosidase production. Analysis of Runx2 Type II-LacZ mice demonstrates that the P1 promoter is transcriptionally most active in mature osteoblasts, but its product, Runx2
Type II is dispensable for embryonic skeletal formation. Lastly, we examine the link between growth control and osteogenic differentiation by tissue-specific deletion of the Mdm2 proto-oncogene in developing skeletal tissues of the mouse embryo. Loss of Mdm2 results in impaired bone formation, with skeletal elements exhibiting lower bone mineral content and higher porosity. *Ex vivo* cultures of calvarial osteoprogenitor cells exhibit severely decreased osteoblastogenesis and bone nodule formation accompanied by a failure to activate Runx2 gene activity. These findings suggest that Mdm2 is required for inhibition of p53 activity that ultimately allows for post-confluent proliferation and induction of Runx2 during maturation of the osteogenic phenotype. Taken together, our findings suggest that Runx2 modulates the commitment of progenitor cells to the osteogenic and chondrogenic lineages, and that Runx2 activity is inextricably linked to mechanisms that control cellular proliferation.
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Chapter I: General Introduction
**Skeletal Formation**

Formation of the vertebrate skeleton is an exquisitely intricate process that requires the coordinated proliferation, migration, and differentiation of a variety of cell types including the cartilage-forming chondrocyte, the bone-forming osteoblast, and the bone- and cartilage-resorbing osteo/chondroclast. During mammalian development, the mesenchymal cells that form the skeleton are derived from distinct embryonic origins. Neural crest cells contribute to the skull and craniofacial bones, lateral plate mesodermal cells form the appendicular skeleton (limbs and extremities), and sclerotomal cells derived from the somites give rise to the axial skeleton (spine and ribs) (Figure 1.1-A).

Despite the distinct embryonic lineages that contribute to skeletal formation, there are similarities in function and gene expression in the skeletal cells derived from these lineages and these cells all participate in several phases of skeletogenesis. Initially, mesenchymal progenitor cells migrate to sites of skeletogenesis, then undergo epithelial-mesenchymal interactions which in turn induce condensation that is followed by overt differentiation either directly into bone-forming osteoblasts (a process termed intermembranous bone formation occurring primarily in the craniofacial skeleton in which there is no cartilaginous template for the future bone) or into chondrocytes which will go on to form a cartilaginous template for future bone formation (Figure 1.1-B).

Bone formation following a cartilaginous template occurs in the majority of the skeleton through a process termed endochondral bone formation. During endochondral bone formation, the mesenchymal condensations that occur at sites of future bone formation
Figure 1.1: Mesenchymal progenitor cells of the somites are induced to differentiate into sclerotome, myotome, and dermomyotome in response to local signalling molecules secreted from the notochord. Cells of the sclerotome are destined to undergo chondrogenesis and form the cartilaginous template for the components of the axial skeleton (A). Mesenchymal progenitor cells undergo several phases of skeletal development. These cells migrate, then undergo epithelial-mesenchymal interactions which induce condensation. Ultimately, these cells undergo differentiation into the various cell types that comprise the mammalian skeleton (B). Adapted from Principles of Bone Biology by JP Bilezikian, LG Raisz and GA Rodan, Academic Press, 2002.
undergo overt chondrogenesis rather than osteogenesis thereby giving rise to a cartilaginous anlage for future mineralization. This temporary cartilage subsequently undergoes a maturation process termed hypertrophy in which the chondrocyte cell body expands and the cartilaginous extracellular matrix is mineralized (the molecular pathways governing these processes are discussed in detail in chapters 2 and 3). This mineralized cartilage is then invaded by vasculature that carries both bone/cartilage-resorbing osteo/chondroclasts as well as precursors of bone-forming osteoblasts to the site of new tissue formation. It is the coordinated activity of these two cell types that ultimately leads to the formation of mineralized bone on the existing cartilaginous template. Bone forming osteoblasts differentiate in situ where they deposit a number of extracellular matrix proteins (primarily type I collagen) that comprise the organic component of the mineralized skeleton. Subsequently, the osteoblast will deposit crystalline salts into the extracellular matrix (primarily calcium and phosphorous in the form of hydroxyapatite) in order to mineralize the organic matrix and provide rigidity to the system. In the past decade, many studies aimed at understanding the genetic activity that underlies these processes have culminated in the discovery and functional characterization of the Runx2 gene, whose product is emerging as the single most important protein in the complex process that is skeletogenesis.

Biology of Runx Proteins

Runx2 is a member of the mammalian family of Runt-related transcription factors that consists of three proteins that share the DNA-binding Runt-homology domain. Genetic
analysis of mice harboring targeted inactivating mutations in these genes has demonstrated their importance in organogenesis as well as cell growth control. Runx1 deficiency leads to early embryonic lethality due to failed hematopoiesis (North et al. 1999; North et al. 2002); Runx2 deficiency results in a block in osteoblast differentiation and a total lack of mineralized tissue (Ducy et al. 1997; Komori et al. 1997; Otto et al. 1997); and Runx3 deficiency leads to impaired neuronal development and hyperplasia of the gastric mucosa (Levanon et al. 2002; Li et al. 2002). Although these findings clearly demonstrate the importance of the Runx transcription factors in development of specific tissues, recent evidence indicates that the role of the Runx proteins is more complex. The co-expression of multiple Runx factors in several early embryonic tissues suggests a functional redundancy that is supported by recent evidence demonstrating the cooperation of Runx2 and Runx3 in cartilage maturation (Taniuchi et al. 2002; Yamashiro et al. 2002; Yoshida et al. 2004). Here we focus on the regulation and function of the Runx2 protein during fate determination in progenitor cells, and also examine some functional interrelationships between Runx2 and Runx1.

Properties and Functions of Runx2

Runx2 was originally identified as an inappropriately activated gene in virus-accelerated T-cell lymphomas. Deregulation of Runx2 expression was found to be a consequence of retroviral insertion into the Runx2 promoter region (Stewart et al. 1997). Further studies from the Stewart group demonstrated that Runx2 overexpression in T-cells results in spontaneous tumor formation in transgenic mice that can be synergistically accelerated in
the presence of a myc transgene, revealing the oncogenic potential of Runx2 (Vaillant et al. 1999). Shortly thereafter, Runx2 was identified independently as a major DNA binding component on the bone-specific osteocalcin promoter, indicating that the normal function of Runx2 is related to osteoblast differentiation (Banerjee et al. 1997). In 1997 two groups independently generating Runx2 null alleles confirmed the importance of Runx2 in osteogenesis as null mice arising from these targeted mutations lacked mature osteoblasts and a mineralized skeleton (Ducy et al. 1997; Komori et al. 1997; Mundlos et al. 1997; Otto et al. 1997). Interestingly, Runx2 null mice successfully form a cartilaginous template for the skeleton, but lack subsequent vascular invasion and turnover of cartilage into bone. Runx2 haploinsufficiency results in a disease in mice that is analogous to the human disorder Cleidocranial Dysplasia (CCD), a disease characterized by supernumerary teeth, defective cranial suture ossification resulting in delayed fontanel closure, and absent or hypoplastic clavicles (Mundlos et al. 1997). Indeed, a variety of mutations in the Runx2 gene have been mapped in CCD patients (Mundlos et al. 1997; Yokozeki et al. 2000; Zhang et al. 2000; Bergwitz et al. 2001; Goseki-Sone et al. 2001; Ito and Zhang 2001; Golan et al. 2002; Otto et al. 2002; Vaughan et al. 2002; Yoshida et al. 2002; Tessa et al. 2003). Molecular analysis of Runx2 function ultimately demonstrated that Runx2 is able to transactivate a plethora of osteoblast phenotypic genes (alkaline phosphatase, MMPs, type I collagen, osteocalcin, Osterix) necessary for proper bone formation. Further, Runx2 acts as a scaffolding protein within the nucleus through its nuclear matrix targeting signal (NMTS). The NMTS is a conserved 31-38 amino acid motif that directs Runx2 to distinct nuclear
domains that support gene expression (Zaidi et al. 2001; Zaidi et al. 2002) and deletion of this domain along with the C-terminus of Runx2 results in a phenotype analogous to that seen in Runx2 null mice (Choi et al. 2001), suggesting that proper subnuclear targeting is required for the functional activity of this protein in promoting osteoblast differentiation.

More recently, findings from Pratap and Galindo (Pratap et al. 2003) have uncovered a role for Runx2 in growth control. They demonstrated that Runx2 levels are stringently regulated with respect to cell cycle entry and exit of osteoprogenitor cells and that these cells exhibit higher proliferation rates in the absence of Runx2. Taken together, these findings suggest that Runx2 supports differentiation of the osteoprogenitor not only through the direct activation of osteoblast phenotypic genes but also by supporting exit from the cell cycle.

**Runx and the Mesenchymal Stem Cell**

Despite the extensive body of literature relating to the function of Runx2 in osteoblast differentiation, little is known about the function or regulation of this gene in the stem cell population that gives rise to osteoblasts, chondrocytes, adipocytes, myoblasts, and fibroblasts. These cells are termed mesenchymal stem cells (MSC) and are derived from the embryonic mesenchyme. For the purposes of this dissertation, we will focus on the embryonic mesenchyme that makes up the somites that in turn give rise to the axial skeleton through the process of endochondral bone formation. The basic anatomic
blueprint during embryogenesis is derived from the segmentation of paraxial mesoderm. Upon gastrulation, these cells segregate to form the presomitic mesoderm that surrounds the neural tube on either side. Through the actions of a number of homeodomain (Hox), and pair-rule (hairy, lunatic fringe) transcription factors, the unsegmented presomitic mesoderm becomes segmented in a series of paired condensations called somites. During somitogenesis, segmentation occurs sequentially by the addition of new somites in a craniocaudal manner with a periodicity that reflects the segmental organization of the embryo. The somites later respond to a number of soluble signaling molecules secreted by the notochord (particularly Sonic hedgehog and Wnt) by undergoing further segmentation into myotome, dermomyotome, and sclerotome, each of which in turn gives rise to specific mesenchymal tissues (Figure 1.1-A). Activation of all three Runx genes is apparent during somitogenesis and persists during formation of the sclerotome. The mesenchymal progenitor cells in the sclerotomal condensations are destined to give rise to the cartilaginous template of the axial skeleton in response to the activity of a number of transcription factors including members of the Pax, Sox, and Nk families. The function of Runx factors during this developmental stage is entirely unknown as deletion of any one Runx protein has no effect on the formation of somites, sclerotome, or the cartilaginous template during endochondral bone formation. It is this observation that has driven the work described in this dissertation in which the function and regulation of
Runx2 are examined in the mesenchymal progenitor cell, and a link between growth control and differentiation into skeletal cells is established through Runx2.

**Aims of the Dissertation**

While significant progress has been made in understanding the functional activity of Runx2, relatively little is known about the regulation of this gene. Initial studies on the Runx2 promoter by Drissi et al. demonstrated that Runx2 activity was suppressed by the osteogenic steroid hormone vitamin D3 as well as by autoregulatory activity in osteoblast cell lines (Drissi et al. 2000; Drissi et al. 2002a; Drissi et al. 2002b). This dissertation is aimed at elucidating pathways that regulate the Runx2 gene during skeletogenesis and understanding the functional consequences of this regulation.

In the subsequent chapters, we address the transcriptional regulation of the Runx2 gene in the developing mammalian embryo using a combination of *in vivo* and *in vitro* approaches. We identify a region of the bone-related Runx2 promoter that appears to be highly active in pre-chondrogenic mesenchymal condensations of the axial skeleton in the developing murine embryo and we show that the Runx2 promoter is negatively regulated by the pro-chondrogenic NK-domain homeobox transcription factor Nkx3.2. Our data suggests that the negative regulation by Nkx3.2 acts to suppress anti-chondrogenic properties of Runx2, thereby promoting chondrogenic differentiation of mesenchymal progenitor cells.
Next, we examine the temporal expression patterns and functional contributions of both Runx1 and Runx2 throughout the differentiation of mesenchymal progenitor cells into mature chondrocytes. To this end, we have developed an *ex vivo* culture system in which mouse embryonic fibroblasts (MEFs) are induced to undergo chondrogenesis in response to high-density culture conditions and bone morphogenetic protein-2 (BMP2) signaling. The ability to isolate MEFs mid-gestation enables the study of *Runx1* gene function during the development of the chondrogenic phenotype *ex vivo* for the first time due to the early, pre-cartilaginous embryonic lethality of mice with inactivating mutations in the *Runx1* gene. We observe that MEFs lacking Runx1 are able to undergo the complete program of chondrocyte differentiation, while MEFs lacking Runx2 are able to form chondrogenic masses *ex vivo*, but cannot undergo final stages of hypertrophy. Further, we show that in absence of Runx2, Runx1 protein levels are unaltered, suggesting that minimal cross-regulation occurs in during this process. Our findings further demonstrate that although both *Runx1* and *Runx2* genes are active in these cells, they exhibit unique expression patterns suggesting that Runx1 and Runx2 may have the capacity to play unique roles during the growth and differentiation of a single cell type.

The fifth chapter of the dissertation is aimed at exploring the contribution of the Runx2 Type II isoform to the process of osteogenesis. This isoform, termed Type II, or til, is under control of the upstream P1 promoter and exhibits a much more restricted expression pattern in comparison to its downstream counterpart, Runx2 Type I (Park et al. 2001; Chen et al. 2002; Choi et al. 2002). These two proteins differ only in that the
Type II isoform contains an additional 19 amino acids at its N-terminus. Despite these additional amino acids, the Runx2 isoforms appear to function identically with regards to their ability to transactivate target promoters (Banerjee et al. 2001) indicating functional redundancy between these proteins. These observations suggest the importance of the two promoter regions at the Runx2 locus may be to gain another level of control of Runx2 expression during different developmental circumstances. To address the specific function of the Type II isoform and the bone-specific activity of the P1 promoter, we have engineered embryonic stem cells and subsequently generated a mouse line containing a targeted gene knockin in which the lac-Z reporter gene replaces the initial exon unique to Runx2 Type II. This targeting strategy enables us to monitor endogenous gene activity at the P1 locus by X-Gal staining while maintaining expression of Runx2 Type I through the downstream P2 promoter. Our results demonstrate that Runx2 P1 promoter appears to be most active in the most mature osteoblasts along cortical bone surfaces in vivo and in the center of mineralizing osteoblast nodules ex vivo. Preliminary phenotype analysis of Runx2 Type II $^-{-}$ mice is underway despite a published report which indicates that loss of this isoform results in defective formation of a variety of skeletal elements and a surprisingly high rate of perinatal lethality (Xiao et al. 2004).

The final portion of the dissertation establishes a novel link that exists between bone formation, Runx2, and the proto-oncoprotein Mdm2. Mdm2 is well known for its function as a critical regulator of p53. By inactivating p53 through several mechanisms, Mdm2 allows for progression of the cell cycle in many primary and immortalized cell
lines. Here we examine the contribution of Mdm2 to skeletogenesis by utilizing an Mdm2 conditional allele generated by Dr. Heather Steinman (Steinman and Jones 2002). We achieved tissue-specific deletion of Mdm2 in the developing skeleton by interbreeding Mdm2 conditional mice to transgenic mice harboring the Cre-recombinase gene under control of the type I collagen promoter (kindly provided by Dr. Barbara Kream at the University of Connecticut). Our findings demonstrate that an increase in Mdm2 activity during osteoblast maturation is required to suppress p53 activity in order for pre-osteoblasts undergo cell division in post-confluent ex-vivo cultures. Upon deletion of Mdm2, pre-osteoblasts fail to form multilayered nodules and subsequently fail to induce Runx2 ultimately resulting in a block in differentiation. These results suggest that pathways governing growth control and differentiation are intricately intertwined in the osteoblast and must be coordinated for the successful establishment of the mammalian skeleton.

Taken together, our findings illustrate the complexities of Runx biology and highlight the importance of Runx2 in the development of multiple mammalian tissues. The proceeding findings of this doctoral thesis offer important insight into the complex regulation and functionality of Runx2 and establish a basis for future research into novel functions of the Runx family of proteins.
Chapter 2:

Activation of the Bone Related Runx2/Cbfa1 Promoter in Mesenchymal Condensations and Developing Chondrocytes of the Axial Skeleton
ABSTRACT

The Runx2/Cbfal transcription factor is expressed at early stages of embryonic development and is required for bone formation. Elucidating the transcriptional mechanisms that support the developmental function of Runx2 is important for understanding the signals mediating skeletal formation. In this study, we examine the spatio-temporal activity in vivo of the Runx2 P1 promoter that controls expression of the bone related isoform. Transgenic mice carrying 3 kb of the Runx2 promoter fused to the LacZ reporter gene exhibit localized promoter activity that parallels the endogenous gene in early mesenchymal condensations shortly after the embryonic turning event. Expression in developing mesenchyme continues throughout the differentiation of these cells into chondrocytes at later embryonic stages and is restricted to the axial skeleton. Taken together, our data support a function for Runx2 in establishment of the prechondrocytic skeleton.
INTRODUCTION

Skeletal formation is a multistep process initiated by condensations of mesenchymal precursor cells that will form the skeletal architecture through both intramembranous and endochondral ossification. The events leading to the development and formation of osseous tissue are governed by a variety of signaling pathways and regulatory factors including hormones, growth factors, cytokines, and transcription factors. Several transcription factors including members of the homeodomain family are required for epithelial-mesenchymal interactions and early skeletal patterning (Thesleff 1998; Olsen et al. 2000; Hall 2001). Runx2, a member of the runt-homology domain family of transcription factors, is the only transcription factor known to be necessary for the formation of the entire mineralized skeleton (Komori et al. 1997; Otto et al. 1997; Choi et al. 2001).

Runx2 shares strong homology with the Drosophila pair-rule genes runt and lozenge that play a role in body patterning (Canon and Banerjee 2000). In mammals, the Runx genes (Runx1, Runx2, and Runx3) have essential roles in organ development. Runx1 is required for proper hematopoiesis (Okuda et al. 1996; North et al. 1999), Runx3 is important for gut development (Levanon et al. 2002; Li et al. 2002), whereas Runx2 is essential for skeletal formation. Haploinsufficiency of Runx2 results in cleidocranial dysplasia (CCD) both in humans and mice (Ducy et al. 1997; Komori et al. 1997; Mundlos et al. 1997; Otto et al. 1997), and targeted disruption of the Runx2 locus causes a total lack of bone
formation due to the maturational arrest of osteoblasts (Choi et al., 2001; Komori et al., 1997; Otto et al., 1997). Runx2 regulates both osteoblast (Banerjee et al., 1997; Ducy et al., 1997) and chondrocyte (Akiyama et al. 1999; Inada et al. 1999; Kim et al. 1999; Enomoto et al. 2000; Takeda et al. 2001; Ueta et al. 2001) maturation and thus, the Runx2 gene must be responsive to both chondrogenic and osteogenic signaling pathways.

The Runx2 locus encodes two principal isoforms that are transcriptionally controlled by two distinct promoter regions. The Type I Runx2 isoform (initiating with the peptide sequence MRIPV) is expressed in a variety of cell types including cartilage and bone (Kim et al. 1999; Banerjee et al. 2001) and is regulated by a downstream promoter (P2). The Type II isoform (initiating with the peptide sequence MASNS) was originally cloned as Cbfa1/til-1 (Stewart et al. 1997) and is under the control of an upstream (P1) promoter (Fujiwara et al. 1999; Drissi et al. 2000; Xiao et al. 2001). This isoform is highly expressed in differentiating osteoblasts and is upregulated in response to the osteogenic BMP-2 signal (Enomoto et al. 2000; Banerjee et al. 2001).

We have previously reported the cloning and characterization of the bone related Runx2 P1 promoter and have identified consensus binding sequences for a number of well known transcriptional regulators including NFkB, AP1 family members, C/EBP, steroid receptors, as well as helix loop helix proteins. Interestingly, the promoter contains functional Runx2 and Vitamin D binding sites that both suppress Runx2 promoter activity (Drissi et al. 2000; Drissi et al. 2002a). Understanding promoter regulatory mechanisms
that mediate skeletal specific expression of Runx2 will provide insight into the requirements for activity and regulation of Runx2 function in skeletal development.

This study addresses the in vivo contributions of a 3 kb segment of the Runx2 promoter to the highly specific spatio-temporal events that occur in the process of skeletal formation. The activity of a Runx2 P1 promoter-LacZ transgene (3 kb-LacZ) was assessed by the detection of the β-galactosidase enzyme throughout the development of the mouse embryo. Consistent with the onset of expression of endogenous Runx2, this promoter confers regulatory information activating transgene expression in early mesenchyme shortly after the embryonic turning event. The transgene continues to express in the perichondrium and in cells of the chondrocytic lineage in the axial skeleton, but is absent from chondrocytes of the appendicular skeleton and from cells of the osteoblastic lineage. Taken together, these results indicate that the promoter of the bone related Runx2 gene contains regulatory information that supports expression in early mesenchymal condensations and specifies temporal and skeletal site selective activation.

MATERIALS AND METHODS

Plasmids

The transgenic vector (Runx2-LacZ) was constructed using a 2.9 kb Bgl II/Pst I fragment from the 5' flanking region of the Runx2/til-1 bone related isoform (Figure 2.2-A) ligated into the pBluescript SK+ backbone vector (Stratagene, La Jolla, CA). The bacterial LacZ gene (β-galactosidase) carrying a bovine growth hormone poly-adenylation signal (kindly
provided by Dr. Stephen Jones, University of Massachusetts Medical School) was then ligated at the immediate 3' end of the promoter fragment into a PstI/Xhol site of the pBluescript polylinker region. In transient transfection assays, a CMV-\textit{LacZ} construct was used to control for transfection efficiencies.

\textit{Transfection Experiments}

ROS 17/2.8 osteosarcoma cells were cultured in F12 media in the presence of 10% fetal calf serum, 2 mM L-glutamine, penicillin/streptomycin, and were maintained in a humidified 37° incubator with 5% CO₂. ROS 17/2.8 cells were transiently transfected using Superfect lipofection reagent (Qiagen, Valencia, CA) in 6 well plates 24 hours after seeding cells at a density of 10⁵ cells per well. The transfection complex was formed using either 2 µg of the \textit{Runx2-LacZ} construct or 0.5 µg of CMV-\textit{LacZ} construct along with carrier DNA for efficient transfection and 8 mL/well of Superfect lipofection reagent in one tenth of the final feeding volume of incomplete DMEM. The cells were then incubated for three hours with the superfect/DNA complex before washing and feeding with fresh completed F12 media. After transfection, cells were incubated for 48 hours and β-galactosidase activity was assessed qualitatively by X-gal staining cells fixed for 5 minutes in 0.5% glutaraldehyde (EM Sciences, Gibbstown, NJ) with the X-gal substrate solution containing 2 mM MgCl₂, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.01% sodium deoxycholate, 0.02% NP-40, and 1 mg/mL X-gal (5-bromo-4-chloro-3-indoly1-β-galactopyranoside, Gold Biotechnology, St Louis, MO) in phosphate buffered saline (PBS, pH 7.3) at room temperature for 10-12 hours.
Generation of Transgenic Mice

The transgenic construct was digested with Not I/Xho I in order to linearize the transgene and remove the backbone vector. The resulting digestion products were electrophoretically separated and the linearized transgene was purified using a gel extraction kit (Qiagen, Valencia, CA). Microinjection of the linearized transgene into fertilized oocytes from C57/BL6xSJL mice (Jackson Labs, Bar Harbor, ME) and transfer of microinjected embryos into pseudopregnant foster mothers was performed by the Transgenic Animal Core Facility at the University of Massachusetts Medical School. Animals were maintained and used in accordance with the Federal Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals under the supervision of the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School. The injections resulted in 65 potential founder mice, 9 of which were found to carry the transgene. Of these nine founder mice, two mice that expressed the transgene were mated to generate transgenic lines (L1 & L2) for analysis.

Genotyping

Genotyping was carried out using Southern blot analysis and PCR with primers spanning the Runx2 promoter/LacZ junction (Fig. 2C). Genomic DNA was isolated from mouse tail clips by digestion with proteinase K followed by phenol-chloroform extraction and ethanol precipitation. For Southern blot analysis (Figure 2.2-C), 10 μg of genomic DNA was digested with Sac I, releasing a 3 kb fragment spanning the promoter/LacZ junction.
Digested DNA was electrophoresed in a 1% agarose gel, transferred onto a nylon membrane, and hybridized with an 1100 bp $^{32}$P labeled cDNA probe spanning the promoter/LacZ junction (Figure 2.2-A). PCR analysis was performed on 100 ng of genomic DNA using forward primer 5'GGAGAGACAGAGGAACACCCATAAG3' and reverse primer 5'CGTGGGAACAAAAGCGGGTGA3' using 29 cycles of one minute at 94°, one minute at 60°, and one minute at 72°. Resulting PCR products were then electrophoresed in a 1% agarose gel and assessed by ethidium bromide staining.

**In vivo analysis of transgene expression**

Embryos were harvested from timed pregnant mothers at two-day intervals. Timed pregnant mothers were sacrificed by cervical dislocation after being anesthetized by isoflurane inhalation. Whole embryos were removed from the mother and fixed in a solution containing 2% paraformaldehyde, 0.2% glutaraldehyde, 5 mM EGTA, 2 mM MgCl$_2$, and 0.1 M PBS from 1 hour to overnight, depending on the size of the embryo. Whole embryos were then stained for β-galactosidase activity or frozen in TBS tissue freezing medium (Triangle Biomed. Sci., Durham, NC) on dry ice after overnight equilibration in 30% sucrose for sectioning in a cryostat (Model OTF, Bright Instruments, Huntingdon, UK). Ten μm sections were cut and allowed to dry at room temperature onto Superfrost positively charged microscopy slides (Fisher Scientific, Pittsburgh, PA) followed by 5-minute fixation in 0.5% glutaraldehyde at room temperature and a 5-minute rinse in PBS prior to staining. Whole embryos and embryo sections were stained for β-galactosidase activity in X-gal substrate solution (see section 4.2) for 2 hours to
overnight at room temperature depending on embryo size. Histological staining for cartilage was performed using safranine-O with iron hematoxylin and fast green counterstains. Alkaline phosphatase activity was localized in tissue sections by exposing frozen and fixed sections to a solution containing 0.5 mg/ml Napthol as MX Phosphate (disodium salt), 1 mg/ml Fast Red salt, and 2.8% N,N dimethyl formamide in 0.1 M Tris maleate buffer, pH 8.4 for 15 minutes at 37°C in the dark. All chemicals were obtained from Sigma Chemical, Madison, WI except where indicated.

RNA Analysis

RNA was isolated from whole embryos using Trizol reagent (InVitrogen, Carlsbad, CA) according to the manufacturer’s protocol. RT-PCR analysis was carried out using 5 μg of total RNA in a reverse transcription reaction with the Superscript reverse transcriptase (InVitrogen) and oligonucleotide-dT primers for messenger RNA. Twenty five percent of the resultant cDNA product was then subjected to 30 cycles of polymerase chain reaction (PCR) using primers specific for the Type II Runx2 isoform or for GAPDH. Twenty five percent of the resulting PCR product was electrophoresed in a 1% agarose gel, transferred onto a Hybond N+ (Amersham Pharmacia, Piscataway, NJ) nylon membrane and hybridized to 32P end labeled oligonucleotidenucleotide probe corresponding to the original PCR primer followed by exposure to X-ray film.
RESULTS

The bone related Runx2 gene is highly expressed prior to skeletal formation in mouse embryos.

To understand utilization of the P1 promoter in its native locus in vivo, we examined the expression of P1 specific mRNAs throughout mouse embryonic development using RT-PCR analysis. We find that in whole embryos, these transcripts can be detected as early as 11.5 dpc with a peak in expression at 12.5dpc, followed by a progressive decrease between 13.5 dpc and birth (at least 10 fold) (Figure 2.1-A). Interestingly, the peak of Runx2 expression in the whole embryo occurs at a very early stage of chondrogenesis, prior to the onset of osteogenesis (around 14.5 dpc). The apparent decrease in Runx2 transcript levels during development may be attributable to the lower representation of skeletal lineage cells in older embryos. We therefore isolated RNA from the heads of embryos between 15.5 dpc and birth to enrich the RNA pool with osseous tissue and minimize the contribution of soft or non-osseous tissue. RT-PCR results show that the expression of P1 related mRNAs increases as the embryos approach birth, with maximal expression in the newborn animal (Figure 2.1-B). These results indicate a transient upregulation of Runx2 expression during chondrogenesis, and at later stages of bone formation an additional increase in expression that reflects the requirement for Runx2 in osteoblast maturation and mineralization.
Figure 2.1: RT-PCR analysis of the expression of the endogenous Runx2 Type II isoform derived from the P1 promoter demonstrates that in whole embryo extracts, expression peaks at E12.5 in the developing embryo (A). When examining Runx2 Type II in osseous tissues, such as the skull, and increase in expression is observed as mature bone is formed after birth (B).
To investigate the spatial and temporal expression patterns of the Runx2 promoter in vivo, we designed a transgenic construct in which a 3 kb promoter region 5' of the transcription initiation site regulates expression of the LacZ reporter gene (Figure 2.2-A). The activity of the Runx2 promoter transgene was initially assessed by transient transfection of Ros 17/2.8 osteosarcoma cells. These cells exhibit significant transgene activity in comparison to cells transfected with a highly expressing CMV driven LacZ reporter construct (Figure 2.2-B). Several transgenic founder mice were generated, identified by Southern blot analysis (Figure 2.2-C) and bred to produce distinct transgenic lines. Based on results obtained from RT-PCR analysis of Runx2 expression in the whole embryo, we analyzed promoter activity in vivo prior to the induction of the endogenous gene at 11.5 dpc and throughout development.

The Runx2 promoter is active in mesenchymal condensations at early embryonic stages prior to sclerotome formation.

To monitor expression of the 3 kb promoter during skeletal development, we analyzed two distinct lines of transgenic mice at various stages during embryogenesis. These two lines have similar expression patterns (Figure 2.3-E and F), and there is no background staining in wild type littermates (Figure 2.3-G). Transgene expression is detected shortly after the embryonic turning event at 8.5 dpc (not shown) in the most caudal somites that begin to give rise to sclerotome around 9.5 dpc (Figure 2.3-A). Promoter activity continues in the developing sclerotome until 11.5 dpc (Figure 2.3-B). At 12.5 dpc, β-gal expression is located solely in the mesenchyme of the sclerotomes that are destined to
Figure 2.2: The 3kb Runx2 P1 transgene (A) exhibits robust promoter activity after transient transfection into Ros 17/2.8 osteosarcoma cells as evidenced by X-gal staining (B). This transgene was then introduced into fertilized *mus musculus* oocytes by pronuclear injection, resulting in the generation of three expressing founder lines identified by Southern blotting using the probe pictured in 2.2-A (C).
Figure 2.3: Examination of Runx2 P1-LacZ expression throughout embryogenesis by X-gal staining at E9.5-E12.5 (A-D) shows β-gal activity in the developing somites. Transgene activity persists in the developing embryo and is reproducible in two independent mouse lines (E,F,&H). Wildtype littermates mice exhibit no background X-gal staining (G).
give rise to the cartilage and bones of the axial skeleton (Figure 2.3-C; 2.3-D, cryosection). These results indicate that the 3 kb Runx2 promoter contains regulatory elements that control Runx2 expression in early mesenchymal cells that will form the vertebrae and ribs.

**The Runx2 promoter supports expression in cells with chondrogenic potential during development of the axial skeleton.**

We examined Runx2 promoter activity in whole embryos (Figure 2.4, A-C) and in cryosections (Figure 2.4, D-I) when mesenchymal condensations of the sclerotome begin to migrate in a ventromedial fashion to form the cartilage anlagen of the ribs. At 13.5 dpc we find intense β-gal activity at the tip of the migrating rib (Figure 2.4, A-C). Histological analysis shows that cells expressing β-gal have not yet produced significant cartilaginous extracellular matrix, reflected by an absence of safranine-O staining (Figure 2.4, F and G, arrows). This finding reveals that activity of the Runx2 promoter continues during the differentiation of mesenchymal cells in the anatomical region that will become the cartilage of the developing rib. Thus, the promoter is highly active in committed chondroprogenitor cells.

At 13.5 dpc cells of the sclerotome are also beginning to segregate along the vertebral column to form the future vertebral bodies (Figure 2.4, H and I, arrows) and intervertebral discs (Figure 2.4 H and I, arrowheads). In this region of the embryo, cells positive for transgene expression are present in areas that are destined to become
Figure 2.4: The Runx2 P1 promoter is expressed in pre-cartilaginous mesenchymal condensations of the axial skeleton. Whole-mount X-gal staining of E 13.5 mice followed by soft tissue clarification in 2% KOH reveals strong transgene activity in the ventromedially migrating cells of the developing rib (A-C). Cryosections of E13.5 transgenic embryos stained with X-gal (E,G,I) or Safranin-O (D,F,H) reveal transgene activity in the caudal, pre-cartilaginous (Safranin-O negative) sclerotome (D-G). Midsaggital sections of the developing vertebral column reveals promoter activity in mesenchymal cells destined to form intervertebral discs (H&I, arrowheads). Condensations destined to form vertebral bodies exhibit weak Safranin-O staining at this time (G,H, arrows).
intervertebral discs (Figure 2.4-I, arrowheads). Thus, the regions that will form ossified vertebrae via endochondral bone formation do not express the 3 kb Runx2 transgene. Taken together our findings reveal that the 3 kb promoter is active in pre-chondrogenic cells of the intervertebral discs and ribs, and that promoter activity is absent from chondrocytes, which form the template for future bone tissue of the vertebral body. We conclude that Runx2 promoter activity demarcates a segmentation event involved in formation of the axial skeleton.

*The 3 kb Runx2 promoter transgene is expressed in mature, but not hypertrophic chondrocytes during later stages of embryonic development.*

Based on prior observations in 13.5 dpc animals, we examined promoter activity during the progressive maturation of chondrocytes in relation to endochondral bone formation. At 15.5 dpc, the cartilaginous anlagen of the skeleton are well established, and ossification centers appear in the ribs, long bones, and flat bones of the skull. Parasagittal sectioning of 15.5 dpc embryos reveals strong transgene activity in the ribs but a complete lack of activity in the alkaline phosphatase positive humerus and developing parietal bone of the skull (Figure 2.5, A-C). This finding demonstrates that the 3 kb promoter is expressed exclusively in the axial skeleton, and not in the appendicular skeleton or in bones, which undergo intramembranous ossification.

Upon closer examination of cells expressing the transgene within the rib, it is evident that the mature chondrocytes of the rib (Figure 2.5-D, arrow, marked by intense safranine-O staining) exhibit β-galactosidase activity (Figure 2.5-E, arrow), but this activity decreases
Figure 2.5: The Runx2 P1 transgene is active in proliferating, but not hypertrophic chondrocytes of the axial skeleton. Cryosections of E15.5 embryos stained with Safranin-O (A,D,G), X-gal (B,E,H), or Alkaline Phosphatase (C,F,I) clearly show transgene activity in the cartilaginous ribs (B, arrowhead) and a lack of activity in the cartilaginous humerus (B, arrow). Longitudinal sections of E15.5 ribs (D-F) exhibit transgene activity in developing chondrocytes (arrows), but no activity in hypertrophic chondrocytes (arrowheads). Midsagittal sections (G-I) of the developing vertebral bodies reveal transgene activity around the developing intravertebral discs (arrows) and an absence of activity in the alkaline phosphatase-positive developing vertebral bodies (arrowheads).
towards the zone of hypertrophic cartilage (Figure 2.5, D & E, arrowhead, marked by a decrease in safranine-O and X-gal staining). No β-gal activity was detected in hypertrophic chondrocytes producing a mineralized matrix (Figure 2.5, E and F, reflected by AP staining). Additionally, we observe that the cells of the perichondrium/periosteum of the rib, which have both chondrogenic and osteogenic potential, express the transgene (Figure 2.5-E). These results demonstrate that activity of the 3 kb Runx2 promoter is reduced during the maturation of chondrocytes.

Similar to our observations in the ribs, cells forming the vertebral bodies of the developing vertebral column begin to express alkaline phosphatase (Figure 2.5-I, arrow), which indicates the presence of hypertrophic chondrocytes at 15.5 dpc in a region that will become ossified tissue (Figure 2.5-G and I). Here, regions exhibiting β-gal activity and alkaline phosphatase staining are mutually exclusive (Figure 2.5-H and I). At the time of birth, transgene activity is retained in the periosteum/perichondrium and cartilage of the axial skeleton (Figure 2.6-A and B). These results indicate that the initial 3 kb segment of the Runx2 gene promoter confers expression to a subset of chondrocytic lineage cells, implying that this promoter is responsive to signals transduced by distinct spatio-temporal regulatory pathways in cells of this lineage.

*The Runx2 P1 promoter is active in the testes of adult transgenic animals.*

Post-natal transgenic mice were examined for expression of the transgene at weaning (3-4 weeks) and at 6 weeks of age by dissection and staining of individual tissues with X-gal.
Figure 2.6: The Runx2 P1 promoter is active in chondrocytes of newborn transgenic mice. The transgene is highly expressed in the cartilaginous portion of the ribs (A, arrowhead) but is silenced in the osseous portion of the rib (A, arrow). (B) Transverse section through the cartilaginous portion of the rib cage. Arrowhead indicates area corresponding to the arrowhead in 2.6-A.
Figure 2.7: Transgene activity is observed in the testes of 6-week old transgenic mice (A), but not in wildtype controls (B). Histological sections of the testes demonstrate that the Runx2 P1 promoter activity is localized to cells lining the seminiferous tubules within the testes.
We examined a broad spectrum of tissues including skeletal tissue (bone, cartilage, and muscle), major organs (liver, kidneys, lungs, heart, skin, and intestine), lymphoid tissues (thymus and spleen), and testes for transgene expression. Of all these tissues, expression was detectable only in the testes, which strongly expressed the transgene in the seminiferous tubules (Figure 2.7, A-D). This finding is consistent with a previous report which identified transcripts of the bone related Runx2 isoform in testis (Ogawa et al. 2000). Our results also indicate that the 3 kb promoter carries insufficient regulatory information for activity in mineralized tissue (hypertrophic cartilage and bone) and in the mature chondrocytes of adult animals. Taken together, our data demonstrate that spatio-temporal activity of the 3 kb Runx2 promoter supports early developmental expression of the endogenous Runx2 gene.

DISCUSSION

In this study we examined the contributions of the bone related Runx2 promoter to spatio-temporal regulation of expression using a P1 3 kb-LacZ transgenic mouse model. We find the Runx2 promoter is highly active at early stages of embryogenesis. Increasing β-gal activity was observed in the somites located in the caudal portion of the developing embryo from 9.5 to 12.5 dpc, consistent with the observation that endogenous bone related Runx2 transcripts reach maximal levels at 12.5 dpc in whole embryo preparations. At 13.5 dpc, LacZ expressing cells migrate ventromedially to form the cartilaginous template of the ribs and vertebral column. From 15.5 dpc until the time of birth, the promoter continues to be active in pre-chondrocytes and mature chondrocytes, and
notably, expression is restricted to the axial skeleton. Taken together, these findings demonstrate a tissue-restricted expression pattern of the bone related Runx2 P1 promoter in mesenchymal populations of chondrocytic progenitor cells, and in early chondrocytes of the axial skeleton. We conclude that the 3 kb Runx2 promoter is responsive to developmental cues that establish the segmentation pattern of the ribs and vertebrae.

The role of Runx2 in bone formation and maturation has been well established through both in vitro and in vivo studies (Banerjee et al. 1997; Ducy et al. 1997; Komori et al. 1997; Mundlos et al. 1997; Otto et al. 1997). In situ hybridization has demonstrated the presence of bone related Runx2 transcripts in osteoblasts and hypertrophic chondrocytes of the growth plate (Kim et al. 1999; Enomoto et al. 2000). In vitro model systems of osteoblast differentiation have clearly defined the role of the bone related isoform of Runx2 in commitment of cells to the osteoblast lineage, as well as in the induction of genes associated with mature osteoblast activity (Freytag et al. 1994; Xiao et al. 1999). Indeed, our results show that Runx2 is dramatically upregulated in ossified tissue at the time of birth. Previous studies have shown that the bone related isoform of Runx2 is upregulated in response to the BMP signaling pathway (Enomoto et al. 2000; Banerjee et al. 2001) and that BMP signaling is involved in multiple stages of chondrogenesis (Murtaugh et al. 1999; Pizette and Niswander 2000; Murtaugh et al. 2001). However, the chondrocyte related promoter activity we observe is unresponsive to BMP/TGF-β signaling (unpublished observations, our laboratories). Our findings indicate that bone
specific gene regulatory sequences responsible for controlling $Runx2$ expression in calcified tissue are located beyond the 3 kb segment used in this study.

Our studies clearly show robust expression of the $Runx2$ promoter in relation to chondrogenesis. We observe promoter activity in prechondrocytic mesenchymal condensations and in the developing chondrocytes of the axial skeleton, but not in the appendicular limbs. Several groups have identified $Runx2$ transcripts in prechondrocytic mesenchyme, as well as in both hypertrophic and pre-hypertrophic chondrocytes of vertebrae and limb bones (Ducy et al. 1997; Inada et al. 1999; Kim et al. 1999; Enomoto et al. 2000). Thus, our results show that the 3 kb $Runx2$ promoter contains regulatory information for activation in a subset of cartilaginous tissues expressing the endogenous gene.

This study represents the first data on developmental expression of the $Runx2$ P1 promoter in vivo using a transgenic model. Previous studies reported the combined activity of the upstream bone related (P1) and downstream (P2) $Runx2$ promoters (Otto et al. 1997). This group inserted the $LacZ$ gene into $Runx2$ exon 2 by homologous recombination, thereby creating a null allele and a reporter that monitors the activity of both $Runx2$ P1 and P2 promoters. These mice exhibit $LacZ$ activity in the developing ribs of 12.5 dpc embryos with a staining pattern consistent with the one shown in this report. In addition, the authors show $\beta$-gal activity in the mesenchyme and periosteum of the developing radius and ulna. Thus, in comparison to the $\beta$-gal activity resulting from
activation of both Runx2 promoters, the bone related Runx2 P1 promoter used in our studies is active in similar cell types but is limited to the mesenchyme and developing cartilage of the axial skeleton. The expression profile observed by Otto et al. in the appendicular skeleton may be derived from activity of the P2 promoter as well as the presence of additional elements that regulate the P1 promoter.

The expression pattern of the Runx2 transgene is strikingly similar to those of the transcription factors Pax1/Pax9 and Nkx3.2, which are involved, in early stages of development of the axial skeleton (Tribioli et al. 1997; Tribioli and Lufkin 1997; Furumoto et al. 1999; Lettice et al. 1999; Peters et al. 1999; Tribioli and Lufkin 1999). Null mutations in these genes cause severe defects in development of the axial skeleton due to improper sclerotome formation and differentiation. Tribioli and Lufkin (Tribioli and Lufkin 1999) demonstrated that in Nkx3.2−/− mice there is a downregulation of the bone related Runx2 isoform only in the axial skeleton and not in the limbs or other areas known to coexpress Nkx3.2 and Runx2. These observations suggest that induction of the Runx2 promoter contributes to early stages of axial skeleton development.

Formation and differentiation of the sclerotome is a process initiated by the Sonic Hedgehog (Shh) signaling molecule, which originates from the notochord. Shh expression is coincidental with the activation of both Nkx3.2 and Pax1 in the presclerotomomal somite (Johnson et al. 1994; Borycki et al. 1998). Interestingly, endogenous Runx2 promoter activity has also been observed in the notochord at the onset
of chondrogenesis (Otto et al. 1997). Therefore, the expression of the bone related Runx2 promoter in developing somites and sclerotome might reflect the convergence of multiple signaling pathways.

Our data support the concept that Runx2 functions in the somite and sclerotome prior to and during chondrogenesis. Thus, the Runx2 P1 promoter used in this study provides a basis for exploring the complexities of Runx2 in regulating initial stages of chondrogenesis and segmentation of the axial skeleton.
Chapter 3:

Nkx3.2 Mediated Repression of Runx2 Promotes

Chondrogenic Differentiation
ABSTRACT

Runx2, a transcription factor known to be essential for osteoblast maturation and skeletogenesis, is also expressed in pre-cartilaginous mesenchymal condensations in the developing embryo. It is therefore necessary to understand the control and consequential regulatory activity of the Runx2 gene within the context of chondrogenic differentiation of a mesenchymal progenitor cell. We identify the homeodomain protein Nkx3.2 as a potent sequence-specific repressor of the Runx2 promoter that acts through a regulatory element 0.1 kb upstream from the site of transcriptional initiation. The biological significance of this repression is established by utilizing BMP-2-induced chondrogenic differentiation of pluripotent C3H10T1/2 cells as a model for the initial events of mesenchymal chondrogenesis. We demonstrate that induction of the chondrogenic phenotype and endogenous Nkx3.2 expression is accompanied by a repression of Runx2 gene activity. Bypassing Runx2 repression by adenoviral-mediated introduction of Runx2 into C3H10T1/2 cells can prevent the induction of chondrogenesis, but cannot reverse the chondrogenic phenotype once it has been initiated, as evidenced by Sox9 and collagen Type 2 expression and extracellular matrix deposition. Our results demonstrate that Runx2 is a direct transcriptional target of Nkx3.2, and that repression of Runx2 at the onset of chondrogenesis is a prerequisite for the activation of a chondrocyte-specific program of gene expression. We postulate that Runx2 is a critical link in BMP-2-mediated initiation of mesenchymal chondrogenesis that results in activation of Sox9 at least in part through the Nkx3.2-dependent repression of Runx2.
INTRODUCTION

During mammalian embryogenesis, the axial skeleton is initially formed as a cartilaginous template prior to conversion into mineralized bone through the coordinated actions of osteoclasts and osteoblasts. The mesenchymal progenitor cells responsible for cartilage formation aggregate around the notochord and are induced to proliferate and subsequently to differentiate in response to the secreted signaling molecules Sonic Hedgehog (Shh) and BMP-2 (Johnson et al. 1994; Fan et al. 1995; Marcelle et al. 1997; Murtaugh et al. 1999). The sequential and cooperative action of these molecules results in the induction of the pro-chondrogenic NK-related homeodomain protein Nkx3.2 in mesenchymal progenitor cells. Nkx3.2 mediates transcriptional repression of target genes through its interactions with BMP-responsive SMAD proteins and histone deacetylase HDAC1 (Kim and Lassar 2003). Activation of Nkx3.2 is required for and closely followed by activation of the master chondrogenic transcriptional regulator Sox9. The activation of Sox9 through Nkx3.2 ultimately leads to activation of chondrocyte phenotypic genes, including type II collagen and aggregcan, and formation of a cartilaginous extracellular matrix (Murtaugh et al. 1999; Murtaugh et al. 2001). The importance of Nkx3.2 in this process is evident from Nkx3.2 null mice, in which mesenchymal progenitor cells of the sclerotome fail to differentiate resulting in malformation or absence of axial skeletal elements and perinatal lethality (Lettice et al. 1999; Tribioli and Lufkin 1999; Akazawa et al. 2000).
Recent findings in our laboratory and others have demonstrated that pre-chondrogenic mesenchymal progenitor cells exhibit Runx2 gene activity as early as 9.5 days post coitum, 3 days prior to overt chondrogenesis in the murine embryo (Ducy et al. 1997; Otto et al. 1997; Lengner et al. 2002; Yamashiro et al. 2002). The Runx family of DNA-binding transcription factors governs cell fate determination in a variety of tissues. Runx factors are essential for hematopoiesis, skeletal development, and development of the digestive and nervous systems (North et al. 1999; Guo et al. 2002; Inoue et al. 2002; Levanon et al. 2002; Li et al. 2002). Runx2 is indispensable for the differentiation of the bone-forming osteoblast, and Runx target genes in this cell lineage have been well characterized (Banerjee et al. 1997; Ducy et al. 1997; Komori et al. 1997; Otto et al. 1997; Choi et al. 2001; Lian et al. 2004). The observation that the Runx2 gene is active in progenitor cells that are destined to undergo chondrogenesis rather than osteogenesis necessitates determining whether Runx2 plays a role in BMP-2 induced chondrogenic differentiation of mesenchymal progenitor cells in addition to the well-characterized function of Runx2 in promoting the osteogenic differentiation of osteoblast precursors later during skeletogenesis.

In this study, we identify the Runx2 gene as a target of Nkx3.2-mediated transcriptional repression and demonstrate that Runx2 is a critical link in the BMP-2 induced pathway of chondrogenesis. We show that Nkx3.2 represses Runx2 activity through an interaction with a regulatory element in the Runx2 promoter. Further, our results suggest that at the onset of BMP-2 induced chondrogenesis of C3H10T1/2 mesenchymal progenitor cells,
an increase in Nkx3.2 activity suppresses Runx2 gene expression. We demonstrate that bypassing the observed suppression of Runx2 at the onset of chondrogenesis inhibits chondrocytic differentiation. Our findings establish that Runx2 is a critical modulator of the commitment of mesenchymal progenitor cells to the chondrogenic lineage.

MATERIALS AND METHODS

Cell Culture and Transient Transfection

C3H10T1/2 and NIH3T3 cells were maintained in Dulbecco’s Modified Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, GA). MC3T3 cells were maintained in α-MEM media supplemented with 10% FBS. ROS17/2.8 cells were maintained in F12 media supplemented with 5% FBS. Transient transfections were performed in 6-well plates at 70% confluence using 5μl of FuGENE6 transfection reagent (Roche, Indianapolis, IN) and 4 μg total DNA per well in accordance with the manufacturers protocol. For Nkx3.2 expression, 100 ng of an Nkx3.2 expression vector in a PCS2 plasmid backbone (a kind gift from Dr. Andrew Lassar at Harvard Medical School) was transfected into each well unless otherwise noted. As a control, 100 ng PCS2 expression vector (empty vector) was transfected into each well. For Sox9 expression, 100 ng of a Sox9 expression construct was introduced into each well of a 6 well plate. Sonic hedgehog (Shh, R&D systems, Minneapolis, MN) and BMP2 (a kind gift from Dr. John Wozney, Wyeth Ayerst, Cambridge, MA) were added exogenously at concentrations of 200ng/mL and 100ng/mL respectively. To monitor transfection
efficiency, transfections included 0.5 μg of a CMV driven LacZ expression vector per well. For Runx2 promoter-reporter assays, transfections included 2 μg of either a Runx2 0.6 kb promoter-luciferase construct, or an empty PGL3 luciferase construct. All results were normalized to activity of the PGL3 empty vector (Promega, Madison WI).

**Luciferase Reporter Assays**

Cells transfected with Runx2 promoter/luciferase reporter constructs were harvested 36-48 hours after transfection and each well was lysed at room temperature for 20 minutes in the presence of 0.5 mL Reporter Lysis Buffer (RLB, Promega Madison, WI). Firefly luciferase activity was quantitated in a luminometer using a 12 second read time immediately after addition of 20 μl cell lysate to 100 μl substrate (Promega Luciferase Assay System). With the exception of Figure 3.1-A, all results were normalized to the luciferase activity resulting from transfection of the promoterless PGL3 luciferase construct (Promega).

**Chondrogenic Induction and Adenoviral Infection of C3H10T1/2 Cells**

Induction of chondrogenesis was carried out by plating C3H10T1/2 cells (between passages 19 and 25) in high-density micromass cultures (10^5 cells in a 10 μL drop of media)(Ahrens et al. 1977; Mello and Tuan 1999; Lengner et al. 2004), followed by a three-hour incubation period in which cells were allowed to adhere. Following adhesion,
micromass cultures were fed with F12 media containing 5% fetal bovine serum and 100 ng/mL recombinant hBMP-2 (kindly provided by Dr. John Wozney at Wyeth-Ayerst, MA). For adeno\-viral transduction experiments, C3H10T1/2 cells were infected with viruses containing either the Runx2 cDNA or the β-galactosidase cDNA as a control at an MOI of 100. To introduce adeno\-virally-expressed proteins prior to chondrogenic induction, C3H10T1/2 cells were infected while proliferating in a monolayer at approximately 80% confluence. At 12 hours after infection, cells were trypsinized and plated in high-density micromass cultures for chondrogenic differentiation as described above. Cultures were harvested 24 hours after induction of chondrogenesis for analysis of gene expression. To deliver adeno\-virally expressed proteins after induction of chondrogenesis, C3H10T1/2 cells were plated in high-density cultures, allowed to adhere, then infected with viruses containing either the Runx2 cDNA or the β-galactosidase cDNA. Immediately after infection, cultures were fed with F12 media containing 5% fetal bovine serum and 100 ng/mL recombinant hBMP-2. Cultures were harvested 24 hours after the induction of chondrogenesis for analysis of gene expression.

**RNA Isolation and Analysis**

RNA was isolated from cultures of C3H10T1/2 cells using Trizol Reagent (Invitrogen, Carlsbad CA) according to the manufacturers protocol. After purification, 5 µg of total RNA was DNAse treated using a DNA-free RNA column purification kit (Zymo Research, Orange, CA). RNA (1 µg) was then reverse transcribed using Oligo-dT primers and the SuperScript 1st Strand Synthesis kit (Invitrogen) according to the
manufacturers protocol. Gene expression was assessed by quantitative real-time PCR (Sox9, Nkx3.2, type II collagen, type I collagen, alkaline phosphatase, and Runx2). Quantitative PCR was performed using either SYBR green 2x master mix (Eurogentec, Belgium) or Fam-conjugated Taqman probes and Taqman 2x Master Mix in the case of Nkx3.2 and Runx2 (Applied Biosciences, Foster City, CA) and a 2 step cycling protocol (anneal and elongate at 60°C, denature at 94°C). All data is expressed as arbitrary expression levels relative to expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Specificity of primers was verified by dissociation of amplicons when using SYBR green as a detector. Primers used for PCR reactions are listed in Table 1.

**Electrophoretic Mobility Shift Analysis (EMSA)**

Expression constructs containing the Nkx3.2 coding region or the empty PCS2 vector were subjected to *in vitro* transcription/translation (IVTT) using the TNT coupled rabbit reticulocyte lysate system (Promega) and SP6 RNA polymerase (NEB, Beverly MA) according to the manufacturer’s protocol. *In vitro* synthesis of recombinant Nkx3.2 was verified by Western blotting against the HA tag present in the PCS2 vector (data not shown). Wild type and mutant oligonucleotidenucIeotides containing the Nkx3.2 consensus site derived from the Runx2 P1 promoter was end labeled by incubating 10 μM of the sense strand of each oligonucleotide with 50 μCi of γ-P\(^{32}\)-ATP in the presence of 10 units polynucleotide kinase (PNK, NEB, MA) for 30 minutes at 37°C. Unlabelled antisense oligonucleotide (30 μM) was then added to the reaction followed by boiling the mixture for 5 minutes. The reaction was then gradually allowed to cool to room
temperature to allow the annealing of sense and antisense strands to occur. Double stranded oligonucleotides were then purified over a G-25 sephadex column to remove unincorporated nucleotides. DNA binding assays were performed by incubating 10 fmol of double stranded oligonucleotide with 2 μg of nuclear proteins from C3H10T1/2 cells along with or without 0.4 μg rabbit polyclonal α-HA antibody (Santa Cruz, Santa Cruz, CA) or a non-specific antibody, α-cMYC (Santa Cruz), at room temperature for 20 minutes. Complexes were visualized after separation on a 16% 40:1 acrylamide:bis-acrylamide gel in the presence of 0.5X TBE buffer followed by autoradiography.

**Western Blotting**

For the detection of Nkx3.2, Runx2, and Lamin B proteins, each well of a 6 well plate was lysed in 400 μl lysis buffer containing 2% SDS, 10 mM DTT, 10% glycerol, 12% urea, 10 mM Tris/HCl (pH 7.5), 1 mM PMSF, 1X Protease inhibitor cocktail (Roche), 25 μM MG132 proteosome inhibitor, and boiled for 5 minutes. Proteins were then quantified using Bradford reagent (Pierce, Rockford, IL) and taking spectrophotometric readings at 590 nm. Concentrations were estimated against a standard curve generated using BSA.

20 μg of total protein was subjected to electrophoresis in a denaturing 10% polyacrylamide gel containing 10% SDS. Proteins were then transferred onto Immobilon-P membranes (Millipore, Billerica, MA) using a semi-dry transfer apparatus. Membranes were blocked in PBS-0.01% Tween-20 containing 2% nonfat-powdered milk (Biorad, Hercules, CA). Proteins were detected by incubating with antibodies at a
concentration of 50 ng/mL in blocking solution. Antibodies used in this study are as follows: Nkx3.2, α-HA epitope mouse monoclonal antibody (Santa Cruz, Santa Cruz, CA); Runx2 mouse monoclonal antibody was a generous gift from Drs. Yoshi Ito and Kosei Ito, National University, Singapore; α-Lamin B mouse monoclonal antibody (Zymed Laboratories, San Francisco, Ca). Primary antibodies were detected with goat α-mouse secondary antibody conjugated to HRP. Secondary antibodies were detected using Western Lightning Chemiluminescence Reagent (Perkin Elmer, Boston, MA).

Chromatin Immunoprecipitation Assays (ChIP)

To cross-link proteins to DNA, C3H10T1/2 cells were incubated for 10 min at room temperature in 1X PBS (3 ml/plate) containing 1% formaldehyde, 25 μM MG-132 (Calbiochem/Sigma), and 1X protease inhibitor (Roche Molecular Biochemicals, Indianapolis, IN). A final concentration of 0.125 M glycine was added to the 1% formaldehyde-PBS solution for neutralization. Cells were collected in PBS after plates were washed twice with ice cold PBS. The cells were then lysed in lysis buffer containing 25 mM HEPES/NaOH (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.1% NP-40, 1 mM DTT, 25 μM MG-132 and 1X complete protease inhibitor. To isolate the nuclei, cells were homogenized in a Dounce homogenizer followed by centrifugation at 1,100 rpm at 4°C. The pelleted nuclei were resuspended in 300 μl (300 μl/100 mm plate) sonication buffer (50 μM HEPES/NaOH (pH 7.9), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, 25 μM MG132, 1X complete protease inhibitor). Samples were sonicated to shear DNA into 0.2-0.6 kb fragments. Cellular
debris was removed by centrifugation at 14,000 rpm for 15 min at 4°C and resulting chromatin-containing solutions were distributed into multiple 1 ml aliquots that were used as the starting material of all subsequent steps.

Chromatin aliquots were precleared with 100 µl of a 25% (v/v) suspension of 2 µg single stranded DNA coated protein A/G and 1 mg/ml BSA. Samples were used directly for immunoprecipitation reaction with 2 µg of α-HA epitope or α-Runx2 (M-70, Santa Cruz Biotechnology) antibody and normal rabbit/mouse IgG as a control. Chromatin immunoprecipitation reactions were allowed to proceed for 2-4 h at 4°C on a rotating wheel. Immune-complexes were mixed with 100 µl of 25% (v/v) pre-coated protein A/G agarose suspension followed by incubation for 1 h at 4°C on a rotating wheel. Beads were collected by brief centrifugation and the immunocomplexes were eluted twice by adding 150 µl of freshly prepared elution buffer (100 mM NaHCO₃, 1% SDS). After reversal of crosslinks at 68°C overnight, the eluate was treated with 100 µg/ml proteinase K followed by phenol-chloroform extraction and ethanol precipitation using 5 µg glycogen as carrier. An aliquot (2-3 µl) of each sample was assayed using quantitative PCR for the presence of specific DNA fragments using primers in the proximal Runx2 promoter. This region contains both the Nkx3.2 binding motif as well as Runx2 autoregulatory motifs. The primers are: Forward 5'-CTC CAG TAA TAG TGC TTG CAA AAA AT-3' and Reverse 5'-GCG AAT GAA GCA TTC ACA CAA-3'. Quantitative real-time PCR was carried out using 2X SYBR Green mix (Eurogentec, Belgium) and a 2-stage cycling protocol (60°C annealing and extension, 94°C denaturation, 40 cycles).
Amplicon specificity was verified by analysis of melting temperature. All data was collected during the linear phase of amplification.
RESULTS

The Runx2 P1 Promoter is Highly Active in C3H10T1/2 Cells and is Suppressed by Nkx3.2.

To gain insight into the regulation of the Runx2 gene in a pluripotent mesenchymal progenitor cell, we transfected a vector containing 600 base pairs of the Runx2 P1 promoter (Drissi et al. 2000; Drissi et al. 2002b) fused to the luciferase reporter gene into C3H10T1/2 cells. The Runx2 promoter (P1-Luc) was transcriptionally active (approximately 10 times that of a promoterless luciferase construct, PGL3, Figure 3.1-A) in this cell line, comparable to the P1-Luc activity observed in osteogenic ROS17/2.8 cells which express high levels of Runx2 protein. This observation is consistent with previous findings that the endogenous Runx2 gene is active in mesenchymal progenitor cells during development in vivo (Ducy et al. 1997; Otto et al. 1997; Lengner et al. 2002; Yamashiro et al. 2002).

We next examined the effect of various signaling molecules and transcription factors known to be critical for mesenchymal chondrogenesis in vivo on the activity of the Runx2 P1 promoter, including Sonic Hedgehog (Shh), Bone Morphogenic Protein 2 (BMP2), Nkx3.2, and Sox9. These molecules act sequentially during murine embryogenesis and are critical at different stages for the expansion and differentiation of mesenchymal progenitor cells into mature chondrocytes (Figure 3.1-B). Our results demonstrate that Shh, BMP-2, and Sox9 exhibit little to no effect on the activity of the Runx2 promoter. In contrast, expression of the transcription factor Nkx3.2 in C3H10T1/2 cells resulted in a
Figure 3.1: The Runx2 P1 promoter is active in mesenchymal cells and is repressed by Nkx3.2. Transient transfection of a 0.6 kb Runx2 P1 promoter-Luciferase into C3H10T1/2 mesenchymal progenitor cells or ROS17/2.8 osteosarcoma cells (A). Examination of the effects of several molecules known to act during mesenchymal chondrogenesis reveals the potent repressive activity of Nkx3.2 on the Runx2 promoter in C3H10T1/2 cells (B). Transfection of Nkx3.2 into C3H10T1/2 cells showing dose-responsive repression of the Runx2 promoter (C).
dramatic, dose dependent suppression of Runx2 promoter activity (Figure 3.1-C). These findings indicate that the robust activity of Runx2 regulatory regions in mesenchymal progenitor cells is negatively regulated by Nkx3.2, a molecule that acts to promote the chondrogenic differentiation of these cells.

**Nkx3.2 Represses the Runx2 Promoter via Interaction with an Nkx3.2 Consensus Binding Sequence.**

We examined the possibility that the Nkx3.2-mediated repression of the Runx2 promoter occurred through direct interaction of Nkx3.2 with its consensus-binding site, HRAGTG (H= A, C, or T; R= A or G) (Kim et al. 2003). Indeed, we identified two consensus binding sites for Nkx3.2 within the Runx2 P1 promoter (at -580 bp and -98 bp from the Runx2 transcription initiation site) (Figure 3.2-A). To define the contribution of these sites to Nkx3.2-mediated repression of the Runx2 promoter, we co-transfected the Nkx3.2 expression construct into C3H10T1/2 cells along with various deletion constructs of the Runx2 promoter. Figure 3.2-A demonstrates that the distal Nkx3.2 binding site is dispensable for repression. However, the -108 bp Runx2 promoter construct still exhibits repression in response to Nkx3.2, and removal of this site (-92 bp deletion) results in a loss of repression, as well as an abrogation of basal promoter activity. When the data is examined as fold repression mediated by Nkx3.2 upon the deletion constructs, it becomes evident that an additional promoter region between -458 bp and -351 bp may be playing a contributing role (Figure 3.2-B). Because there is no acceptable Nkx3.2 consensus
Figure 3.2: Localizing the repressive effects of Nkx3.2 on the Runx2 P1 promoter. Co-transfection of Nkx3.2 along with various deletion constructs into C3H10T1/2 cells (A). When data from 3.2-A is plotted as fold repression, two repressive domains are revealed between -351/-458 and -92/-108 (B). The latter region contains an Nkx3.2 consensus site illustrated in 3.2-A.
binding sequence in the –458/-351 region, this effect may occur independently of Nkx3.2 DNA binding.

To validate the interaction between Nkx3.2 and its consensus-binding site at –98 bp in the Runx2 promoter, electrophoretic mobility shift assays (EMSAs) were performed using a 24 bp oligonucleotide containing the Nkx3.2 site (Figure 3.3-A). This site lies downstream of a purine rich region of the promoter in a highly conserved area that contains binding sequences for a number of transcriptional regulators including HLH, ATF, Vitamin D, as well as Runx2 itself. Nkx3.2 binds to this site in a sequence specific manner as mutation of this site completely abrogates binding activity (Figure 3.3-B compare WT to MT). The specificity of this complex was confirmed using supershift analysis of the Nkx3.2 interaction with the wild type oligonucleotide. Incubation of the radiolabelled wild type oligonucleotide results in the formation of a specific protein-DNA complex only in the presence of Nkx3.2 expression vector (Figure 3.3-C). The addition of mouse polyclonal α-HA antibody completely supershifts this complex. To further confirm that Nkx3.2 is occupying this site in the native promoter, CHiP assays were performed 24 hours after transfection of Nkx3.2 into C3H10T1/2 cells. Using antibodies against endogenous Runx2 or HA-tagged Nkx3.2, we were able to immunoprecipitate the Runx2 promoter using an α-HA antibody, but not using an α-Runx2 or non-specific (IgG) antibody, demonstrating that Nkx3.2 is indeed occupying the Runx2 promoter (Figure 3.3-D). These results demonstrate that the Nkx3.2 transcription factor interacts
Figure 3.3: Nkx3.2 interacts with a regulatory motif in the Runx2 promoter. The Runx2 promoter contains an Nkx3.2 consensus binding site 100bp upstream of the transcriptional start site (A). This sequence was used to create wildtype or mutated oligonucleotides for electrophoretic mobility shift assays (EMSA). EMSA demonstrates that Nkx3.2 can bind the wildtype, but not mutant oligonucleotides (B). The specificity of the observed protein-DNA interaction was verified by supershift assay using an anti-HA antibody against the tagged Nkx3.2 protein (C). Chromatin immunoprecipitation (ChIP) assay in C3H10T1/2 cells transfected with HA-tagged Nkx3.2 confirms the physical interaction between Nkx3.2 and the endogenous Runx2 promoter.
with a functional consensus-binding site in the Runx2 P1 promoter approximately 100 bp upstream from the site of initiation of transcription.

**The Proximal Nkx3.2 Binding Site in the Runx2 Promoter is Required for Maximal Repressive Effects of Nkx3.2.**

To determine whether the proximal Nkx3.2 binding site is responsible for mediating the observed repression of the Runx2 promoter in the presence of Nkx3.2, we introduced the mutation used for EMSA analysis into both the -600 bp and the -108 bp Runx2 promoter constructs. Mutation of this site in the -600 bp promoter resulted in a significant abrogation of the Nkx3.2-mediated repression of the -600 bp Runx2 promoter (Figure 3.4-A, from 5 to 2 fold). The persistence of repression of the mutated -600 bp promoter in the presence of Nkx3.2 may be due the Nkx3.2-responsive region between -458 bp and -351 bp observed in Figure 3.2-B. To minimize the effects of other potential promoter regions through which Nkx3.2 may act, we introduced the mutation of the Nkx3.2 binding sequence into the 108 bp Runx2 promoter construct (Figure 3.4-B). While this construct has less basal promoter activity than the 600bp construct (see Figure 3.2-A), Nkx3.2 still exhibits strong repressive activity upon this promoter fragment (Figure 3.4-B). Mutation of the Nkx3.2 binding site in this context abolishes Nkx3.2-mediated repression of the Runx2 promoter confirming that Nkx3.2 is exerting repressive effects through this site.
**Figure 3.4:** Introduction of an Nkx3.2 binding-incompetent mutation of the Nkx3.2 consensus site in the Runx2 promoter abrogates Nkx3.2-mediated repression. Introduction of the mutation identified in figure 3.3 into the 0.6kb P1-Luc construct results in a partial loss of promoter activity (A). When this mutation is introduced into the 108bp P1-Luc construct, Nkx3.2 repressive activity is abolished (B). Whole cell lysates of C3H10T1/2 cells transfected with either HA-Nkx3.2 & CMV-EGFP or vector (PCS-2) & CMV-EGFP were FACs sorted to isolate GFP positive cells, followed by protein extraction and western blotting against the HA tag and endogenous Runx2 protein demonstrating an Nkx3.2-induced reduction of Runx2 protein levels (C).
We next addressed whether the observed Nkx3.2 mediated-repression of the Runx2 promoter affects physiologic Runx2 levels in C3H10T1/2 cells. These cells were transfected with the Nkx3.2 expression vector and endogenous Runx2 protein levels were examined. Expression of Nkx3.2 was verified by western blotting against the HA epitope tag fused to the Nkx3.2 protein (Figure 3.4-C). In the absence of Nkx3.2, Runx2 is strongly expressed in C3H10T1/2 cells. Upon forced expression of Nkx3.2, we find a 34% apparent reduction in Runx2 protein, which represents greater than a 75% loss of total Runx2 in transfected cells (when accounting a 40% transfection efficiency). Taken together, these results demonstrate that Nkx3.2 suppresses the activity of the Runx2 promoter in pluripotent mesenchymal progenitor cells through its interaction with a functional Nkx3.2 binding element in the proximal Runx2 promoter.

Nkx3.2-mediated Repression of Runx2 is Abrogated in Cells Committed to the Osseous Lineage.

To gain insight into the biological significance of Nkx3.2 repression of the Runx2 gene, we asked whether the observed repression was cell type specific. We found that Nkx3.2-mediated repression of the Runx2 promoter was much greater in undifferentiated mesenchymal cells (C3H10T1/2, NIH3T3) in comparison to cells already committed to the osteoblast lineage (MC3T3, ROS17/2.8) (Figure 3.5). This phenomenon may reflect a biological role for Runx2 repression by Nkx3.2 in undifferentiated mesenchyme, as Runx2 activity in committed osteoblasts is critical for their ability to differentiate and
Figure 3.5: Nkx3.2-mediated repression of the Runx2 promoter is abrogated in cells committed to the osseous lineage. Co-transfection of Nkx3.2 and 0.6kb Runx2 P1-Luc constructs into undifferentiated mesenchymal cells (NIH3T3, C3H10T1/2) or committed osseous cells (MC3T3, ROS17/2.8) showing that the repressive effects of Nkx3.2 are much greater in uncommitted mesenchyme.
these cells may therefore have passed a differentiation checkpoint after which Nkx3.2 can no longer exert repressive effects upon the Runx2 gene.

**Nkx3.2 and Runx2 Genes exhibit Reciprocal Expression Patterns During Chondrogenic Differentiation.**

To assess the significance of the specificity of Nkx3.2-mediated repression of the Runx2 gene in undifferentiated mesenchymal cells, we examined the expression of patterns of endogenous Runx2, Nkx3.2, Sox9 and type 2 collagen genes during chondrogenic differentiation of C3H10T1/2 cells. These cells proliferate as undifferentiated fibroblasts, and, like primary mesenchymal progenitor cells, are capable of differentiating into several cell types including adipocytes (Zehentner et al. 2000; Chan et al. 2003), osteoblasts (Spinella-Jaegle et al. 2001; Chan et al. 2003), myoblasts (Singh et al. 2003), and chondrocytes (Denker et al. 1999; Haas and Tuan 2000), depending on the environment in which they are cultured. Here C3H10T1/2 cells were induced to undergo chondrogenesis by plating them in high-density micromass cultures in the presence of BMP2 (Denker et al. 1999).

Gene expression was monitored over a 4-day period after induction of chondrogenesis (Figure 3.6). Strong induction of Nkx3.2 expression was observed by one day of culture and was closely followed by activation of the expression of the chondrogenic transcriptional regulator Sox9 and of the cartilage-specific extracellular matrix protein, collagen type 2, demonstrating that these cultures have entered the pathway of
Figure 3.6: Runx2 and Nkx3.2 exhibit reciprocal expression patterns during mesenchymal chondrogenesis. C3H10T1/2 cells were induced to undergo chondrogenic differentiation in response to high-density culture and BMP-2 treatment. Gene expression was monitored over a four day time period using quantitative RT-PCR. Chondrogenic genes Nkx3.2, Sox9, and collagen type II are induced 24 hours after induction of chondrogenesis. Conversely, Runx2 gene expression is suppressed by the 24 hour time point.
chondrocytic differentiation. In contrast, Runx2 expression was strongly repressed by one day of culture; consistent with our findings that Nkx3.2 activity suppresses expression of the Runx2 gene. Taken together these findings demonstrate that a loss of Runx2 gene activity is associated with the transition of the C3H10T1/2 mesenchymal progenitor cell into the chondrogenic lineage.

**Runx2 Acts to Prevent Mesenchymal Progenitor Cells from undergoing Chondrogenesis.**

Based on our observations of Runx2 gene expression during chondrogenesis of C3H10T1/2 cells, we tested the hypothesis that there is a requirement for Runx2 suppression for induction of chondrogenesis. We therefore introduced exogenous Runx2 into C3H10T1/2 cells by adenoviral infection prior to induction of chondrogenesis in order to circumvent the suppression of Runx2 observed at the onset of chondrogenic differentiation. Proliferating C3H10T1/2 cells were infected with adenovirus containing either Runx2 cDNA or LacZ cDNA as a control, and gene expression was analyzed 24 hours after the induction of chondrogenesis with BMP2 and high-density culture. Adenoviral infection maintains high levels of Run2 expression 24 hours after induction of chondrogenesis in Runx2 infected cells when compared to LacZ infected control cultures (Figure 3.7-A). Remarkably, BMP2 treated micromass cultures infected with Runx2 fail to significantly activate Sox9 or type 2 collagen genes (Figure 3.7-A) and are unable to produce significant cartilaginous extracellular matrix, as assessed by alcian blue staining of sulfonated proteoglycans present in cartilage (Figure 3.7-B, upper panel).
Figure 3.7: Overexpression of Runx2 in C3H10T1/2 cultures prevents entry into the chondrocytic lineage. Runx2-containing Adenoviral infection of C3H10T1/2 cells prior to initiation of chondrogenesis prevents induction of collagen type II, and Sox9, while only marginally increasing osteogenic markers collagen type I and alkaline phosphatase (A). Alcian blue and alkaline phosphatase staining of chondrogenic cultures infected with Ad-Runx2 or Ad-LacZ reflects the observed changes in gene expression (B). When Ad-Runx2 is introduced after initiation of chondrogenesis in C3H10T1/2 cultures, Runx2 is no longer capable of altering chondrogenic gene expression (C).
Surprisingly, when analyzing expression of Nkx3.2 in these cultures we found that it was significantly increased in the presence of Runx2, supporting the hypothesis that Nkx3.2 acts upstream of Runx2 and suggesting that a feedback loop exists in which the Nkx3.2 gene becomes induced to suppress Runx2 activity (Figure 3.7-A). Our results clearly demonstrate that in an undifferentiated mesenchymal progenitor cell, Runx2 is able to inhibit chondrogenic differentiation, and Runx2 repression may be a pre-requisite for entry into the chondrocytic lineage.

Given the role of Runx2 in promoting osteogenesis, we examined whether the inhibition of chondrogenic differentiation in adenoviral-Runx2 infected C3H10T1/2 cultures was associated with increased osteogenesis. We examined adenoviral cultures for expression of the osteogenic genes alkaline phosphatase (AP) and type I collagen and observed that mRNA levels of these genes were indeed increased in the presence of Runx2 adenovirus (Figure 3.7-A). When examining the activity of the alkaline phosphatase enzyme in these cultures by colorimetric reaction we observe that AP-positive cells are localized primarily to cells in monolayer at the edges and top surface of the nodule (Figure 3.7-B, lower panel). These observations suggest that while sustained Runx2 activity under pro-chondrogenic culture conditions prevents the majority of these cells from undergoing chondrogenesis, it's expression is capable of inducing an osteogenic response in a subset of these cells.
Interestingly, we find that if Runx2 is introduced by adenoviral infection after the onset of chondrogenic differentiation it is unable to suppress chondrocyte phenotypic genes (Figure 3.7-C). This phenomenon may reflect the passing of a critical checkpoint during the chondrogenic differentiation of mesenchymal cells after which the activity of Runx2 cannot reverse the differentiation process once the program of chondrogenesis has begun. Taken together, these findings indicate that suppression of Runx2 gene expression by Nkx3.2 is a requirement for the progression of chondrogenic differentiation of mesenchymal progenitor cells.

DISCUSSION

In this study we identify the Runx2 gene as a direct target for repression by the NK-family homeodomain factor Nkx3.2. We establish the biological significance of this repression by demonstrating that inhibition of Runx2 expression at the onset of BMP-2 induced chondrogenesis of C3H10T1/2 mesenchymal progenitor cells is a pre-requisite for cartilage formation. While prior studies have shown that osteogenic differentiation of progenitor cells in response to BMP-2 treatment requires Runx2 activity, our results demonstrate for the first time that BMP-2 induced chondrogenic differentiation of mesenchymal progenitor cells requires suppression of Runx2. This finding provides direct evidence that Runx2 is also a critical regulator of BMP-2 induced chondrogenic fate determination in pluripotent mesenchymal cells.
We identified Nkx3.2 as a potential regulator of the Runx2 gene based on the pattern of Runx2 promoter activity in pre-cartilaginous mesenchymal condensations of the axial skeleton as early as E9.5 in the murine embryo (Lengner et al. 2002). Cells comprising the mesenchymal condensations first proliferate, then undergo chondrogenic differentiation in response to the coordinated and sequential actions of soluble signaling molecules sonic hedgehog and BMP-2 (Marcelle et al. 1999; Murtaugh et al. 1999; Murtaugh et al. 2001). The expression of the Runx2 gene in these condensations precedes the activation of Nkx3.2 and overt chondrogenesis. Chondrogenic differentiation of these cells is dependent upon activation of Nkx3.2 by Shh and BMP-2, which ultimately leads to induction of Sox9 (Murtaugh et al. 1999; Murtaugh et al. 2001; Zeng et al. 2002). Runx2 expression is absent from mature chondrocytes: thus the repression of Runx2 by Nkx3.2 at the onset of chondrogenesis is a key regulatory event. Later during endochondral bone formation, Runx2 is induced again during the final stages of hypertrophic cartilage mineralization to promote terminal differentiation prior to turnover of mineralized cartilage into bone.

Significantly, we find that the Nkx3.2 regulatory element (CACTT) at -108 bp in the Runx2 P1 promoter is located in a region that is essential for basal transcriptional activity. This element lies in close proximity to Runx and other regulatory sites and marks the 5' boundary of the minimal promoter region required for transcriptional activity. This Nkx3.2 site confers potent, dose-dependent transcriptional repression in the presence of Nkx3.2. Our findings show that Nkx3.2 physically interacts with the Nkx3.2 binding
element within the Runx2 promoter, and that this interaction is required for effective repression of Runx2 promoter activity. These results make Runx2 the first bona fide target of Nkx3.2 to be identified to date.

Based on our prior observations of Runx2 promoter activity in vivo and our findings in this study demonstrating that the Runx2 gene is a target of Nkx3.2-mediated transcriptional repression, we investigated the biological significance of Runx2 gene activity in pre-chondrogenic mesenchymal progenitors as they undergo BMP-2 induced chondrogenesis. Although the mechanisms that support Runx2 gene activity in these progenitor cells are unknown, several lines of evidence suggest that Runx2 is responsive to sonic hedgehog signaling. In this study we observe a minor (10%) activation of Runx2 promoter activity in response to Shh treatment of C3H10T1/2 cells. The lack of a robust response to Shh may reflect the fact that these cells have endogenously active Runx2. In support of this, other studies have observed an induction of Runx2 as a result of Shh treatment (Takamoto et al. 2003) and have observed that the Runx2 gene is active in the notochord (the source of soluble Shh during embryogenesis) in vivo (Otto et al. 1997). Thus, the Runx2 gene appears to be responsive to signaling pathways that are essential for early skeletal development.

Later during development of the axial skeleton, BMP-2 acts in concert with Shh to activate Nkx3.2 and induce cartilage formation (Zeng et al. 2002). The sequence of events is recapitulated in this study using the C3H10T1/2 model of mesenchymal
chondrogenesis. Upon BMP-2 treatment and high-density culture, Nkx3.2 is induced and Runx2 is concomitantly repressed, supporting our findings that Nkx3.2 is an inhibitor of Runx2 gene expression, and suggesting that the activity of Runx2 must be suppressed prior to activation of the chondrogenic program of gene expression. This conclusion is further supported by the finding that adenoviral overexpression of Runx2 prior to chondrogenesis inhibits induction of Sox9, collagen type II, and the chondrogenic phenotype. The observation that elevating Runx2 levels after induction of chondrogenesis does not suppress expression of chondrocyte phenotypic genes indicates that a developmental ‘checkpoint’ has been passed. Subsequently, the ability of Runx2 to maintain an undifferentiated progenitor is lost.

While these findings underscore the importance of Runx2 repression at the onset of mesenchymal chondrogenesis, the function of Runx2 in undifferentiated mesenchyme remains unclear. These progenitor cells express Runx2 during proliferative expansion both in vivo and in vitro. This observation raises the question whether Runx2 maintains an undifferentiated phenotype during expansion of the progenitor population. In this study, circumvention of Runx2 repression at the onset of BMP-2 induced cartilage formation efficiently inhibits activation of chondrocyte phenotypic genes and extracellular matrix deposition. It is therefore possible that these cells either remain undifferentiated in the presence of exogenous Runx2 and BMP-2 or, alternatively become osteogenic. However, we did not observe induction of the bone phenotypic marker osteocalcin (not shown) and observed only a minor induction of alkaline phosphatase (an
early osteogenic marker) that was restricted to cells at the periphery and on the surface of the micromass cultures. While other studies have observed induction of Runx2 and osteogenic gene expression upon BMP-2 treatment of C3H10T1/2 cells in monolayer cultures, we find that the osteogenic phenotype cannot be induced in high-density micromass cultures despite the high levels of exogenous Runx2 and BMP-2. These findings suggest that cell-cell contact in high-density cultures predisposes cells to undergo chondrogenesis in response to BMP-2 signaling.

Our findings further suggest that a feedback loop may exist in which high levels of Runx2 positively affect the Nkx3.2 gene in order for Nkx3.2 to repress Runx2 and thereby promote chondrogenesis. This conclusion is based on the observation that BMP-2 treated micromass cultures infected with Runx2 adenovirus exhibit increased Nkx3.2 expression in comparison to lac-Z infected controls. These findings are of particular interest in light of the observation that the ability of Nkx3.2 to repress Runx2 gene expression is severely abrogated in committed osteoprogenitor cells, suggesting that Runx2 may act as a switching mechanism for differentiation induced by BMP signaling. In an undifferentiated mesenchymal progenitor cell, Runx2 may maintain a pluripotent state until receipt of a BMP-2 signal. Depending on the microenvironment of the progenitor cell, BMP-2 can induce Nkx3.2 to suppress Runx2 and promote chondrogenic differentiation of the pluripotent progenitor (Figure 3.8). Once chondrogenesis has begun and Sox9 is activated, Runx2 cannot reverse the differentiation process. If the BMP-2 signal is osteogenic, the progenitor cell will further induce Runx2 and activate Osterix,
Figure 3.8: Model for Runx2 Modulation of BMP-induced Differentiation. Previous findings have shown in vivo and in monolayer cultures of mesenchymal progenitor cells that BMP-2 induces the osteogenic phenotype via activation of Runx2. Runx2 is necessary for activation of Osterix and other osteoblast phenotypic genes. BMP2 itself can also lead to Osterix accumulation, however this seems occur independent of transcriptional activity of the Osterix gene (dashed arrow). In condensing mesenchyme or high-density micromass cultures, BMP-2 activity activates Nkx3.2, which in tum suppresses Runx2 transcription. This repression of Runx2 allows for activation of Sox9 and expression of chondrocyte phenotypic genes. Activation of Nkx3.2 by BMP-2 therefore indirectly leads to Sox9 expression and chondrogenesis.
another essential pro-osteogenic transcription factor whose activities, along with those of Runx2, are responsible for osteoblast formation (Nakashima et al. 2002; Lee et al. 2003; Ohyama et al. 2004). In this context, the committed pre-osteoblast has passed another ‘checkpoint’ in differentiation where the activity of Nkx3.2 is no longer competent to repress Runx2 gene expression and therefore no longer able to promote entry into the chondrogenic lineage.

In conclusion, we have identified a novel role for Runx2 as a critical component of the differentiation program that is initiated by activation of the sonic hedgehog and BMP signaling pathways and ultimately results in the chondrogenic differentiation of mesenchymal progenitor cells and formation of the cartilaginous template of the vertebrate skeleton.
Chapter 4:

Primary Mouse Embryonic Fibroblasts: A Model of Mesenchymal Cartilage Formation
ABSTRACT

Cartilage formation is an intricate process that requires temporal and spatial organization of regulatory factors in order for a mesenchymal progenitor cell to differentiate through the distinct stages of chondrogenesis. Gene function during this process has best been studied by analysis of in vivo cartilage formation in genetically altered mouse models. Mouse embryonic fibroblasts (MEFs) isolated from such mouse models have been widely used for the study of growth control and DNA damage response. Here we address the potential of MEFs to undergo chondrogenic differentiation. We demonstrate for the first time that MEFs can enter and complete the program of chondrogenic differentiation ex vivo, from undifferentiated progenitor cells to mature, hypertrophic chondrocytes. We show that chondrogenic differentiation can be induced by cell-cell contact or BMP-2 treatment, while in combination, these conditions synergistically enhance chondrocyte differentiation resulting in the formation of 3-dimensional cartilaginous tissue ex-vivo. Temporal expression profiles of pro-chondrogenic transcription factors Nkx3.2/Nkx3.2 and Sox9 and cartilaginous extracellular matrix proteins collagen type II and X demonstrate that the in vivo progression of chondrocyte maturation is recapitulated in the MEF model system. We utilize this model system for analysis of chondrogenesis in MEFs that are nullizygous for Runx1, Runx2, or both Runx family members. Our findings suggest that these genes play distinct, non-overlapping roles in chondrogenesis. We find that MEFs lacking Runx2 enter into the chondrogenic lineage and form cartilage tissue ex vivo. These cells, however, fail to mineralize the extracellular matrix and do not become fully hypertrophic. Runx1 null MEFs are able not only to enter the
chondrogenic lineage, but are also capable of achieving hypertrophy \textit{ex vivo}. These cells form less cartilage than wildtype counterparts, seemingly due to a decreased proliferative capacity. Further, deletion of both Runx family members does not result in abrogation of chondrogenic differentiation or the ability of MEFs to activate chondrocyte-specific genes in response to BMP2 treatment and high-density culture. These results indicate that Runx1 and Runx2 play non-redundant roles in mesenchymal chondrogenesis. Our findings establish the MEF as a powerful tool for the study of gene function during chondrogenesis. The MEFs capacity for chondrogenic differentiation has further enabled us to examine the contribution of \textit{Runx} gene function during commitment to this lineage.
INTRODUCTION

Chondrogenic differentiation of mesenchymal cells and the subsequent formation of cartilaginous tissue are governed by an exquisitely regulated series of events in which both spatial and temporal regulation of gene activity must be tightly controlled to achieve the formation of viable tissue. Currently, analysis of chondrocyte differentiation is limited to in vitro analysis of primary chondrocytes as well as immortalized cell lines and to in vivo analysis using mouse and rat models. In vitro systems for chondrogenic differentiation have classically used a combination of BMP-2 treatment and high-density culture in order to induce chondrogenesis. These culture conditions have proven effective in both the immortalized C3H10T1/2 pluripotent mesenchymal progenitor cell line (Denker et al. 1999; Carlberg et al. 2001; Nochi et al. 2004) as well as in primary cultures of bone marrow stromal cells (Mackay et al. 1998; Muraglia et al. 2003).

In vivo genetic approaches represent a powerful tool for understanding the spatio-temporal organization of cartilaginous tissue as well as for understanding the contribution of specific genes to the process of chondrogenesis through the use of transgenic and targeted gene deletions in the mouse (de Crombrugghe et al. 2001; Akiyama et al. 2002). Chondrocytes, however, do not begin to undergo differentiation in the developing embryo until the latter half of gestation, making the analysis of chondrogenesis in embryonic lethal murine knockout models technically challenging. In order to analyze
the contribution of genes whose deletion results in embryonic lethality to the process of chondrogenesis, we have turned toward the developing mouse embryo for a source of cells that have chondrogenic potential and can be isolated prior to overt chondrogenesis during the latter half of gestation. We have identified the mouse embryonic fibroblast (MEF) as a candidate for such a cell type based on several very appealing properties.

The MEF is a cell isolated from mid-gestation mouse embryos and cultured *ex-vivo*. These cells are capable of undergoing a limited number of population doublings before entering crisis and senescence-like growth arrest (Espejel and Blasco 2002; Rossi et al. 2003). Their ease of isolation and the ability to derive these cells from mice harboring various genetic alterations (both targeted and non-targeted) has made the MEF ideal model system for studying aspects of cell growth control and functional genetics (Lowe et al. 1994; Kamijo et al. 1997; Rzonca et al. 2004; Steinman et al. 2004). Since many murine knockout lines, including those harboring inactive Runx alleles, exhibit embryonic lethality, the MEF provides an invaluable system for studying cellular processes in such lines. Few reports however, have utilized the MEF as a tool for the study of cell differentiation. The ability of the MEF to undergo adipogenic differentiation *ex vivo* has been demonstrated by several groups (Alexander et al. 1998; Yun et al. 2002; Landsberg et al. 2003). Since adipocytes share a common mesenchymal origin with several other cell types, including osteoblasts and chondrocytes, we set out to develop a model system of mesenchymal chondrogenesis using the MEF. Here we address the ability of the MEF to undergo a program of chondrogenic differentiation, thereby
providing a tool that enables the study of a mesenchymal progenitor cell's entry into and journey through the chondrogenic pathway as well as identifying a source of cells that can be expanded significantly for the purpose of tissue generation. In addition, we examine the ability of MEF cultures lacking either or both Runx1 and Runx2 genes to enter and complete the program of chondrogenic differentiation.

Both Runx1 and Runx2 genes exhibit distinct spatio-temporal expression patterns during chondrogenesis (Drissi et al. 2004; Yamashiro et al. 2004). Based on transgenic and knock-in animal models it has been shown that the Runx2 gene is active in mesenchymal progenitor cells in the developing embryo as well as in hypertrophic chondrocytes. While it's role in maturation of the hypertrophic chondrocyte is well understood (Stricker et al. 2002; Iwamoto et al. 2003; Komori 2003; Zheng et al. 2003; Smits et al. 2004; Yoshida et al. 2004), the presence of Runx2 in pre-chondrocytic mesenchyme is not understood. Since Runx2 null mice exhibit no apparent defect in the differentiation of these mesenchymal cells, we hypothesize that Runx1 may play a role redundant to that of Runx2 during the proliferation and differentiation of these cells. Runx1 null mice die prior to the migration and differentiation of mesenchymal progenitor cells from hemorrhage and a block in hematopoiesis (Wang et al. 1996; North et al. 2002) making the study of chondrogenesis in these mice infeasible. Runx1 is co-expressed with Runx2 in mesenchymal condensations (Yamashiro et al. 2002; Lian et al. 2003) suggesting that these family members may play redundant roles in mesenchyme. To address this possibility we utilize a Runx1 conditional allele (Runx1^{fix/flx}) that has been bred onto a
Runx2 null allele. We examine the effects of the loss of either one or both of these Runx factors on chondrogenic differentiation of the mesenchymal mouse embryonic fibroblast.

MATERIALS AND METHODS

MEF Isolation and Culture

MEFs were isolated from mouse embryos derived from C57/B6 mothers between 10 and 12 days of gestation (E10-E12). Briefly, each embryo was sheared in an 18-gauge syringe in the presence of 1 mL 0.25% trypsin/1 mM EDTA (Gibco, Carlsbad CA) per embryo and incubated at 37°C for 15 minutes. Trypsin was inactivated by addition of DMEM (Gibco) containing 15% FBS (HyClone, South Logan UT) and cells were then plated in 10 cm culture dishes and allowed to adhere for 24 hours. Non-adherent cells were then discarded and cells in the adherent fraction are termed MEFs. MEFs were expanded by passaging pre-confluent cultures at a 1:5 ratio prior to induction of differentiation. Differentiation experiments were carried out using cells between passage 3 and 5. Animals were treated in accordance with IACUC guidelines.

Runx1 null MEFs were generated by crossing Runx1

null mice (a kind gift from Dr. Nancy Speck, Dartmouth NH) against one another and harvesting MEFs as stated above, followed by infection with Cre-recombinase containing adenovirus (University of Iowa Gene Vector Core) or control Lac-Z containing adenovirus at an MOI of 100. Runx2 null MEFs were generating by crossing Runx2

null mice (a kind gift from Dr. Tosihisa
Komori, Japan) against one another and harvesting MEFs. Runx1/Runx2 null MEFs were generating by crossing Runx1<sup>fl/+</sup> Runx2<sup>+/−</sup> mice against one another and harvesting MEFs as previously stated. Runx1<sup>fl</sup>Runx2<sup>−/−</sup> and control MEFs were then infected with Cre-recombinase containing adenovirus (University of Iowa Gene Vector Core) or control Lac-Z containing adenovirus at an MOI of 100.

**Chondrogenic Induction**

In order to stimulate chondrogenic differentiation, we sought to recapitulate the in vivo environment of mesenchymal condensations in the embryo using various culture conditions. Cells were either plated onto 6 well plates in high-density micromass cultures (10<sup>5</sup> cells in a 10 µL drop of media) (Ahrens et al. 1977; Mello and Tuan 1999) or as a monolayer (10<sup>5</sup> cells/well) both in the presence or absence of 100 ng/mL recombinant hBMP-2 (kindly provided by Dr. John Wozney at Wyeth-Ayerst, Cambridge MA). High-density culture provides cell-cell contact in three dimensions similar to that which occurs in vivo in condensing mesenchyme prior to induction of chondrogenesis. BMP-2 was chosen because it has been shown to be a potent chondrogenic factor for mesenchymal stem cells in vitro (Denker et al. 1999; Schmitt et al. 2003) and plays an important role in the chondrogenesis of condensed mesenchyme in vivo (Zeng et al. 2002).

**RNA Isolation and Analysis**

RNA was isolated at various times during a 12-day culture period using Trizol Reagent (Invitrogen, Carlsbad CA) according to the manufacturers protocol. After purification, 5
ug of total RNA was DNase treated using a DNA-free RNA column purification kit (Zymo Research, Orange, CA). One µg of RNA was then reverse transcribed using Oligo-dT primers and the SuperScript 1st Strand Synthesis kit (Invitrogen) according the manufacturers protocol. Gene expression was assessed by semi-quantitative (collagen type II and X, 25 cycles) and quantitative real-time PCR (Sox9, Nkx3.2/Nkx3.2, alkaline phosphatase, osteocalcin, Indian hedgehog). Quantitative PCR was performed using SYBR green 2x master mix (Eurogentec, Belgium) and a 2 step cycling protocol (anneal and elongate at 60°C, denature at 94°C). Data are expressed as arbitrary expression level relative to GAPDH expression level. Specificity of primers was verified by dissociation of amplicons. Primers used for PCR reactions are listed in Table 1. Results are representative of three or more independent experiments.

**Histology**

MEF cultures were fixed in 4% paraformaldehyde for 10-15 minutes prior to histological analysis. Alcian blue staining was performed using 1% Alcian blue GX8 (Sigma) solution, pH 8, overnight. Safranine-O staining was performed using a 0.1% Safranine-O solution for 10 minutes at room temperature. Von Kossa stain for mineralized tissue was performed using a 3% silver nitrate solution and staining for 10 minutes in direct sunlight. Alkaline phosphatase enzymatic activity was detected by colorimetric reaction using a 0.1 M Tris maleate buffer (pH8.4) containing 0.05% Napthol as Mx Phosphate disodium salt, 2.8% N,N dimethyl formamide, and 0.1% Fast Red salt. Staining was
carried out at 37°C for 10 minutes (Gold Biotechnology, St Louis, MO) in phosphate buffered saline (PBS, pH 7.3) at room temperature for 10-12 hours.

**Scanning Electron Microscopy**

Cartilage spheroids were fixed by immersion in 2.5% (v/v) glutaraldehyde in 0.5 M Na cacodylate-HCl buffer (pH 7.2) for 1 hr at room temperature; the fixed samples were washed three times in the same buffer. Following the third wash the cells were post fixed for 1 hr in 1% osmium tetroxide (w/v) in the same buffer, washed three more times in the same buffer and left overnight at 4°C in fresh buffer. The next morning the samples were dehydrated through a graded series of ethanol to 100% and then critical point dried in liquid CO₂. The bottoms of the dishes were isolated and affixed to aluminum SEM stubs using silver conductive paste and sputter coated with Au/Pd (80/20). The specimens were then examined using an ETEC autoscan scanning electron microscope at 20 Kv accelerating voltage.

**RESULTS**

*BMP2 and High Cell Density are Required for Entry of Mouse Embryonic Fibroblasts into the Chondrogenic Lineage*

We initially established appropriate culture conditions for induction of chondrogenesis by MEFs as reflected by several established markers for progression of the chondrocyte
phenotype. Cells were plated to grow either as a monolayer or as a high-density micromass culture in the presence or absence of BMP2 (Figure 4.1). In the absence of BMP2, entry of MEFs into the chondrogenic lineage occurred in response to cell-cell contact in monolayer culture post-confluence, as assessed by the transcriptional activity of the Nkx3.2 gene. Nkx3.2 is the earliest known marker of chondrogenic commitment in undifferentiated mesenchyme (Lettice et al. 1999) and its activation is a prerequisite for induction of Sox9, the canonical 'master regulator' of chondrogenesis whose activity induces expression of various chondrocyte phenotypic genes, such as collagen type II (Coll II) (Lefebvre et al. 1998; Bi et al. 1999; Zeng et al. 2002). Nkx3.2 and Sox9 expression was induced in post-confluent monolayer cultures between days 9 and 12 (Figure 4.1-A); however, this did not translate into significant cartilaginous extracellular matrix (ECM) production, as evidenced by lack of Coll II expression (Figure 4.1-C). The addition of BMP-2 to monolayer cultures resulted in a more rapid induction of Nkx3.2 and Sox9 that lead to a modest induction of Coll II transcription post-confluence. These findings indicate that while BMP-2 treatment of monolayer MEF cultures can induce the chondrogenic program after cells have achieved confluence, the chondrogenesis is inefficient.
Figure 4.1: Chondrogenic induction of mouse embryonic fibroblasts (MEFs). RNA was isolated from MEFs cultured under various conditions and analyzed for chondrocyte phenotypic gene expression by quantitative RT-PCR at the indicated times. Monolayer cultures (MO, open symbols) induce pro-chondrogenic transcription factors Nkx3.2 and Sox9 in post-confluent cultures (A). Addition of BMP2 to these cultures results in a more rapid induction of these genes (MO, solid symbols). Micromass cultures (MM, open symbols) moderately induce Sox9 and Nkx3.2 in the absence of BMP2 (B). Addition of BMP2 to these cultures (MM, solid symbols) results in an earlier and more robust induction of these genes. Type II collagen, an cartilaginous ECM component and Sox9 target gene, is strongly induced only during micromass culture in the presence of BMP2 (C). Cartilaginous extracellular matrix is visualized with alcian blue and safranin-O stains which have an affinity for negatively charged ECM secreted by chondrocytes (D).
High-density culture (micromass) of MEFs in the absence of BMP-2 resulted in the induction of Nkx3.2 and Sox9 expression (Figure 4.1-A), and similarly to monolayer cultures, this activation lead to weak induction of the chondrogenic phenotype observed by 9 days of culture, as judged by collagen II expression (Figure 4.1-C). In contrast, addition of BMP-2 to high-density micromass cultures synergistically activated expression of the Nkx3.2 and Sox9 genes by 3 days of culture (Figure 4.1-B). Unlike BMP-2 treated monolayer cultures or untreated monolayer/micromass cultures, the Nkx3.2/Sox9 induction in BMP-2 treated micromass cultures resulted in a robust activation of the Coll II gene. These results demonstrate that cell-cell contact and the BMP-2 signal cooperate for the transcriptional activation of the chondrocyte-specific transcription factors Nkx3.2 and Sox9, and their induction synergistically drives robust chondrogenesis in MEFs, recapitulating the early stages of mesenchymal chondrogenic differentiation.

We next examined the organization of the extracellular matrix secreted by chondrogenic MEFs via incorporation of Alcian blue and Safranine-O staining for sulfonated proteoglycans typical of cartilage and found that after 6 days of high-density culture (MM) cartilaginous matrix was only deposited when BMP-2 was added to the culture medium (Figure 4.1, D). To further characterize the chondrogenic differentiation of MEFs and the formation of cartilaginous tissue, we performed cell morphology studies on BMP-2 treated MEFs in micromass culture. Scanning electron microscopy demonstrates that after 8 days of culture, there is an accumulation of extracellular matrix
Figure 4.2: Scanning electron microscopy of MEFs. MEFs cultured at high density in the presence of BMP2 can be seen secreting proteoglycan fibrils (arrows) as well as collagen sheets (arrowheads) characteristic of chondrocytes at low (A) and high (B) magnification.
proteins that are deposited over cells. Individual cells are observed secreting proteoglycan strands (Figure 4.2, arrows) and collagen fibers (Figure 4.2, arrowheads). Thus, chondrogenic induction of MEFs results in the formation of a cartilaginous spheroid with tissue-like organization.

**Chondrocytic MEF Cultures Undergo Hypertrophy**

Having established that MEFs are competent to enter the chondrogenic lineage, we asked whether these cultures were able to complete the program of chondrocyte maturation by undergoing cellular hypertrophy, characterized by the expansion of the chondrocytic cell body, the expression of type X collagen (Coll X), and the induction of alkaline phosphatase (AP) enzymatic activity. At the final stage of hypertrophic cartilage formation transcription of the osteocalcin gene (OC) is initiated and the extracellular matrix is mineralized (reviewed by (Gerstenfeld and Shapiro 1996). Real time and semi-quantitative RT-PCR performed on MEF cultures demonstrates that both high-density culture conditions and the presence of BMP-2 are required for efficient hypertrophy (Figure 4.3). Cultures not treated with BMP-2 exhibited minimal Coll X gene activity (Figure 4.3-A) and were unable to activate AP or OC (Figure 4.3, B&C). In monolayer cultures, BMP-2 modestly stimulated these phenotypic genes. In contrast, the combination of high-density culture and BMP-2 treatment resulted in a synergistic activation of CollX, AP, and OC between 6 days and 9 days of culture. The temporal expression of these phenotypic genes distinctively delineates a second stage of chondrogenic differentiation achievable by the MEF. Importantly, the chondrogenic
Figure 4.3: Chondrogenic MEF cultures become hypertrophic. RT-PCR analysis of collagen X expression, a hallmark of chondrocyte hypertrophy (A). Alkaline phosphatase expression is a prerequisite for ECM mineralization, and it is synergistically induced in BMP2 treated micromass cultures (B). Osteocalcin expression is indicative of a mature, actively mineralizing matrix (C). Indian hedgehog (Ihh) activity is also restricted to BMP2 treated micromass cultures during the later stages of chondrocyte maturation. All quantitative PCR data expressed as transcript level relative to GAPDH transcript level.
differentiation of MEFs ex vivo reflects the development of the chondrocytic phenotype from mesenchymal progenitor to hypertrophic chondrocyte. In the growth plate in vivo, hypertrophic chondrocytes express and secrete Indian hedgehog (Ihh), a signaling molecule that acts upon pre-hypertrophic chondrocytes and regulates their rate of maturation (Long et al. 2001; Kobayashi et al. 2002). Analysis of Indian hedgehog expression demonstrates that this gene is responsive only to a combination of BMP-2 and micromass culturing, and its induction between 6 and 9 days of culture suggests that these cultures have entered the hypertrophic pathway, and are limiting the rate of tissue maturation via the anti-hypertrophic Ihh signal (Figure 4-D). Therefore, induction of Ihh by cell-cell contact combined with BMP-2 treatment further establishes the necessity for cooperation of these two signals in the progression of the chondrogenic phenotype.

In order to correlate chondrocyte phenotypic gene expression with the formation of mature cartilaginous ECM, alkaline phosphatase enzymatic activity was visualized using a colorimetric assay. Alkaline phosphatase activity was present at low levels throughout the monolayer in untreated day 10 cultures, while addition of BMP-2 to the monolayer resulted in more intense AP activity. Untreated micromass cultures exhibited focal AP activity, while BMP-2 treatment of micromass cultures caused very intense staining throughout the micromass spheroid, consistent with greater ECM production (Figure 4-A). Next, toluidine blue stain was employed to visualize the negatively charged ECM that is a hallmark of mature cartilage. Toluidine blue staining only provided contrast to BMP-2 treated micromass cultures, indicating the presence of a negatively charged ECM. To visualize the mineralization of the ECM characteristic of the final stage of hypertrophic
Figure 4.4: BMP2 induced MEF differentiation results in hypertrophic cartilage formation. Visualization of the ECM synthesized by MEFs (A). Alkaline phosphatase activity is detected by colorimetric reaction in the ECM at 10 days of culture shows intense activity in BMP2 treated Micromass cultures (A, top panels). Toluidine blue is a metachromatic stain that indicates total cellularity of MEF cultures and reveals negatively charged cartilaginous ECM (darker blue staining) in Micromass cultures treated with BMP2 (A, lower panels). Mineralization of hypertrophic ECM can be visualized at the final stages of MEF micromass cultures treated with BMP2. Silver nitrate stains mineral deposits in the ECM (black) that surrounds individual chondrocytes in lacunae.
cartilage formation, we performed silver nitrate staining according to the von Kossa method after 15 days of micromass culture in the presence of BMP-2 (Figure 4.4-B). This stain reveals that the chondrocytes have acquired a hypertrophic phenotype, as each individual cell is in a lacuna surrounded by a mineralized extracellular matrix, stained black.

Runx2 Null MEFs Fail to Mineralize the Cartilaginous ECM

In order to examine the contribution of Runx2 to chondrocyte phenotypic gene expression, MEFs isolated from Runx2 null embryos were induced to undergo chondrogenesis using BMP2 and high-density culture conditions. We verified an absence of Run2 protein at all timepoints during differentiation (Figure 4.5-A). Furthermore, we observe a co-expression of Runx2 and Runx1 during the initial three days of culture (Figure 4.5-A). Interestingly, observation of Runx2 levels in wildtype cells shows that Runx2 protein declines concomitantly with the onset of chondrocyte phenotypic gene expression (Figure 4.5-B and 4.7). Runx2 protein levels are later strongly induced along with markers of late hypertrophy, alkaline phosphatase and osteocalcin (Figure 4.6-B and 4.7). In contrast, Runx1 is induced at the onset of high-density culture and protein levels are maintained only for the first three days of culture. Runx1 protein levels were not affected by the loss of Runx2 (Figure 4.5-A) suggesting cross-regulation between Runx2 and Runx1 does not occur during induction of the chondrogenic phenotype. These results confirm previous observations of Runx2 expression patterns during chondrogenesis of C3H10T1/2 cells in Chapter 3 in which Runx2 is transiently suppressed in the
Figure 4.5: Chondrogenic differentiation of Runx2 null MEFs. MEFs were isolated from E12 Runx2 null embryos and their wildtype littermates and induced to undergo chondrogenesis in response to high density micromass culture in the presence of BMP2. Whole cell extracts were analyzed by western blotting at various timepoints demonstrates a decrease in Runx2 protein at the onset of chondrogenesis in wildtype cultures, and a total absence of Runx2 protein in null MEFs (A). Runx1 protein in these cultures is dramatically induced at the beginning of the culture period and then declines as cultures mature (A). Analysis of pro-chondrogenic transcription factor expression by quantitative RT-PCR (B) indicates that Nkx3.2 activation is abrogated in the absence of Runx2 (B).
mesenchymal progenitor cell as it enters the chondrogenic lineage in response to BMP treatment and high-density culture conditions.

When analyzing expression of chondrocyte phenotypic genes in the absence of Runx2, it became evident that Nkx3.2 levels were decreased in the absence of Runx2 (Figure 4.5-B), supporting our previous results in C3H10T1/2 cells in Chapter 3 demonstrating that adeno-viral mediated overexpression of Runx2 induces the Nkx3.2 gene. Although lack of Runx2 activity did not influence expression of Sox 9 or collagen type II on a per cell basis (Figure 4.5-B and 4.7), its absence appeared to result in the formation of a larger cartilaginous area, as evidenced by alcian blue staining (Figure 4.6-A), indicating that the absence of Runx2 activity in these cells results in increased proliferation prior to the onset of differentiation. During the final stages of chondrocyte hypertrophy, Runx2 null MEFs were unable to activate osteocalcin or alkaline phosphatase, genes known to be dependent on Runx2 activity for their expression (Figure 4.7). Interestingly activation of type X collagen (a marker of early hypertrophy) still occurred in the absence of Runx2. This finding is in contrast with previous findings that have suggested that collagen type X expression is dependent on the combined activities of Runx2 and BMP2. These findings provide new insight into the precise point of action of Runx2 during chondrogenic differentiation and suggest that Runx2 functions to mineralize the ECM of hypertrophic chondrocytes by supporting the activity of genes related to mineralization such as alkaline phosphatase and osteocalcin similarly to the way Runx2 supports the activity of these genes during the mineralization of osteoblast-generated ECM.
Figure 4.6: Cartilage formation by Runx2 null MEF cultures. Alcian blue incorporation into chondrogenic Runx2 null and wildtype MEFs cultured at high density in the presence of BMP2 (A, upper and middle panels). In the absence of BMP2, there is no cartilaginous ECM production from either Runx2 wildtype or null high-density MEF cultures (A, lower panels). Alkaline phosphatase (AP) enzymatic activity in BMP-2 treated high-density MEF cultures demonstrate the requirement for Runx2 in efficient AP production (B).
Figure 4.7: Extracellular matrix gene expression in chondrogenic Runx2 wildtype and null MEF cultures. The absence of Runx2 has little effect on chondrocyte-specific genes collagen type II (A, circles) and collagen type X (A, squares). Genes associated with mineralization of the chondrocyte ECM such as osteocalcin (B, circles) and alkaline phosphatase (B, squares) are not activated in MEF cultures nullizygous for the Runx2 gene.
Runxl Activity does not Contribute to Chondrocyte Phenotypic Gene Expression

We next examined the effects of Runxl loss to the process of chondrogenesis. In order to bypass the early embryonic lethality or Runxl deletion in vivo, we harvested MEFs from Runxl conditional mice (Runxl<sup>flox/flox</sup>, kindly provided by Dr. Nancy Speck, Dartmouth, NH) and their wildtype littermates and infected them with Cre-expressing adenovirus (Ad-Cre) prior to chondrogenic induction. Cre-expression in Runxl<sup>flox/flox</sup> MEFs results in a complete loss of Runxl activity in these cells (Figure 4.8-A). In contrast to Runx2 null MEFs, Runxl null MEFs were able to activate all phenotypic genes (Sox9, Nkx3, Figure 4.8-B and collagen type II&X, OC, and AP, Figure 4.9-A) comparable to wildtype MEFs at all stages of chondrogenesis on a per cell basis. Collagen type X appears to maintain increased activity at the 9 day timepoint in Runx2 knockout cultures cultures in comparison to wildtype cultures for reasons not understood. Runxl null cells, however, formed significantly smaller cartilaginous nodules as assessed by alcian blue and alkaline phosphatase staining (Figure 4.9-B) These findings suggest that the Runxl induction observed at the onset of high-density culture (Figure 4.5-A, time 0) supports the proliferative expansion of mesenchymal cells or pre-chondrocytes prior to differentiation. Our results thusfar suggest that Runxl and Runx2 perform distinct functions during the mesenchymal chondrogenesis.

To rule out any possibilities that Runxl and Runx2 cooperate during mesenchymal-chondrogenic differentiation, we isolated MEFs from Runx2<sup>−/−</sup> Runxl<sup>flox/flox</sup> mouse embryos and infected them with Cre-expressing adenovirus during proliferative
Figure 4.8: Chondrogenesis of Runx1 null MEFs. Runx1 conditional MEFs were infected in vitro with a Cre-containing adenoviruses prior to initiation of the experiment. Quantitative RT-PCR shows an absence of Runx1 message in Cre-infected cultures when compared to LacZ-adenoviral infected controls (A). Cre-infected cultures are therefore treated as Runx1 null, and LacZ-infected cultures as wildtype. Analysis of pro-chondrogenic transcription factor gene expression in Runx1 wildtype or null cultures indicate Runx1 loss has no effect on the expression of these genes.
Figure 4.9: Extracellular matrix formation and gene expression in Runx1 null and wildtype MEF cultures. Analysis of ECM gene expression (Coll 2, Coll X, AP, and OC) indicates that Runx1 loss does not affect their activity (A&B). Alcian blue staining for sulfonated proteoglycans and colorimetric staining for alkaline phosphatase activity reveals significantly smaller cartilaginous nodules in the absence of Runx1 (C).
Figure 4.10: Chondrocyte phenotypic gene expression is not dependent on cooperation between Runx1 and Runx2. Runx2<sup>−/−</sup> Runx1<sup>lox/lox</sup> MEfS were infected with the Cre adenovirus in order to inactivate Runx1 followed by high-density culture and BMP2 treatment to promote chondrogenic differentiation. These cultures progress through chondrogenesis despite the lack of both of these Runx factors.
expansion to ablate Runx1 activity \textit{ex vivo}. These cells were subsequently induced to undergo chondrogenic differentiation in high-density cultures treated with BMP2. Quantitative RT-PCR analysis of these cultures demonstrates that these Runx factors do not cooperate to drive chondrogenic differentiation, as they remain able to activate chondrocyte-phenotypic genes collagen type 2, collagen type X, and Nkx3.2 (Figure 4.10). These findings support our previous observations suggesting that Runx1 and Runx2 play distinct, non-redundant roles during the chondrogenic differentiation of undifferentiated mesenchymal progenitor cells.

**DISCUSSION**

The results of this study demonstrate that the mouse embryonic fibroblast has the ability to undergo a complete program of chondrogenic differentiation resulting in the formation of a cartilaginous spheroid \textit{ex vivo}. These cells begin as undifferentiated, rapidly proliferating fibroblast-like cells, that then readily differentiate into chondrocytes in response to BMP-2 and high density culture conditions. The MEFs produce a true cartilaginous extracellular matrix, and finally reach a state of hypertrophy, mineralizing the ECM. These events recapitulate chondrocyte maturation during endochondral bone formation \textit{in vivo}.

The finding that neither BMP-2 nor cell-cell contact alone are sufficient to drive chondrogenesis demonstrates the importance of cooperation between 3-dimensional extracellular contact and soluble BMP-2 signaling in the formation of cartilaginous
tissue. The culture conditions that promote the differentiation of these cells have striking effects on the transcriptional regulation of chondrocyte-specific transcription factors Nkx3.2, Sox9 and Indian Hedgehog. *In vivo*, BMP2 regulates both initiation of chondrogenic differentiation through Nkx3.2 (Murtaugh et al. 2001; Zeng et al. 2002), which is the earliest marker of a pre-chondroyte and a pre-requisite for activation of Sox9, as well as the rate of hypertrophy through Indian hedgehog (Pathi et al. 1999; Zeng et al. 2002). Thus, the finding that the BMP2 signal is required in addition to high-density culture for the chondrogenic differentiation of MEFs suggests that these cells are in a pluripotent, undifferentiated state while proliferating *ex vivo*. In contrast, previous studies using limb bud derived cells as a model of chondrogenesis respond to high density culture conditions and do not require the BMP2 signal, indicating that limb bud cells may already be committed to the chondrocyte lineage.

*In vivo*, hypertrophic chondrocytes secrete Indian hedgehog, which acts upon chondrocytes in the growth plate in order to attenuate the rate of hypertrophy. Under our culture conditions, MEFs have the ability to complete the program of chondrocyte maturation within 9 days of culture and express hypertrophic markers such as collagen type X, alkaline phosphatase, and osteocalcin, which have all been detected in cells of mineralized cartilage *in vivo*. Importantly, mature chondrocytic MEF cultures express the soluble regulator of the rate of hypertrophy, Indian Hedgehog, reflecting the fidelity of signaling events that occur during chondrocyte differentiation *in vivo*.
Recent findings from our group and others (see Chapter 3) have demonstrated that the Runx family of transcription factors is expressed in undifferentiated mesenchymal progenitor cells prior to the onset of skeletogenesis \textit{in vivo}. Studies from our group have demonstrated that overexpression of Runx2 in C3H10T1/2 cells prior to the induction of chondrogenesis with high-density culture and BMP-2 results in inhibition of cartilage formation, suggesting that Runx2 suppression at the onset of chondrogenesis may be a pre-requisite for differentiation. Further studies in our lab examining the expression of Runx1 during chondrogenesis \textit{in vivo} demonstrated that this family member is co-expressed in mesenchymal condensations along with Runx2, but unlike Runx2, Runx1 expression continues in active chondroblasts, as well as in the perichondrium (a source of chondro- and osteoprogenitor cells). Utilizing the MEF model system of chondrogenic differentiation, we have examined changes in Runx1 & 2 expression and protein levels as well as the functional consequences of nullizygosity for either or both of these proteins during entry into the chondrogenic lineage.

We demonstrate that Runx2 protein levels decrease during entry of wildtype MEFs into the chondrogenic lineage, followed by a strong induction of Runx2 protein and message during chondrocyte hypertrophy. Furthermore, we demonstrate that MEFs lacking Runx2 enter the chondrogenic lineage and form cartilage \textit{ex vivo} more efficiently than their wildtype counterparts. While chondrogenic ECM gene expression is unaltered in Runx2 null MEFs on a per cell basis, these cells form larger chondrocytic nodules, indicating that more cells are entering the chondrogenic lineage. The observation that
Runx2 null MEFs express significantly lower levels of Nkx3.2 during chondrogenesis is consistent with our previous observations in Chapter 3 demonstrating that overexpression of Runx2 in C3H10T1/2 cells increases Nkx3.2 gene expression. These findings support the hypothesis that Runx2 acts to negatively regulate entry of mesenchyme into the chondrogenic lineage. Further, our results provide evidence for the existence of a feedback loop between Runx2 and Nkx3.2 in which Nkx3.2 acts to suppress Runx2 in order to drive chondrogenesis: in the absence of Runx2, Nkx3.2 levels are reduced, whereas high levels of exogenous Runx2 induce Nkx3.2 expression for the progression of the chondrogenic phenotype.

Unlike Runx2, Runx1 protein levels increase at the onset of chondrogenesis, then gradually decline as chondrocyte maturation progresses. This observation suggests that Runx1 may play a positive role during the induction of mesenchymal chondrogenesis, and MEFs lacking Runx1 form less cartilage ex vivo that wildtype cells. Taken together, our findings indicate that Runx1 and Runx2 have distinct functions during cartilage formation. This hypothesis is supported by our observation that MEFs lacking both Runx1 and Runx2 are still able to undergo chondrogenic differentiation, ruling out the possibility that these two factors cooperate to drive chondrogenesis.

The capability of the MEF to undergo chondrogenesis makes it a powerful tool for analyzing the contribution of specific genes to the process of chondrogenesis and particularly applicable to the study of genes whose alteration results in embryonic
lethality in genetically altered mice. The ability of the embryonic fibroblast to be expanded *ex vivo* makes it particularly attractive for generation of cartilage, since mature chondrocytes are difficult to isolate and reluctant to proliferate while maintaining the chondrocytic phenotype *ex vivo* (Kuriwaka et al. 2003; Malpeli et al. 2004). Previous studies have characterized adipogenic differentiation of MEFS (Alexander et al. 1998; Yun et al. 2002; Landsberg et al. 2003). Here our studies establish the MEF as a pluripotent cell by demonstrating its ability to enter a second differentiation pathway, chondrogenesis. A source of human embryonic fibroblasts may someday allow us to generate cartilage for therapeutic purposes through the advances of molecular/cell biology and tissue engineering.
Chapter 5: Targeted Inactivation of Runx2 Type II
INTRODUCTION

The Runx family of transcription factors exhibits a high degree of homology in their DNA binding domains and their genomic structure. The three mammalian Runx genes each encode two major isoforms that are controlled by two distinct promoter regions (i.e., P1 and P2), with approximately 80 kb of genomic sequence separating their respective start codons. The upstream isoform, termed Type II, or *til-1*, initiates with the amino acid sequence MASNS and is driven by the P1 promoter, while the downstream isoform, termed Type I, initiates with the amino acid sequence MRIPV and is driven by the P2 promoter. These isoforms differ only in the utilization of one additional exon in the Type II isoform that becomes spliced to downstream exons common to both isoforms. This additional exon makes the Type II isoform 19 amino acids longer than Type I, although these additional amino acids serve no known function (Harada et al. 1999; Banerjee et al. 2001). We speculate that the evolution of two promoter regions reflects a demand for a higher order of transcriptional regulation, and that co-expression of both Type I and Type II isoforms in mature osteoblasts meets a requirement for differential regulation of Runx2 protein during distinct stages of skeletal development (Choi et al. 2002).

Towards gaining further insight into the regulation and function of the Runx2 Type II isoform, we have generated a knock-in mouse model in which exon 1, the only coding region unique to the Type II isoform, is replaced with the *Lac-Z* reporter gene. This model will allow for analysis of the functional contribution of this isoform to
skeletogenesis while also providing an *in situ* reporter assay for the examination of P1 promoter activity in its native context. Preliminary analysis of these mice indicates that the lac-Z reporter gene is indeed expressed in mature osteoblasts that are actively forming new bone. The *ex vivo* culture of osteoprogenitor cells derived from these mice will enable the study of the responsiveness of the endogenous upstream P1 promoter to a variety of cytokines and growth factors, and, in particular, BMP-2, whose activity is known to elevate Runx2 message and protein levels in osteoprogenitor cells *in vivo*. The mechanism by which BMP2 elevates Runx2 remains elusive as *in vitro* and transgenic studies (see Chapter 2) aimed at analyzing activity of the proximal 3 kb segment of the Runx2 P1 promoter have demonstrated that the BMP-2 responsiveness and osteogenic specificity of this promoter do not lie within this region. While a phenotypic analysis of a similar Runx2 Type II null animal has recently been published (Xiao et al. 2004), we are pursuing the analysis of homozygous Type II knockin mice independently because there are critical differences in the two targeting approaches that may influence the outcome of the results.

**MATERIALS AND METHODS**

**Cloning**

Genomic DNA for use in the Runx2 Type II targeting vector was isolated from AB2.2 murine embryonic stem cells. Target DNA for generating arms of homology was amplified using *Pfu* ultra DNA polymerase (Invitrogen) and polymerase chain reaction. Genomic DNA fragments used for arms of homology in the targeting vector were ligated to the *LacZ* reporter gene, the PGK-neomycin positive selection marker, and the PGK-
thymidine kinase negative selection marker (Figure 5.1) (all kind gifts from Dr. Stephen Jones, University of Massachusetts Medical School) using T4 DNA ligase (Invitrogen). The final targeting vector was created using the pBluescript vector backbone (Stratagene). Transformation of constructs was carried out using XL-10 Gold ultracompetent *E. coli* (Stratagene). Ultimately, 10 μg of complete targeting vector was linearized and electroporated into 1X10⁷ AB2.2 murine embryonic stem cells followed by positive and negative selection. Resulting clones were then cultured in 96 well plates in duplicate. One set of duplicate plates was screened by Southern blot for successful homologous recombination using an external 3’ probe and PCR-based analysis (See Genotyping section and Figure 5.2). Properly targeted clones were isolated from the second set of duplicate plates, expanded, and used in blastocyst injections performed by the University of Massachusetts Medical School Gene Targeting Core Facility. Resulting chimeric mice were backcrossed to wildtype B6 mice until germline transmission was achieved. These crosses yielded one female mouse heterozygous for the *til-l* knock-in (TK) allele. This founding female was backcrossed to a wildtype B6 male to expand the colony.

**Genotyping**

ES cells and mice were genotyped by Southern blotting and PCR. Genomic DNA was isolated from cells or mouse tissue after overnight incubation at 55°C in lysis buffer containing proteinase K. DNA was then isolated by phenol-chloroform extraction. Following purification, genomic DNA was resuspended in TE buffer, pH8, and was either digested for Southern blotting or used as a template for
Figure 5.1: Targeting strategy for gene replacement of Runx2 Type II exon 1 with the LacZ reporter gene. The targeting vector contains a 2.2 kb 3' arm of homology and a 3 kb 5' arm of homology which partially encompasses the Runx2 P1 promoter (3 kb P1). The LacZ reporter gene and a Neomycin drug resistance gene flanked by loxP sites were inserted between the arms of homology, that, upon homologous recombination with the wildtype allele, replace exon 1 as well as the splice donor site required for splicing into downstream exons common to Runx2 Type I and II. The targeted allele produces β-galactosidase as a result of P1 promoter activity and Runx2 Type I as a result of P2 promoter activity.
PCR. Southern blotting for the presence of the targeting construct was done using an internal probe against the LacZ reporter gene (Figure 5.2, IP). Verification of homologous recombination was performed by southern blotting using BamHI digest and an external probe that lies in the intronic region outside of the 3' arm of homology at the Runx2 locus (Figure 5.2, EP). This probe picks up a ~15 kb genomic BamHI fragment on the wildtype allele, and a smaller, ~7 kb fragment resulting from the introduction of a BamHI site from the neomycin cassette after homologous recombination on the mutant allele (Figure 5-2, panel A). Homologous recombination was verified at the 5' terminus of the targeted allele using PCR primers extending from just upstream of the 5' terminus of the 5' arm of homology into the LacZ coding region (Figure 5.2, 5'-lacZ PCR). Routine genotyping was then carried out using two sets of PCR primers. The first amplifies the targeted exon in the wildtype allele (Figure 5.2, Exon1 PCR) (F: 5'-atg ctt cat tcg cct cac aaa c-3', R: 5'-ccc aaa aga agc ttt gct gac-3'), generating a product in both wildtype and heterozygotes. The second amplifies the LacZ coding region (Figure 5.2, lacZ PCR) (F: 5'-cca act taa tcg cct tgc agc aca tc-3', R: 5'-cgt ggg aac aaa cgg cgg att gac-3'), generating a product in both heterozygotes and nullizygotes (Figure 5.3-B).

Histological Analysis

Newborn mice were sacrificed by decapitation and eviscerated followed by overnight fixation in a solution containing 2% paraformaldehyde, 0.2% glutaraldehyde, 5 mM EGTA, 2 mM MgCl2, and 0.1 M PBS. Whole embryos or individual skeletal elements
were then stained for β-galactosidase activity. Whole mount images of β-gal staining were taken prior to dehydration in a graded ethanol series and infiltration with paraffin wax. Histological sections were taken at 10 μm and mounted on glass slides with Permount mounting media. Ex vivo osteoblast cultures were fixed in 4% paraformaldehyde for 10 minutes at room temperature and stained for β-gal activity or alkaline phosphatase activity directly in 6-well tissue culture plates. Alkaline phosphatase activity was visualized in cultures by exposure to a solution containing 0.5 mg/ml Napthol as MX Phosphate (disodium salt), 1 mg/ml Fast Red salt, and 2.8% N,N dimethyl formamide in 0.1 M Tris maleate buffer, pH 8.4 for 15 minutes at 37°C in the dark. All chemicals were obtained from Sigma Chemical, Madison, WI except where indicated.

Primary Calvarial Osteoblast Cultures

Calvarial osteoblasts were isolated from E19 embryos by enzymatic digestion of calvarial bones. Briefly, calvaria were minced and subjected to three sequential digestions (8, 10, and 26 min) with collagenase P (Roche Molecular Biochemicals, Indiana, IN) at 37°C. Osteoblasts in the second and third digest were collected and resuspended in α-MEM supplemented with 10% FBS (Hyclone). Cells were plated at a density of 1 x 10⁶ cells/six-well plate (Owen et al. 1990). Differentiation was initiated post-confluence by the addition of ascorbic acid and β-glycerol phosphate. Cultures were harvested at various timepoints and stained for β-galactosidase activity.
Figure 5.2: Genotyping strategy for identification of the Runx2 Type II-LacZ allele. The presence of the targeting vector in ES cells after selection with neomycin and G418 was confirmed by Southern blotting with an internal probe against the LacZ gene (IP). Homologous recombination was then confirmed at the 3' end of targeted allele by Southern blotting BamHI digested genomic DNA with an external probe (EP). Homologous recombination was confirmed at the 5' end of the targeted allele using a genomic PCR strategy (5'-lacZ PCR) in which the forward primer lies outside the 5' terminus of the 5' arm of homology and the reverse primer lies within the coding sequence of the LacZ gene. Routine genotyping of the mouse colony was carried out using two PCR reactions. The first (Exon1 PCR) detects the presence of Runx2 exon 1, and the second (lacZ PCR) detects the presence of the LacZ gene.
RESULTS

_Homozygosity for the TK Knock-in Allele does not Result in Embryonic Lethality_

To determine whether the introduced mutation results in an embryonic lethal phenotype, several pregnancies resulting from the mating of heterozygous mice were brought to term, and resulting litters were analyzed for the absence of the Runx2 Type II exon 1 (Figure 5.3, panel B). Nullizygous mice were born at the expected Mendelian ratios demonstrating that loss of Runx2 Type II does not result in embryonic lethality (Figure 5.3, +/-). Further, Runx2 Type II null mice form a complete mineralized skeleton that exhibits no apparent defects. We continued to monitor the development of Runx2 Type II null mice and their wildtype littermates, and by two months of age all of the nullizygous mice were visibly smaller than their wildtype littermates, indicating that loss of Runx2 Type II does affect the maintenance of body mass in mature mice (Figure 5.4). The underlying physiological cause of the observed decrease in body mass is under investigation. These findings demonstrate that Runx2 Type II is not essential for development or skeletal formation, but may play a role later in life. Our findings are conflicting with those of Xiao et al. who report lethality within the first 2 weeks post-partum due to the absence of Runx2 Type II (Xiao et al. 2004). This group was, however, unable to identify the cause of lethality.
Figure 5.3: Genotyping of the Runx2 Type II-LacZ allele. Southern blotting of genomic DNA derived from ES cells that have passed both positive (neomycin) and negative selection for the presence of a correctly targeted knock-in allele (A). Genomic DNA was digested with BamH\text{I} and hybridized with the external probe pictured in figure 5.2 (EP). The wildtype allele (WT) yields a 15 kb band, while the targeted allele yields a 7 kb band resulting from the introduction of a BamH\text{I} site from the targeting vector. Routine genotyping of the Runx2 Type II-LacZ mouse colony by PCR amplification of Runx2 exon 1 and LacZ (B). Wildtype mice (+/+) are positive only for exon 1, heterozygotes (+/-) are positive for both exon 1 and LacZ, and homozygous knock-in mice (-/-) are positive only for the LacZ gene.
Figure 5.4: Deletion of Runx2 Type II results in decreased body mass. Pups from 5 litters of Runx2 Type II<sup>+</sup>/<sup>LacZ</sup> X Runx2 Type II<sup>+</sup>/<sup>LacZ</sup> matings were weighed at one month of age. Runx2 Type II<sup>LacZ/LacZ</sup> (knockout) mice were consistently smaller than their wildtype or heterozygous littermates.
Replacement of Runx2 Exon 1 with LacZ results in Osteoblast-specific β-galactosidase Activity.

In order to evaluate the effects of various signaling molecules on Runx2 P1 promoter activity, we replaced the only exon unique to Runx2 Type II with the LacZ reporter gene (Figure 5.1). Whole mount staining of newborn tissues for β-galactosidase activity indicates that expression of the Runx2 P1 promoter is limited to mineralized skeletal elements, including bones formed by both intramembranous (Figure 5.5, Calvaria) and endochondral ossification (Figure 5.5, Ribs, Sternebrae). Examination of soft tissues and embryos prior to skeletogenesis revealed no additional sites of promoter activity (data not shown). Histological sections of longbones (Figure 5.6) reveal β-gal positive osteoblasts (OB) lining the bone surface beneath the periosteum (PO). The positioning of these osteoblasts along the bone surface indicates that these cells may be actively involved in bone formation. Interestingly, osteoblasts that have become embedded within the mineralized matrix of the cortical bone (CB) thereby becoming osteocytes (OCY) are negative for β-galactosidase activity.

Runx2 Type II Expression is Restricted to the Later Stages of Osteoblast Maturation.

To further define the time frame in which the Runx2 Type II gene is expressed, bone marrow stem cells (BMSC) were harvested from 6-week old Runx2 Type II -/LacZ mice and culture ex vivo. None of these cells were β-galactosidase positive after the initial 4-day period of adhesion. We next induced osteogenic differentiation in confluent cultures of BMSCs by the addition of ascorbic acid and inorganic phosphate to the cultures. No
Figure 5.5: Whole mount analysis of newborn Runx2 Type II +lacZ mice reveals restricted β-galactosidase activity in mineralized tissues. X-gal staining of organs and skeletal elements reveals that LacZ expression is limited to the mineralized skeleton, including bones formed by endochondral ossification (Ribs and Sternebrae) and bones formed by intramembranous ossification (Calvaria).
Figure 5.6: Histological analysis of longbones isolated from newborn Runx2 Type II +/LacZ demonstrating the specificity for Runx2 Type II expression in mature osteoblasts. Longbones stained with X-gal were sectioned and counterstained with Eosin to reveal mineralized cortical bone (CB). LacZ positive osteoblasts (OB) were seen localized to the cortical bone surface below the periosteum (PO). Osteocytes (OCY) embedded in the mineralized matrix are LacZ negative.
Figure 5.7: Ex vivo culture of primary bone marrow stem cells induced to undergo osteogenic differentiation demonstrates that Runx2 Type II expression is limited to the most mature osteoblasts in mineralizing nodules. X-gal staining of osteogenic BMSC cultures reveals clusters of LacZ positive osteoblasts (left panels) that are at the center of multilayered colonies which exhibit strong alkaline phosphatase activity (right panels).
β-galactosidase positive cells were observed until several days after the formation of multilayered osteoblast nodules. In these multilayered nodules, we observed Runx2 Type II gene activity only in the most mature cells residing in the center of the nodules (Figure 5.7, left panels). The presence of high levels of alkaline phosphatase concentrated in the center of the β-galactosidase positive osteoblasts supports in vivo observations indicating that Runx2 Type II gene activity is restricted to activated osteoblasts involved in mineral deposition (Figure 5.7, right panels).

DISCUSSION

Here we report on the generation of a mouse model in which the Type II, bone-related isoform of Runx2 is replaced by the LacZ coding sequence. This model has allowed us to assess the contribution of this isoform to the process of skeletogenesis and, more importantly, has created a reporter allele for readout of endogenous Runx2 P1 promoter activity. Our results demonstrate that this particular Runx2 isoform is dispensable for embryonic development and for skeletal formation. This result is not surprising since several studies have demonstrated that Runx2 Type I and Runx2 Type II are co-expressed in most skeletal progenitor cells during development (Sudhakar et al. 2001; Chen et al. 2002; Choi et al. 2002). Further, mice homozygous for the Runx2 Type II exon replacement develop to maturity, but exhibit reduced body length and mass in comparison to their wildtype littermates. The cause of this defect, and whether it is related to osteoblast differentiation and skeletal maintenance is under investigation.
Interestingly, our findings differ from those of Xiao et al. (Xiao et al. 2004) who report that deletion of Runx2 Type II results in a high rate of perinatal lethality (80% lethality by day 1). The mice generated by this group present with decreased body mass similar to that observed in our animals, and also exhibit minor defects in bone formation, particularly in trabecular bone. The skeletal defects observed by Xiao et al are not sufficient to explain the observed lethality raising the question as to what is the cause of death in their mouse model, which seemingly exhibit phenotypic normalcy otherwise. It is possible that the mild skeletal phenotype observed by Xiao et al. is due to compensation by the downstream Runx2 Type I isoform as Runx2 Type II knockout animals do exhibit an approximately 30% increase in Runx2 Type I mRNA levels. Whether our animals will exhibit similar defects in trabecular bone formation remains to be determined and necessitates micro-CT (3-dimensional X-ray microtomographical reconstruction) analysis.

Unlike the mouse model generated by Xiao et al., our mouse model harbors the \textit{LacZ} coding sequence thereby facilitating the analysis of endogenous Runx2 P1 promoter activity. We demonstrate here that the \textit{LacZ} allele becomes activated only in the most mature, active osteoblasts in the developing skeleton and in primary osteoblast cultures \textit{ex vivo}. This result is consistent with findings in the literature demonstrating that the transcript derived from the P1 promoter is restricted to mature osteoblasts (Park et al. 2001; Sudhakar et al. 2001; Choi et al. 2002). The generation of the Runx2 Type II-\textit{LacZ}
allele will be an invaluable tool for the identification and functional analysis of factors that modulate Runx2 gene expression and skeletogenesis.
Chapter 6:

Activity of the Mdm2 Oncogene Supports Runx2 Expression and Osteoblast Differentiation During Embryonic Skeletogenesis
ABSTRACT

The Mdm2 oncogene can promote cell proliferation through it's ability to negatively regulate the activity of the p53 tumor suppressor. Loss of Mdm2 results in p53 accumulation and ultimately results in exit from the cell cycle or apoptosis. Mdm2 null mice exhibit early embryonic lethality that can be rescued by deletion of p53. Mdm2/p53 null mice are phenotypically normal, indicating that the primary function of Mdm2 is to regulate p53. We have observed an increase in Mdm2 expression during the process of osteoblast differentiation that has led us to examine the role of Mdm2 during the process of skeletogenesis. To address this question, we have utilized a murine genetic system for tissue-specific Mdm2 ablation. In this system a conditional Mdm2 allele (Mdm2 flox) is excised during embryogenesis by Cre recombinase under control of the type I collagen promoter (Coll I-Cre). We find that deletion of Mdm2 in pre-osteoblasts upon activation of the Coll I-Cre transgene results in impaired skeletal formation. Bones of Mdm2 flox/flox Coll I-Cre (Mutant) embryos are shorter, exhibit increased porosity, and have less mineral content than their Mdm2 flox/+ Coll I-Cre (Wildtype) littermates. To characterize the molecular basis for the decreased bone quality observed in these animals, we isolated calvarial pre-osteoblasts and induced them to differentiate ex vivo. In these cultures, mutant pre-osteoblasts proliferate identically to pre-osteoblasts isolated from wildtype littermates and both cell types reach confluence concomitantly. In post-confluent cultures, however, the Coll I-Cre transgene becomes active in multilayering pre-osteoblasts resulting in Mdm2 excision and subsequently preventing the increase in Mdm2 expression observed in wildtype cultures. The loss of Mdm2 in these cells
prevented multilayered nodule formation \textit{ex vivo} due to inhibition of post-confluent cell division. Further, mutant cultures that have ablated Mdm2 activity fail to activate the master osteogenic transcriptional regulator Runx2, and therefore do not induce osteoblast associated phenotypic genes such as alkaline phosphatase and osteocalcin. Thus, Mdm2 is genetically upstream of the mechanism mediating the bone-specific induction of Runx2. In addition, Mdm2 performs a novel osteogenic function that supports progression of osteoblast differentiation and post-confluent nodule formation.
INTRODUCTION

The Mdm2 proto-oncogene was initially identified due to its amplification on mouse double minute chromosomes present in spontaneously transformed 3T3 cells (Cahilly-Snyder et al. 1987). Initial investigations by the Oren and Levine laboratories identified Mdm2 as a binding partner for p53, and subsequent work from these groups revealed that Mdm2 negatively regulates p53-induced transcription of the Mdm2 gene. During times of cellular insult, p53 activates Mdm2 gene expression by binding to a p53 response element within the first intron of the Mdm2 gene (Juven et al. 1993). Induction of Mdm2 protein levels lead to an increase in Mdm2-p53 complex formation that interferes with the ability of p53 to transactivate target genes, including Mdm2. Thus, Mdm2 not only regulates p53 activity, but by doing so also regulates its own gene expression. Mdm2 has been shown to inhibit p53 in several ways. First, Mdm2 can interfere with the ability of p53 to transactivate target genes by binding to and stearically hindering the N-terminal activation domain of the p53 protein (Juven-Gershon and Oren 1999). Secondly, Mdm2 can function as an E3 ligase to coordinate the monoubiquitinlylation of p53, which in turn acts as a signal for polyubiquitinylation by other factors such as p300/CBP (Grossman et al. 1998; Lai et al. 2001). Finally, Mdm2 shuttles polyubiquitinylated p53 from the nucleus into the cytoplasm where it is degraded by the 26S proteosome (Freedman and Levine 1998). The importance of Mdm2 in negatively regulating p53 activity is perhaps best illustrated by the finding that the early embryonic lethal phenotype of Mdm2-null mice can be fully rescued by the concomitant deletion of p53 (Jones et al. 1995).
Given the importance of p53 as the principal ‘guardian of the genome’ during times of DNA damage and cellular insult, it is not surprising that inactivating mutations in the p53 gene frequently result in tumor formation. The intimate relationship between Mdm2 and p53 therefore suggests that the activity Mdm2 may also be affected in cancers, and in fact recent findings have demonstrated that deregulated Mdm2 gene expression serves as an initiating event for tumor development. Gene amplification and overexpression of Mdm2 have been shown to transform cells, disrupt coupling of S-phase to mitosis, interfere with cell cycle arrest and apoptosis, as well as induce tumor formation in vivo (Leach et al. 1993; Lundgren et al. 1997; Jones et al. 1998; Bartel et al. 2002). These findings have made Mdm2 an attractive target for cancer therapeutics. Small-molecule inhibitors known as ‘Nutlins’ developed by Hoffman-La Roche (Basel, Switzerland) have been shown to bind with high-affinity to the hydrophobic cleft of Mdm2 (a site normally occupied by p53) and block Mdm2-p53 association to either induce a cell cycle arrest or initiate programmed cell death in tumor-derived cell lines (Vassilev et al. 2004). Similarly, Hybridon (Cambridge, MA) has generated Mdm2 antisense racemic mixtures that induce growth arrest or apoptosis in prostate cancer cell lines via p53-dependent and p53-independent mechanisms (Zhang et al. 2003). The drawback of these studies is that loss of Mdm2 function was not examined in primary cell lines or normal tissues in vivo. If Mdm2 loss is detrimental to normal tissue, is Mdm2 inhibition a feasible strategy for tumor suppression in vivo?
Although the importance of Mdm2 regulation of p53 during early stages of embryogenesis is clear from the apoptotic response that results from Mdm2 loss, little is known with regards to the requirement for maintaining the p53-Mdm2 autoregulatory loop in developing tissues later during gestation. Until now, knowledge of the consequences of Mdm2 loss or p53 hyperactivity have been elusive since attempts at generating p53 overexpressing transgenic mice have resulted in early embryonic lethality, as does ablation of the Mdm2 gene. Here we describe for the first time the effects of Mdm2 loss on normal tissue development during murine embryogenesis. We utilize a conditional Mdm2 allele that becomes inactivated in developing skeletal elements in response to the activity of a transgene in which Cre recombinase is under the transcriptional control of the type I collagen promoter (Coll-Cre).

Our results demonstrate that there are spatially and temporally specific responses to the loss of Mdm2. An early (E10) activation of the Coll-Cre transgene results in a significant apoptotic response in primitive neuro-progenitor and mesenchymal progenitor cells. Surprisingly, later activation of the Coll-Cre transgene (post-E14) in the skeleton does not result in an apoptotic response, but rather inhibits osteoblast differentiation causing an osteoporotic phenotype in vivo. This phenotype can be attributed to an increase in activated p53, which ultimately results in failure of osteogenic precursors to induce the transcriptional regulator, Runx2, whose activities are directly responsible for the activation of osteoblast phenotypic genes and the formation of mineralized tissue. Our results clearly demonstrate that Mdm2 plays a critical role in the growth and
differentiation of normal tissues during development, and that the consequences of Mdm2 deletion are cell-type dependent, reflecting a differential requirement for the Mdm2-p53 autoregulatory loop. Our findings suggest that the potential use of Mdm2 inhibitors as cancer therapeutics may be confounded by the requirement for Mdm2 activity in the homeostasis of normal tissues.

MATERIALS AND METHODS

Mouse Lines

Mdm2 conditional mice were developed in our laboratory using homologous recombination. The resulting allele contains two loxP sites flanking exons 11 and 12. Upon Cre-mediated recombination, exons 11 and 12 are excised, rendering the allele inactive (Figure 6.1-A, described in Steinman and Jones 2002). Mdm2 excision was mediated by crossing Mdm2 conditional (Mdm2^{flx/flx}) mice to transgenic mice in which the 3.6 kb type I collagen promoter governs the expression of the Cre recombinase enzyme (Coll-Cre) (a generous gift from Dr. Barbara Kream at the University of Connecticut) (Figure 6.1-A). Visualization of tissues in which the Cre recombinase activity has recombined target alleles was facilitated by mating Coll-Cre transgenic mice to R26R reporter mice (Jackson Laboratories, Bar Harbor, Maine) in which Cre expression results in the removal of a loxP-flanked DNA segment that prevents expression of a LacZ gene (Figure 6.1-A). All animals were appropriately cared for in accordance with the University of Massachusetts IACUC protocol.
**Whole Mount and Histological Analysis**

Embryos were harvested from timed pregnant mothers at various timepoints during gestation followed by fixation in 4% paraformaldehyde. Tissues destined for histological sectioning were dehydrated in a graded series of ethanol and xylene, followed by infiltration with paraffin wax. Tissues from R26R/Coll-Cre crosses were fixed in 4% paraformaldehyde, equilibrated overnight in 30% sucrose, and embedded in OCT for cryosectioning. Some embryos were fixed in 4% paraformaldehyde followed by whole mount staining for β-galactosidase activity. Paraffin sections were cut at 7 μm and counterstained with eosin. Frozen sections were cut at 10-12 μm, stained for β-galactosidase activity, and counterstained with eosin. β-galactosidase activity was visualized by staining whole embryos, cryosections, or calvarial cultures in a solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂, 0.2% NP-40, 0.01% sodium deoxycholate, and 1mg/mL X-Gal in PBS, pH 7.4 at 37°C for 1-6 hours. Calvarial cultures and paraffin sections were analyzed for mineral content using the method of Von Kossa. Briefly, sections were exposed to a solution of 3% silver nitrite under direct sunlight for 15 minutes after which mineral deposits were visualized as a black precipitate under bright field microscopy. Alkaline phosphatase activity in calvarial cultures was visualized by colorimetric enzymatic reaction to a solution containing 0.5 mg/mL naphthol as MX phosphate disodium salt (Sigma), 2.8% N,N dimethylformamide (Sigma), 0.1 M Tris-maleate buffer (pH 8.4), and 1mg/mL Fast Red salt (Sigma). Reaction was carried out at 37°C for 10 minutes. TUNEL staining was
performed on dewaxed paraffin sections using a fluorescein-conjugated in situ cell death detection kit (Roche) according to the manufacturer's protocol.

Skeletal Preparations

Skeletals were prepared for visualization of cartilage and bone using alcian blue and alizarin red stains, respectively. Embryos were eviscerated followed by overnight fixation in 100% ethanol. The next day, cartilaginous elements were stained overnight in a solution containing 4 parts ethanol, 1 part glacial acetic acid, and 0.3 mg/mL alcian blue 8GX (Sigma). The next day, soft tissues were dissolved for 6 hours in a 2% KOH solution followed by an overnight staining in a 1% KOH solution containing 75 µg/mL alizarin red S (Sigma). Skeletals were then destained in 20% glycerol, 1% KOH for several days and stored in 50% glycerol 50% ethanol.

Calvarial Osteoblast Preparations

Calvarial osteoblasts were isolated from E19 embryos by enzymatic digestion of calvarial bones. Briefly, calvaria were minced and subjected to three sequential digestions (8, 10, and 26 min) with collagenase P (Roche Molecular Biochemicals, Indiana, IN) at 37°C. Osteoblasts in the second and third digest were collected and resuspended in α-MEM supplemented with 10% FBS (Hyclone). Cells were plated at a density of 1 x 10⁶ cells/six-well plate (Owen et al. 1990). Differentiation was initiated post-confluence by the addition of ascorbic acid and β-glycerol phosphate. Cultures were harvested at
various timepoints and stained for β-galactosidase activity, mineral content, alkaline phosphatase activity, or total cellularity using toluidine blue.

**RNA Isolation and Analysis**

RNA was isolated from tissue or cell cultures using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturers protocol. After purification, 5 μg of total RNA was DNAse treated using a DNA-free RNA column purification kit (Zymo Research, Orange, CA). One μg of RNA was then reverse transcribed using Oligo-dT primers and the SuperScript 1st Strand Synthesis kit (Invitrogen) according to the manufacturers protocol. Gene expression was assessed by semi-quantitative (Cre, Mdm2, GAPDH, 25 cycles) and quantitative real-time PCR (Runx2, AP, OC, collagen type I, GAPDH). Quantitative PCR was performed using SYBR green 2x master mix (Eurogentec, Belgium) and a 2 step cycling protocol (anneal and elongate at 60°C, denature at 94°C). Specificity of primers was verified by the dissociation temperature of amplicons. Primers used for PCR reactions are listed in Table 1. Results are representative of two or more independent experiments.

**Protein Analysis**

Total protein was isolated from calvarial cultures in the presence of direct lysis buffer (2% SDS, 10 mM DTT, 10% glycerol, 12% urea, 10 mM Tris/HCl (pH 7.5), 1 mM PMSF, 1X Protease inhibitor cocktail (Roche), 25 μM MG132 proteosome inhibitor) followed by heating for 5 minutes at 100°C. 40 μg total protein was then electrophoresed
through a 10% acrylamide gel followed by transfer onto a PVDF (Immobilon) membrane. Membranes were blocked in PBST containing 2% non-fat dry milk (Biorad) prior to incubation with antibodies. Antibodies were incubated with membranes in the presence of PBST containing 2% non-fat dry milk 1 hour at room temperature. Excess primary antibody was removed with three 10-minute washes of PBST. Secondary antibodies were incubated with membranes for 30min-1hour at room temperature followed by three 10-minute washes with PBST to remove excess antibody. Proteins were visualized on the membrane by exposure to Western Lightning (Perkin Elmer, Wellesley, MA) chemiluminescent reagent. The Runx2 antibody was a generous gift from Drs. Yoshi Ito and Kosei Ito at the National University of Singapore. All antibodies were used at a concentration of 50 ng/mL.

**RESULTS**

*Activity of the Collagen Type I Promoter during Embryogenesis*

To understand the contribution of the *Mdm2* proto-oncogene to the development of connective tissues, we have utilized a conditional *Mdm2* allele in which the majority of the coding region (exons 11 and 12) is flanked by loxP recombination sites (Figure 6.1-A). Deletion of these exons renders the allele inactive in tissues expressing a transgene in which the 3.6 kb type I collagen promoter controls expression of Cre recombinase. We initially verified the expression of Cre in skeletal elements of adult mice to insure that excision will occur (Figure 6.1-B). To identify regions in the developing embryo that
Figure 6.1: Mouse models for tissue-specific deletion of Mdm2 in the developing skeleton. A transgene in which the type I collagen promoter drives expression of Cre recombinase (A, top construct) was used to excise a stop codon in R26R reporter mice (A, second construct) as well as to excise exons 11 and 12 of Mdm2 (A, third construct) yielding an Mdm2 null allele (A, fourth construct). Cre expression in the Coll-Cre transgenic mouse was verified by RT-PCR analysis of RNA isolated from adult tissue (B).
have undergone Cre-mediated excision, we bred Coll-Cre transgenic mice to R26R reporter mice that activate β-galactosidase in response to Cre recombination. Our findings demonstrate that this promoter becomes activated at two distinct points during development. The initial activation of the Coll-Cre transgene occurs early in development (E9) and is localized to the caudal portion of the embryo (Figure 6.2). This region of the developing embryo contains both neuroprogenitor and mesenchymal progenitor cells that are destined to give rise to the caudal axial skeleton and spinal cord. Later in development (around E 14), the Coll-Cre transgene undergoes a robust activation in relation to the formation of connective tissue. This activation results in excision in both the skin and developing skeletal elements (Figure 6.2). These findings indicate that the observed biphasic transgene activation will result in Mdm2 deletion in several tissues upon crossing the transgene onto Mdm2 conditional mice.

**Early Transgene Activation Results in Loss of Caudal Tissue due to Apoptosis**

Having established the pattern of excision caused by expression of the Coll-Cre transgene, we crossed Mdm2 conditional (Mdm2^flx/flx) mice to Coll-Cre transgenic mice containing one conditional Mdm2 allele (Mdm2^flx/+Coll-Cre). The resulting litters contained Mdm2^flx/flxColl-Cre mice that are designated as mutant (MT) as well as Mdm2^flx/+Coll-Cre mice that exhibit no defects and are referred to as wildtype (WT).
Based upon the observation that Cre-mediated excision occurred early in development, we examined the effects of this excision in embryos between E10.5 and E19.5. Loss of Mdm2 in this developing caudal region resulted in a complete absence of tissue surrounding the somites. Embryos exhibited caudal runting, exposure of somitic mesenchyme at the surface of the embryo, absence of a tail, and a severe invagination in the posterior dorsal region encompassing the lumbar vertebrae (Figure 6.3). Skeletal preparations of E19 embryos revealed highly dysplastic axial skeletal elements in the mutant embryo with fused cartilaginous lumbar vertebrae that have failed to undergo turnover into mineralized bone (Figure 6.4).

To define the events resulting in malformation of the caudal axial skeleton, E10 embryos were sectioned and tissue morphology was examined. At E10, wildtype embryos had clearly segmented somites that would give rise to axial skeletal components including vertebrae and ribs (Figure 6.5, arrows). The somites are surrounded by primitive neural tissue and developing dorsal root ganglia (Figure 6.5, arrowheads). In contrast, mutant embryos lack developing neural tissue and posterior dorsal root ganglia, leaving somites externalized. To understand the mechanism underlying this tissue loss, we performed TUNEL staining on serial sections. Our results show that there is a dramatic increase in TUNEL-positive apoptotic cells in the caudal somites and surrounding tissue of mutant embryos in comparison to wildtype littermates. These observations are consistent with well established findings demonstrating that Mdm2 loss induces p53-mediated apoptosis early in development of the mammalian embryo (Jones et al. 1995).
Figure 6.2: Lineage tracing of cells expressing the Coll-Cre transgene during embryogenesis. Cre recombinase activity irreversibly excises a stop codon in R26R reporter mice thereby activating constitutive LacZ expression from the Rosa locus. Cre activity initiates in the caudal region of the embryo by E10 (A). No additional Cre-mediated excision occurs until between E14.5 and E15.5 when the type I collagen promoter becomes activated in relation to the formation of connective tissues (A&B). Histological sections of embryos at E12.5 and E14.5 stained with X-gal and counterstained with eosin reveal the dramatic induction of Cre recombinase activity in developing connective tissues (B).
Figure 6.3: Cre-mediated excision of Mdm2 in the caudal region of the embryo prior to E10 results in loss of tissue. The defect is visible by E10.5 (left panels), and ultimately results in failed tail formation and a dermal invagination at the site of tissue loss (right panels).
Figure 6.4: Activation of the Coll-Cre transgene in the caudal region of Mdm2 flox/flox mice results in malformation of the caudal elements of the axial skeleton, including the absence of tail vertebrae. Coll-Cre Mdm2 flox/flox (MT) mice fail to form mineralized vertebral bodies in comparison to Coll-Cre Mdm2+/+ (WT) mice (arrows). The cartilaginous vertebrae in mutant mice appear to have fused (arrows) and lack an ossification center. Cartilage is stained blue with alcian blue and bone red with alizarin red.
Figure 6.5: Tissue loss resulting from Mdm2 deletion is due to apoptosis. Saggital sections (top and center panels) of WT E10.5 embryos showing segmented somites (arrows) and dorsal root ganglia (arrowheads). MT embryos lack developing neural tissue (arrowheads) leaving somites externalized (arrow). TUNEL staining of above sections reveals a marked increase in the number of apoptotic cells in MT embryos (lower panels).
Excision of Mdm2 in Skeletal Tissues Results in Impaired Bone Formation

Despite cellular loss in the caudal portion of the embryo in response to an early activation of the Coll-Cre transgene, the appendicular skeleton and bones of the skull were unaffected by the initial Cre activation. Activation of the Coll-Cre transgene in the limbs and skull did not occur until the latter half of gestation (beginning at E14.5). These skeletal elements were more porous in mutant animals (Figure 6.6-A) and all appendicular bones examined were shorter in comparison to wildtype littermates (Figure 6.7-A&B). Further, appendicular bones from mutant animals showed significantly less mineral deposition than wildtype littermates, as assessed by silver nitrate staining (Figure 6.8). These findings indicate that Mdm2 loss in developing skeletal tissues negatively affects multiple parameters of bone quality. Unlike the consequences of Mdm2 deletion in undifferentiated progenitor cells after Coll-Cre induction, skeletal elements from mutant embryos exhibited no increase in apoptotic cell number (Figure 6.9). These observations led us to examine the growth and differentiation of osteoprogenitor cells ex vivo.

Activation of the Mdm2 Gene is Required for Efficient Osteoblast Differentiation Ex Vivo

To understand the underlying cause of the decrease in bone quality in Mdm2^{flx/flx}/Coll-Cre mice, we isolated calvarial osteoprogenitor cells from E19 WT and MT embryos as well as from R26R/Coll-Cre embryos and visualized the pattern of Cre-mediated excision in these cultures. Calvarial osteoprogenitors were induced to undergo osteogenic
Figure 6.6: Coll-Cre Mdm2

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mice exhibit decreased bone density and long bone length when compared to Coll-Cre Mdm2+/+ littermates. Skulls of E19 embryos stained with alizarin red for mineralized bone and alcian Blue for cartilage (A) reveals extremely porous calvarial bones in mutant mice. Longbones of MT mice also exhibit increased porosity and a decrease in overall bone length (B)
Figure 6.7: Bone-related loss of Mdm2 results in decreased mineralized and total bone length. Longbones were isolated from Coll-Cre Mdm2 $^{lox/lox}$ and Coll-Cre Mdm2$^{+/+}$ E19 embryos and were stained with alizarin red and alcian Blue. Bone lengths for six embryos of each genotype were taken by measuring length between chondyles (A) or by measuring the mineralized portion only (B).
Figure 6.8: Mdm2 loss in the developing skeleton compromises mineral deposition. silver nitrate staining for mineral on sections of E19 longbones revealing higher mineral content in Coll-Cre Mdm2+/+ (WT) mice in comparison to Coll-Cre Mdm2 flox/flox (MT) littermates.
Figure 6.9: Mdm2 loss in the developing skeleton does not induce apoptosis. Sections of developing ribs (top panels, H&E stained corresponding to arrowheads on lower panels) from E19 embryos followed by TUNEL staining for apoptotic cells (lower panels, arrows) reveals no increase in apoptosis in skeletal tissues lacking Mdm2 (right panels) compared to control samples (left panels). β-galactosidase activity (inset) in Coll-Cre R26R mice marks cells in which recombination has occurred.
differentiation ex vivo by allowing cultures to proliferate for several days followed by the addition of ascorbic acid and inorganic phosphate to the media post-confluence. The addition of ascorbic acid to the cultures osteoprogenitors stimulates post-confluent proliferation resulting in the formation of multilayered nodules that later become mineralized. Interestingly, Mdm2 was expressed at very low levels in wildtype cultures during the period of proliferation and became strongly activated in post-confluent cultures during multilayering and extracellular matrix maturation until mineralization (Figure 6.10-A). Mutant cultures failed to induce Mdm2 gene expression at any stage of culture (Figure 6.10-A), and examination R26R/Coll-Cre cultures indicated that Cre-mediated recombination of Mdm2 occurred exclusively in maturing osteogenic nodules (Figure 6.10-B). These results indicate that the increase of Mdm2 gene expression occurred in differentiating osteoblasts within multilayering nodules.

Analysis of both wildtype and mutant-derived osteoprogenitor cultures indicates that these cells achieved confluence simultaneously (Figure 6.11, left panels), and BrdU incorporation indicated that there were no proliferation defects in mutant cultures prior to confluence (data not shown). In addition, X-gal staining of R26R/Coll-Cre cultures indicated that few osteoprogenitor cells had activated the transgene prior to isolation from the embryo (Figure 6.10-B, left panel). After osteoprogenitor cells reached confluence, wildtype cultures underwent robust nodule formation, while mutant cultures were unable to form a significant number of multilayered nodules, and subsequently only a small fraction of these cells were able to undergo differentiation and activate alkaline
Figure 6.10: Mdm2 induction during osteoblast differentiation occurs in mature cells and is inhibited in cultures derived from Coll-Cre Mdm2 flox/flox (MT) mice. RT-PCR analysis of Mdm2 messenger RNA levels throughout ex vivo osteoblast differentiation showing an increase in Mdm2 levels concomitant to an increase in osteogenic differentiation in Coll-Cre Mdm2+/+ (WT) cells (A). Cultures derived from Coll-Cre Mdm2 flox/flox (MT) do not exhibit an increase in Mdm2 mRNA. Analysis of β-galactosidase activity in Coll-Cre R26R mice reveals that Cre activity is inducing recombination primarily in multilayering nodules of mature osteoblasts (B).
Figure 6.11: Loss of Mdm2 during osteoblast maturation results in failure to form osteogenic nodules ex vivo. Cultures of Coll-Cre Mdm2+/+ (WT) osteoprogenitors undergo post-confluent proliferation to form alkaline phosphatase secreting osteoblast nodules that ultimately become mineralized (silver nitrate). In contrast, Mdm2 $^{flox/flox}$ (MT) cultures reach confluence, but fail to multilayer and form osteogenic nodules, and subsequently are unable to secrete alkaline phosphatase or a mineralized extracellular matrix.
phosphatase activity (Figure 6.11, Center panels). Consistent with our in vivo findings, the wildtype osteoblasts ultimately formed heavily mineralized nodules while mutant cultures failed to deposit significant mineral in the extracellular matrix (Figure 6.11, right panels).

Quantitative analysis of osteogenic gene expression revealed that both wildtype and mutant cultures activate early osteogenic genes type I collagen and alkaline phosphatase, but gene activity is abrogated in mutant cultures shortly after achieving confluence (Figure 6.12). Osteocalcin, a marker of late osteoblast differentiation, is not activated in mutant cultures at any time during the culture period (Figure 6.12). Since osteoblast differentiation and expression of these osteoblast phenotypic genes is entirely dependent on the activity of the Runt-related transcription factor Runx2 (Ducy et al. 1997; Komori et al. 1997; Otto et al. 1997), we next examined the impact of Mdm2 loss on the activity of this gene. Interestingly, both Runx2 protein and mRNA levels are lost in cultures concomitantly with the loss of Mdm2 (Figure 6.13 A&B). Taken together, our results indicate that Mdm2 activity is required for post-confluent proliferation of osteoblast cultures, and that this post-confluent cell division is required for the subsequent activation of the master osteoblast transcriptional regulator Runx2. Failure of cultures lacking Mdm2 to activate the Runx2 gene ultimately results in inhibition of osteoblast differentiation and inactivity of osteoblast phenotypic genes.
Figure 6.12: Osteoblast phenotypic gene expression is abrogated after Mdm2 loss in ex vivo cultures of osteoprogenitor cells. ColI-Cre Mdm2+/+ (WT) osteoblasts initially activate alkaline phosphatase and Type I collagen gene expression at confluence, followed by a dramatic induction of osteocalcin gene expression prior to mineralization. Mdm2^floxflox (MT) cultures induce alkaline phosphatase and Type I collagen gene expression at confluence, but are ultimately unable to support the activity of these genes or osteocalcin after Mdm2 loss.
Figure 6.13: Mdm2 activity is required for activation of the osteogenic master transcriptional regulator, Runx2. Quantitative RT-PCR analysis of Runx2 expression demonstrates that after Mdm2 loss in osteogenic nodules in Mdm2 ^{lox/lox} (MT) cultures, these cells cannot support the increase in Runx2 gene activity observed in Coll-Cre Mdm2^{+/+} (WT) (A). The lack of Runx2 gene activity results in dramatic loss of Runx2 protein in Mt cultures (B).
Inhibition of p53 Activity by Mdm2 is Required for Osteogenic Differentiation

Given the prevailing role of Mdm2 as a negative regulator of p53, we hypothesized that the induction of Mdm2 during formation reflected a requirement for a decrease in p53 activity to facilitate post-confluent cell division. Interestingly, examination of total p53 levels revealed no change in total p53 protein in mutant cultures. (Figure 6.14, upper panel). Upon closer examination of activated p53 using a phospho-Ser15 specific antibody, a marked induction in P-Ser15 p53 was observed in mutant cultures when compared to Mdm2-retaining cultures (Figure 6.14, lower panel). This increase in phosphorylated p53 corresponded to a more rapid and stronger induction of p21 gene activity during osteoblast differentiation, but this was not associated with an increase in apoptosis (Figure 6.15-A&B). These findings support the hypothesis that Mdm2 activity in osteoblasts regulates p53 activity, and suggest that this regulation occurs at the level of post-translational modification of p53.

To determine definitively whether the effects of Mdm2-loss on osteoblast differentiation are due exclusively to an excess of p53 activity, we examined differentiation of osteoprogenitor cells harvested from calvaria of p53−/−Mdm2−/− mice.

Mice lacking both p53 and Mdm2 were phenotypically normal at the time of calvarial osteoprogenitor cell harvest (E19) (Figure 6.16). These embryos did not exhibit the shortening of limbs and increased porosity observed in Mdm2flx/flxColl-Cre animals, suggesting that unregulated p53 is in fact the cause of the defective bone formation seen
Figure 6.14: Mdm2 loss during osteoblast differentiation results in an increase in p53 activation. Analysis of p53 protein levels in ex vivo osteoblast cultures from Coll-Cre Mdm2+/+ (WT) or Mdm2 flox/flox (MT) cultures showing no difference in total p53 levels (A). Activated p53 (phospho-serine 15) is increased in the absence of Mdm2 (B).
Figure 6.15: Mdm2 loss results in increased p21 gene activity not apoptosis in calvarial osteoblast cultures lacking Mdm2. TUNEL staining of post-confluent osteogenic cultures reveals no significant apoptotic events in either Wt or Mt cells (A). Activity of the cell cycle inhibitor p21 was analyzed by q-RT-PCR analysis in primary osteoblast cultures (B). Coll-Cre Mdm2+/+ (Wt) cells exhibit a gradual increase in p21 messenger RNA levels reaching maximal expression in mineralized cultures. Coll-Cre Mdm2 flox/flox (Mt) cultures dramatically increase p21 activity levels two-fold over Wt cultures between confluence and extracellular matrix formation.
Figure 6.16: Concomitant deletion of both p53 and Mdm2 rescues dysplasia of the axial skeleton and the retardation in longbone growth observed in Coll-Cre Mdm2\textsuperscript{floxed/floxed} embryos. Skeletons from E19 mice were stained with alizarin red and alcian blue. Axial skeletons (A) and longbones (B) were indistinguishable between p53 null and p53/Mdm2 null animals.
in Mdm2\textsuperscript{flx/flx}Coll-Cre skeletons. The loss of p53 in the absence of Mdm2 also rescues the dysplasia of the axial skeleton observed in Coll-Cre Mdm2\textsuperscript{flx/flx} embryos, demonstrating that the apoptotic event underlying that defect was due to de-regulation of p53.

We next isolated calvarial osteoblasts from both p53\textsuperscript{-/-} and p53\textsuperscript{-/-} Mdm2\textsuperscript{+/+} mice and induced these cells to differentiate \textit{ex vivo}. These cultures achieved confluence simultaneously when plated at equal density and underwent robust post-confluent proliferation (Figure 6.17, toluidine blue) resulting in dramatic extracellular matrix formation (Figure 6.17, alkaline phosphatase) and mineralization (Figure 6.17, silver nitrate). Further, these cultures had restored Runx2 activity and subsequently exhibited normal expression of osteoblast phenotypic genes (Figure 6.18 and 6.19). Surprisingly, cultures lacking p53 or both p53 and Mdm2 were still capable of strong p21 induction, indicating that in this system, p21 expression is not dependent on p53. Mdm2 was also strongly induced during p53 null osteoblast differentiation, again indicating that p53-independent mechanisms induce Mdm2 gene expression in the osteoblast. Taken together these results further suggest that the earlier induction of p21 observed in the absence of Mdm2 is not responsible for the failure of these cultures to undergo post-confluent cell division. Our findings demonstrate that loss of Mdm2 during osteoblast differentiation results in an inappropriate activation of p53 ultimately leading to a failure to undergo post-confluent proliferation prior to differentiation. The regulation of p53 by Mdm2 is therefore critical for proper skeletal development and disruption of the p53-
Mdm2 regulatory loop ultimately results in failure to activate the master osteogenic transcriptional regulator, Runx2, whose activities are required for osteoblast differentiation and bone formation.
Figure 6.17: Primary osteoblasts lacking either p53 or both p53 and Mdm2 undergo robust proliferation in post-confluent culture. Calvarial osteoblasts were isolated from p53\(^{+/−}\) or p53\(^{+/−}\) Mdm2\(^{+/−}\) mice and plated at even densities. Cultures achieved confluence simultaneously (toluidine Blue) and were induced to differentiate (alkaline phosphatase). Both p53\(^{+/−}\) and p53\(^{+/−}\) Mdm2\(^{+/−}\) cultures formed large amounts of mineralized tissue ex vivo (silver nitrate).
Figure 6.18: Primary osteoblasts lacking either p53 or both p53 and Mdm2 exhibit normal bone-specific gene expression patterns. QRT-PCR analysis of osteogenic cultures showing dramatic induction of bone-specific genes in the absence of p53.
Figure 6.19: Primary osteoblasts lacking either p53 or both p53 and Mdm2 support Runx2 and p21 gene activity. Messenger RNA levels in these cultures reveal normal Runx2 expression in the absence of p53. The p21 gene is induced despite the absence of p53 in primary osteoblast cultures.
DISCUSSION

While maintenance of the Mdm2-p53 autoregulatory loop has been shown to be critical for implantation of developing mouse embryo (Jones et al. 1995), the importance of Mdm2 in controlling p53 during normal tissue development is not known. We demonstrate here for the first time that deletion of Mdm2 has tissue-specific consequences that are ultimately a result of deregulated p53 activity. We have achieved biphasic deletion of a Mdm2 conditional allele (Steinman and Jones 2002) during embryogenesis by activation of a Cre recombinase-containing transgene controlled by the activity of the type I collagen promoter (Coll-Cre). This promoter is activated early in both mesenchymal and neural progenitor cells in a localized region of the developing embryo. Activity of this transgene in mice homozygous for an Mdm2 conditional allele resulted in widespread apoptosis. This finding is consistent with prior studies demonstrating that Mdm2 loss in the pre-implantation mouse embryo results in lethality due to apoptosis (Jones et al. 1995; de Rozieres et al. 2000; Chavez-Reyes et al. 2003) and suggests that such undifferentiated embryonic cells preferentially undergo apoptosis rather than induce cell-cycle arrest in response to p53 hyperactivity.

Lineage tracing to mark cells that have undergone Cre recombination revealed a second, more widespread activation of the Coll-Cre transgene later during development (E13.5-E14.5) in relation to the differentiation of connective and skeletal tissues. Strong transgene activity was observed in osteoblasts and bones where Mdm2 loss led to impaired bone formation. Bones of Mdm2\textsuperscript{flx/flx} Coll-Cre mice were shorter, more
porous, and exhibited decreased mineral content in comparison to wildtype animals due to inefficient activation of osteoblast specific genes responsible for secretion and mineralization of extracellular matrix. Coll-Cre Mdm2 flox/flox primary calvarial pre-osteoblasts inactivate Mdm2 in response to activation of type I collagen in post-confluent cultures resulting in an increase of phosphorylated p53 protein levels and p21 gene expression. After post-confluent inactivation of Mdm2, these cultures fail to undergo the proliferation required to multilayer and form osteogenic nodules. Consequently, Runx2 and the array of osteogenic genes regulated by Runx2 fail to become induced thereby inhibiting osteoblast differentiation.

We initially hypothesized that the inhibition of post-confluent proliferation was due to increased p53 activity and subsequent induction of p21 resulting in cell-cycle arrest. To address this hypothesis, we examined osteogenic differentiation in the absence of both Mdm2 and p53. The concomitant loss of both of these proteins rescues the defects observed in Mdm2 flox/flox Coll-Cre mice, demonstrating that p53 activity is indeed the underlying cause of the observed defect. Interestingly however, p21 gene expression is still dramatically upregulated during osteogenic differentiation in both p53 null and p53/Mdm2 double null osteoblasts, indicating that p21 is not responsible for the inhibition of post-confluent proliferation and subsequent skeletal abnormalities observed in Mdm2 flox/flox Coll-Cre mice. Further, this result demonstrates that p21 induction is not p53-dependent during osteogenesis, consistent with the findings of Funato et al. (Funato et al. 2001) who demonstrate that the observed p21 induction during osteoblast
differentiation is mediated by E2A and co-factor CBP and that this induction is p53-independent.

Taken together, our findings indicate that maintenance of the Mdm2-p53 autoregulatory loop is required for skeletal development and suggest that the induction of Mdm2 during osteogenic differentiation acts to suppress p53 activity. Furthermore our results demonstrate that in the absence of Mdm2, increased p53 activity prevents pre-osteoblasts from undergoing proliferation prior to osteogenic differentiation. Our findings suggest that this period of proliferation is a pre-requisite for osteogenesis, as cultures that lack Mdm2 and fail to enter this proliferative period are unable to activate the Runx2 gene, and therefore are unable to undergo osteogenic differentiation. The major novel finding emerging from these studies is that there is a requirement for an intact p53-Mdm2 autoregulatory loop during normal tissue formation in the developing mouse embryo. Further, our findings suggest that Runx2 may act as a link between proliferation and differentiation of an osteoblast (Pratap et al. 2003), as Runx2 gene activity is inextricably linked to the fidelity of the p53-Mdm2 autoregulatory loop.

The results presented in this study indicate that the use of Mdm2 inhibitors as a treatment to induce growth inhibition in malignant tumors may have detrimental consequences on the formation and maintenance of skeletal tissue and the effects of such compounds should be carefully assessed prior to administration to human subjects.
Chapter 7:

General Discussion
The Runx family of transcriptional regulators is well known for the tissue-specific roles Runx proteins play in cell fate determination and progression of differentiation during mammalian embryogenesis. Evolutionary pressure demanded that Runt homology proteins develop distinct functions in divergent eukaryotic species. In mammals, the three Runx genes exhibit sequence homology and some functional redundancy but their expression is highly tissue-specific in order to accommodate cell-type specific functions. These three mammalian Runx genes evolved from a single gene that initially occurred in bilaterians where it is involved in body patterning and cell fate determination. In chordates, the Runx genes evolved a second promoter and then underwent gene duplication (Rennert et al. 2003). While evolutionary pressures demanded that the mammalian Runx factors have distinct, tissue-specific functions, these genes have conserved protein motifs and regulatory elements in their promoter regions, suggesting that a common function for Runx factors may persist in vertebrates. Studies from many groups examining Runx expression have identified several tissues that support the expression of multiple Runx genes, including early embryonic, undifferentiated mesenchymal progenitor cells (Lengner et al. 2002; Stricker et al. 2002; Taniuchi et al. 2002; Yamashiro et al. 2002). These observations support a hypothesis that the Runx proteins may play redundant roles early during development.

At its onset, the research summarized in this dissertation was aimed primarily at understanding the regulation of the Runx2 gene in osteoblast lineage cells, however it rapidly became clear that Runx2 regulation was distinct from that of typical osteoblast-
specific genes. We initially generated transgenic animals in which the Runx2 P1 promoter controls expression of the LacZ reporter gene. Surprisingly, our findings demonstrated that this promoter region is inactive in osteoblast lineage cells known to express Runx2 and is unresponsive to the osteogenic BMP2 signal, suggesting that the osteoblast-related locus control region of the Runx2 gene lies outside of the proximal 3 kb used in these studies. Interestingly, activity of this promoter was strongly induced as early as E8.5 during gestation in progenitor cells surrounding the notochord, consistent with previous observations of endogenous Runx2 gene activity in the notochord prior to skeletogenesis (Otto et al. 1997). This finding suggested that Runx2 plays a role in undifferentiated progenitor cells that are not destined to undergo osteogenesis and that the Runx2 gene is responsive to signaling pathways active early during the development of the mammalian embryo. Interestingly, Runx2 transgene expression in these mice displayed a pattern reminiscent of the segmented expression seen for runt transcripts in drosophila embryos (Gergen and Wieschaus 1985; Gergen and Wieschaus 1986).

These initial observations led to the investigation of pathways upstream of the Runx2 gene in mesenchymal progenitor cells and further, led us to examine the function of Runx2 in these early embryonic tissues. Based on the segmented expression of the Runx2 P1 promoter in developing somites at E9, several candidate factors were examined for their effect on Runx2 gene activity. Ultimately, the homeodomain factor Nkx3.2 was identified as a repressor of the Runx2 P1 promoter that interacts with a consensus Nkx3.2 binding sequence in the minimal promoter region. Chromatin immunoprecipitation
analysis further demonstrated that Nkx3.2 binds the Runx2 promoter in C3H10T1/2 mesenchymal progenitor cells in order to suppress Runx2 expression. During chondrogenic differentiation of these C3H10T1/2 cells we observed a sharp decrease in Runx2 expression that coincided with an equally intense activation of the Nkx3.2 gene, suggesting that repression of Runx2 played a role in the chondrogenic commitment of a progenitor cell. Overexpression studies demonstrated that Runx2 could in fact inhibit activation of the chondrocyte-specific program of gene expression if it is not adequately repressed. Interestingly, these cells also express Runx1 and, to some extent Runx3, but these genes exhibit dissimilar expression patterns during chondrogenesis and their promoters do not contain the Nkx3.2 consensus sequence found in the Runx2 promoter.

To examine a potential functional cooperation between Runx1 and Runx2 in the mesenchymal progenitor cell, we developed a culture system in which chondrogenic differentiation of mouse embryonic fibroblasts is promoted through stimulation with BMP2 in high-density micromass cultures. Like C3H10T1/2 cells, MEFs differentiate along the chondrogenic lineage, expressing stage-specific chondrocytic genes and undergoing hypertrophy to form a mineralized extracellular matrix. MEFs and C3H10T1/2 demonstrate very similar patterns of Runx1 and Runx2 expression. Runx2 is suppressed at the onset of chondrogenesis only to become strongly induced in late-stage chondrocytes where it plays a well-characterized role that it shares with Runx3 in promoting hypertrophy and mineralizing the extracellular matrix (Yoshida et al. 2004). Conversely, Runx1 levels were strongly induced at the onset of chondrogenic
differentiation, primarily in response to the three-dimensional environment of the micromass cultures (Figure 4.5A, T=0) and then declined as cultures entered final stages of chondrocyte hypertrophy. The distinct patterns of Runx1 and Runx2 expression during chondrogenesis suggest that these two factors may play non-redundant functions during chondrogenic differentiation. We therefore utilized the MEF cell culture model to analyze chondrogenic differentiation in the absence of Runx1 or Runx2. Consistent with our previous findings, Runx2 null MEFs exhibited increased cartilage formation ex vivo. Runx1 null MEFs, while still able to complete the program of chondrogenic differentiation, formed smaller cartilaginous nodules ex vivo. This result suggests that the increase in Runx1 levels at the onset of chondrogenesis supports some proliferative expansion prior to differentiation. Together, these findings indicate that Runx1 and Runx2 play distinct roles in chondrogenic differentiation. We note that coexpression of Runx1, 2, and 3 in undifferentiated cells in the early embryo may still be masking common functions of the Runx factors. The elucidation of these functions may ultimately demand deletion of all three factors.

In addition to the role of Runx genes in fate determination, these factors have also been closely linked to cell growth control. Translocations of the Runx1 gene result in hyperproliferation and a block in the differentiation of myeloid progenitor cells (Yergeau et al. 1997; Zent et al. 1997), Deletion of Runx3 results in gastric cancer in mice and men (Yergeau et al. 1997; Zent et al. 1997), pro-viral insertions that activate the Runx2 gene cooperate with myc in virus-accelerated lymphomas (Stewart et al. 1997) and Runx2 has
been shown to inhibit proliferation in osteoprogenitor cells, a novel role distinct from its function in activation of osteoblast phenotypic genes (Pratap et al. 2003). Based on these findings, we chose to examine the interrelationship between Runx2, bone formation, and the Mdm2-p53 autoregulatory loop. It is thought the p53 tumor suppressor undergoes continual degradation through the actions of Mdm2 during normal cell proliferation. Upon cellular insult, such as DNA damage, Mdm2 degradation of p53 is terminated, allowing for p53 accumulation and activation (Alarcon-Vargas and Ronai 2002; Vousden and Lu 2002; Chene 2003). Active p53 can then induce cell cycle arrest or apoptosis; how p53 makes this decision is not understood. While p53 inactivation/Mdm2 hyperactivation commonly result in tumorigenesis, the consequences of p53 hyperactivation/Mdm2 inactivation are not known as mice lacking Mdm2 die around E5 due to p53-dependent apoptosis (Jones et al. 1995; Montes de Oca Luna et al. 1995; de Rozieres et al. 2000). Further, exogenous p53 overexpression in vivo has invariably led to lethality. It has been suggested that a hypermorphic p53 allele that results in increased p53 tetramer stabilization leads to premature ageing and osteoporosis in vivo (Tyner et al. 2002). These findings led us to examine the effects of tissue-specific Mdm2 loss on bone formation and Runx2 activity.

By utilizing the Cre-lox system for conditional gene deletion, we were able to inactivate Mdm2 in immature osteoblasts in response to activation of a transgene containing the Cre recombinase coding sequence under transcriptional control of the type I collagen promoter. Surprisingly, deletion of Mdm2 in the pre-osteoblast did not result in
apoptosis *in vivo* or *in vitro* as it does earlier during murine embryogenesis. Instead, Mdm2 *floxed* Coll-Cre Transgenic mice displayed decreased limb length and bone mineral content. *Ex vivo* calvarial pre-osteoblast differentiation experiments demonstrated that the collagen-Cre transgene is active in multilayering osteoblast nodules, and that loss of Mdm2 in these cells prevents them from undergoing the post-confluent proliferation required for nodule formation. These cultures fail to activate Runx2 and the osteoblast phenotypic program of gene expression. These findings demonstrate that fidelity of the Mdm2-p53 regulatory loop is critical for proliferation of the pre-osteoblast and that this proliferation is, in turn, a pre-requisite event for activation of *Runx2* gene expression. Thus, this study establishes the interconnectedness of cellular proliferation and differentiation and demonstrates that *Runx2* gene activity is dependent on the Mdm2-p53 autoregulatory loop.
<table>
<thead>
<tr>
<th>Primer Name</th>
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<th>Reverse Primer (5’-3’)</th>
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Zehentner, B.K., U. Leser, and H. Burtscher. 2000. BMP-2 and sonic hedgehog have contrary effects on adipocyte-like differentiation of C3H10T1/2 cells. DNA Cell Biol 19: 275-81.


