ROLE OF ACTIVATING TRANSCRIPTION FACTOR-2 IN CONTROL OF POCKET PROTEIN EXPRESSION AND REGULATION OF CHONDROCYTE GROWTH

By

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To my wife, Kristi, for her unconditional love and support.

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Endochondral ossification is the process of skeletal bone growth through the formation of a cartilage template that subsequently undergoes mineralization to form trabecular bone. Genetic mutations affecting the proliferation or differentiation of chondrocytes results in skeletal abnormalities. Activating transcription factor-2 (ATF-2) modulates expression of cell cycle regulatory genes in chondrocytes, and mutation of ATF-2 results in a dwarfed phenotype.

The overall aim of these studies was to further examine the role ATF-2 plays in cell cycle progression. Previous data had identified sites of ATF-2 binding located in promoters of the pocket protein family of cell cycle regulators. Initial studies indicated changes in pocket protein expression in response to loss of ATF-2 function. Mutation of ATF-2 lead to reduced expression of retinoblastoma (pRb), a member of the pocket protein family. pRb regulates cell cycle progression and influences cellular proliferation and terminal differentiation. Using an *in vitro* chondrogenesis model, we observed that pRb influenced cell cycle exit and differentiation.

Transgenic mice expressing a conditional truncation of pRb in chondrocytes were produced to further delineate the role of pRb in endochondral development. Although no gross skeletal abnormalities were observed conditional mutants displayed reduced size at birth. The

observed size difference was overcome with age. Histological examination of the growth plate demonstrated changes in growth plate architecture of neonates in response to pRb mutation. Growth plates of older animals were re-organized indicating the observed alterations in growth plate structure were transient. Immunohistchemical localization of proliferative markers indicated that pRb conditional deletion allowed prolonged chondrocyte proliferation in newborn mice. These studies suggest that pRb may influence chondrocyte proliferation and differentiation, but its role is largely insignificant in overall endochodral development.

CHAPTER 1
INTRODUCTION

The vertebrate skeleton is comprised of cartilage and bone and serves three distinct functions. First, the skeleton serves as the framework for the body, and provides support and protection for the internal organs. Second, the skeleton is a storage center for vitamins and minerals. Bone is a depot for calcium and phosphorous utilized by the body. Third, the skeleton is the center for production of blood cells from the bone marrow. Both white and red blood cells are produced within the bone marrow cavities of the skeleton, giving the skeleton an added function in aiding immune response.

The skeleton is the product of different embryonic cell lineages. Cells from distinct lineages form high-density mesenchymal condensations at sites specific for skeletal elements. The condensed mesenchyme then undergoes differentiation to chondrocytes and osteoblasts (Hall and Miyake, 1992). Two distinct processes control further skeletal growth: intramembranous and endochondral ossification. The bones of the skull are formed through intramembranous ossification, while the long bones comprising much of the appendicular skeleton develop through the process of endochondral ossification.

Most of what we know regarding skeletal growth and development is the result of observations of human bone disorders and experimental animal studies. Correlation between the function of genes and specific embryological events has been determined through the use of transgenic and knockout animals. Genetic studies of inherited disorders involving bone and cartilage have identified novel genes and pathways and furthered our knowledge of the underlying molecular mechanisms of skeletal development and growth.

Given the number of inherited genetic disorders implicated in skeletal growth, this dissertation seeks to elucidate further the molecular mechanisms involved in activating

transcription factor-2 (ATF-2) regulation of bone growth. Moreover, these studies seek to identify genes important in bone growth that are altered in response to loss of ATF-2 function and further characterize their function in bone growth.

Skeletal Development

Morphogenesis of the skeleton is the result of cell migrations from sites of embryonic origin to locations of specific skeletal elements and condensation into the mesenchymal precursors of cartilage and bone. Different portions of the skeleton are derived from distinct embryonic cell lineages. Genes that control morphogenesis largely encode for transcription factors that control cellular fate decisions and migration events.

The craniofacial skeleton is derived from the cranial neural crest, and is comprised of the bones of the skull and face. The majority of the bone of the face and skull are of neural crest origin (Bronner-Fraser, 1994). These cells migrate from the dorsal aspect of the neural tube to the frontonasal mass forming cartilage and bone tissue. Cranial neural crest commitment to skeletal fate is controlled through interactions with the overlying epithelium. Epithelial cells produce cytokine-based signals that stimulate signaling pathways and expression of various transcription factors within the mesenchyme. As a result of the activated state of the mesenchyme, factors are secreted that in turn control the proliferation and differentiation of the overlying epithelium.

A multitude of genes are therefore vital for craniofacial development. These include genes that encode for transcription factors, cytokines, growth factors, and proteolytic enzymes. Transcription factors important for development include: homeobox-containing transcription factors such as *Msx1* and *Hoxa1* (for review see Alappat et al., 2003), polycomb group genes, basic helix-loop-helix factors such as *Twist* (Howard et al., 1997), and *PAX* genes encoding

paired-box containing transcription factors (for review see Lang et al., 2007). Bone morphogenic proteins (BMPs) (Ripamonti et al., 2005), the transforming growth factor-β (TGF-β) family (Chai et al., 2003), and fibroblast growth factors (FGFs) (Chen and Deng, 2005; Nie et al., 2006) are important cytokines and growth factors implicated in craniofacial development . Extracellular matrix degrading enzymes such as the matrix metalloproteases (MMPs) and their inherent inhibitors (tissue inhibitor of metalloprotease, TIMPs) are essential in normal craniofacial development (Letra et al., 2007; Egeblad et al., 2007). Genetic defects or mutations in these or other related factors produce developmental disorders of the skull and face such as craniosynostosis (premature closure of skull sutures), cleft palate, lack of teeth, and mandible defects. Alterations in signaling, including BMPs, TGFs, FGFs *sonic hedgehog* (Shh) among others, disrupt normal development and lead to anomalies. Furthermore, the communication between the overlying epithelium and mesenchyme is critical in formation and patterning of structures such as teeth. Clearly, the processes that govern craniofacial development are complex and rely heavily on patterning and cell fate determination to insure normal development.

Paraxial mesoderm tissue is the embryonic precursor to the axial skeleton, which consists of the vertebral column and the dorsal portion of the rib cage. Somites are formed following segmentation of the paraxial mesoderm that subsequently differentiates into sclerotomes (for review see Monsoro-Burq, 2005). Somitic derived tissues also include the dermis of the skin and skeletal muscle of the body wall and limbs (for review see Christ et al., 2007). Genes that control the segmentation of the paraxial mesoderm largely influence somitic development. Somite formation occurs bilaterally in a rostro-caudal timed sequence. Expression of *hairy-1* (Palmeirim et al., 1997) modulates *Notch* signaling by regulating expression of *lunatic fringe*.

The importance of *Notch* signaling in somite development is evident in the numerous mouse knockouts that affect the pathway. Knockouts of *Notch1* (Conlon et al., 1995) and other modulators of *Notch* signaling including *Delta-like1* (*Dll1*) (Hrabe de Angelis et al., 1997), *Dll3* (Kusumi et al., 1998), and *lunatic fringe* (Zhang and Gridley, 1998) display abnormal somite phenotypes resulting in vertebral and rib defects.

Differentiation of cells within the somite is necessary for formation of sclerotomes and further development of skeletal elements. These signals induce epithelial-mesenchymal transformation and proliferation of somatic cells and their migration to form the sclerotomes. The sclerotome arises from the ventral portion of the somites and gives rise to the cartilage of the vertebral body and the dorsolateral portion of the ribs (Christ et al., 2000). *Shh* signaling, originating from the notochord and floorplate of the neural tube is vital for sclerotome formation as *Shh*-null embryos are devoid of vertebrae and the dorsolateral portion of the ribs (Chiang et al., 1996). *Shh* signaling conditons sclerotomal cells to be competent for responding to BMP actions and subsequent differentiation into chondrocytes (Murtaugh et al., 1999). *Pax* genes 1 and 9, specific to the cells of sclerotome, are induced by *Shh* signaling. *Pax 1* mutants display skeletal defects in the vertebral column, scapula, and sternum (Chalepakis et al., 1991; Wilm et al., 1998).

Anterior to posterior patterning of the axial skeleton is controlled by expression of homeotic (*Hox*) genes. These genes control the identification of individual vertebrae through an overlapping expression domain along the vertebral column (Burke et al., 1995; Favier and Dolle, 1997). Disruptions in *Hox* gene expression patterns result in the loss or addition of vertebral elements or alterations into shapes resembling other skeletal elements (Zakany et al., 1997; Chen et al., 1998).

Skeletal tissues of the limb are derived from lateral plate mesoderm (Cohn and Tickle, 1996). Other tissues such as nerves, muscles, and blood vessels are derived from the developing somites and migrate into the limb buds. Interactions between the developing mesenchyme and overlying epithelium control the patterning and shaping of the limb (Ng et al., 1999). Limb outgrowth is controlled by FGF signaling originating from specialized epithelium covering the limb bud composing the apical ectodermal ridge (AER). Patterning of the limb is further accomplished through the actions of *Shh* and *Wnt7a* signaling and expression of transcription factors such as LIM-homeodomain protein (Lmx1b) and *engrailed* (for review see Robert and Lallemand, 2006). The cartilage anlagen are formed in a proximal to distal sequence coincident with the development of the limb bud. The more proximal bones such as the femur and humerus are formed first, while the phalanges and metatarsals are formed last. The cartilage is formed as continuous rods through a series of divisions and segmentations that give rise to the limb skeleton (for review see Tuan, 2004). BMP signaling, segmentation and eventual apoptosis are vital for the formation of the joints (for review see Rosen, 2006 and Khan et al., 2007).

Disruptions in the genetic or signaling mechanisms that control limb bud outgrowth or patterning are manifested in various ways. Shortening of the bones of the fingers and toes (brachydactylies) and the absence or fusion of joints have been shown to be the result of disruptions of normal BMP signaling. Disruptions in *Shh* signaling are manifested as the occurrence of extra digits (polydactyly) or the fusion of digits (syndactyly), among others.

Skeletal Bone Growth

The bones of the skeleton are mineralized in various modes depending on the type of bone being formed. Intramembranous ossification is manifested via mesenchymal cell differentiation into osteoblasts and bone, formed in the absence of a cartilage precursor. The flat

bones of the skull, face and scapula are mineralized through the process of intramembranous ossification. Perichondral ossification is the most common form of ossification which utilizes a cartilage precursor. Perichondral ossification starts with the transformation of the perichondrium into the periosteum (Scott-Savage and Hall, 1980).

Endochondral ossification is the primary mode of mineralization within the long bones of the skeleton. The process of endochondral ossification involves a cartilaginous template that is replaced by bone matrix. The overall process of endochondral ossification involves the tightly regulated coordination of chondrocyte proliferation, differentiation into hypertrophic chondrocytes, apoptosis and ultimately calcification of the remaining cartilage matrix.

Mesenchymal progenitor cells follow the chondrocyte lineage following condensation. Chondrocytes within the resting zone of the growth plate are arrested in the cell cycle forming a suppository of cells necessary for proliferation. Resting zone chondrocytes are stimulated to initiate proliferation through multiple signaling cascades. Chondrocytes are stimulated to proliferate and become organized in a columnar fashion. These chondrocytes also begin the production of type II collagen. Later, chondrocytes cease proliferating and begin differentiation to pre-hypertrophic chondrocytes, which undergo further increases in cellular volume to become hypertrophic chondrocytes which initiate the production of type X collagen. The post-mitotic, terminally differentiated hypertrophic chondrocytes then undergo apoptosis, leaving behind the calcified cartilage matrix. Osteoblasts are then recruited to begin deposition of bony matrix over the mineralized cartilage residue.

Several factors are critical in allowing normal longitudinal bone growth. Both the proliferation of chondrocytes and expanse of cellular volume are essential factors in skeletal growth. The proliferation of chondrocytes is a critical step in providing an adequate number of

cells for growth. The increase in cellular volume established during hypertrophy also contributes to the longitudinal lengthening of the bone. Genetic disruption of normal chondrocyte proliferation results in appendicular skeletal abnormalities (for review see Shum et al., 2003). Mutations affecting chondrocyte differentiation and subsequent hypertrophy results in skeletal malformations.

Role of ATF-2 in Skeletal Bone Growth

Activating transcription factor (ATF)-2 is a basic helix-loop-helix DNA binding protein that belongs to the ATF/CREB family of transcription factors. Members of the ATF/CREB family regulate transcription of target genes through interactions with cyclic AMP-response elements (CREs) located within target gene promoters. Members of the ATF/CREB family can form homo- or hetero-dimers that modulate transcription of regulated genes.

The importance of ATF-2 in skeletal growth was demonstrated by the production of transgenic mouse mutants. Two independent groups produced ATF-2 mutations that had severe phenotypes. Maekawa et al. (1999) produced ATF-2 null mice through gene targeting. These mice displayed perinatal lethality due to severe respiratory distress similar to meconium aspiration syndrome. Reimold et al. (1996) first described the phenotype of ATF-2 mutant animals harboring a gene disruption created by the introduction of an antibiotic resistance cassette resulting in the truncation of ATF-2 protein expression. In this case, ATF-2 mutants displayed ataxia, hyperactivity and diminished hearing. ATF-2 mutant mice also displayed a disruption in endochondral ossification at the epiphyseal growth plate similar to human hypochondrodysplasia and achondrodysplasia.

Within the developing bone, ATF-2 mRNA is expressed within the resting and proliferating zones suggesting a potential role in regulating chondrocyte proliferation. Indeed,

chondrocyte proliferation was decreased in ATF-2 mutant animals assayed by incorporation of [^{3}H]-thymidine (Reimold et al., 1996).

Chondrocyte proliferation is stimulated by parathyroid hormone related peptide (PTHrP) and TGF-β signaling (Beier et al., 2001). PTHrP signaling is mediated through binding of the PTHrP ligand to its putative receptor. During endochondral development PTHrP is expressed in the perichondrium, while the PTHrP receptor is expressed at low levels by proliferating chondrocytes and at higher levels in pre-hypertrophic chondrocytes (Lanske et al., 1996; Vortkamp et al., 1996). Genetic ablation of PTHrP results in animals that exhibit perinatal lethality and severe abnormalities in bones that form during endochondral ossification including shortening of the snout, mandible, and limbs (Karaplis et al., 1994). Shortening of the bones results from premature transition of proliferative chondrocytes to hypertrophic chondrocytes. Interestingly, when PTHrP is overexpressed in chondrocytes animals are born with shoirtened limbs due to decreased mineralization and delayed maturation of chondrocytes (Weir et al., 1996). These data illustrate the importance of PTHrP signaling in the endochondral process, and further underscore the necessity for the organization and control of chondrocyte proliferation, differentiation and apoptotic cell death in bone elongation.

PTHrP is a mediator of Ihh activity within the growth plate. Ihh is expressed in the maturation region between proliferative and hypertrophic regions of the growth plate. Ihh inhibits chondrocyte differentiation by inducing expression of PTHrP (Lanske et al., 1996; Vortkamp et al., 1996). Deletion of Ihh results in the loss of PTHrP mRNA expression and a predominance of hypertrophic chondrocytes coupled with a decrease in chondrocyte proliferation (St-Jacques et al., 1999). A negative feedback loop is then established where Ihh maintains chondrocyte proliferation through the induction of PTHrP expression which in turn delays Ihh

production. Again, the delicate balance between chondrocyte proliferation and differentiation is evident in the intricacies of the PTHrP/Ihh negative feedback loop.

TGF-β and PTHrP regulate the proliferation of growth plate chondrocytes by modulating cyclin-D1 expression (Beier et al., 2001). Cyclin D1 regulates cellular proliferation by regulating cell cycle progression through its interaction with cyclin-dependent kinases (CDKs). The cyclin-CDK complex is then activated and phosphorylates cell cycle regulatory proteins such as retinoblastoma (pRb). The role of cyclin D1 in chondrocyte proliferation is evident in the skeletal phenotype exhibited in cyclin D1 mutant mice (Fantl et al., 1995; Sicinski et al., 1995). ATF-2 has been shown to regulate cyclin D1 promoter activity and expression (Beier et al., 1999). Cyclin D1 expression is necessary to drive cell cycle progression through the modification of cell cycle inhibitors. Proliferation of chondrocytes is altered in response to ATF-2 loss due to lack of cyclin D1 expression and subsequent inability of cyclin D1-CDK complexes to phosphorylate and inactivate cell cycle regulator proteins. Inactivation of cell cycle inhibitors is paramount in cell cycle progression and ultimately cell proliferation. This may account for the reduction in chondrocyte proliferation observed in ATF-2 mutant animals (Reimold et al., 1996). Chondrocyte proliferation is also stimulated by TGF-β treatment (Beier et al., 2001; Ferguson et al., 2000; Pateder et al., 2000; Ionescu et al., 2003; Dong et al., 2005) by induction of cyclin D1 expression through β-catenin signaling (Li et al., 2006). ATF-2 has the ability to regulate cell cycle progression by modulating expression of target genes important for cell cycle progression. Loss of ATF-2 would influence cell cycle progression through the reduction of cell cycle inhibitor expression (Luvalle et al., 2003) or by limiting the expression of genes necessary for inactivation of cell cycle inhibitors, resulting in reduced proliferation due to sequestration of transcription factors necessary for cell cycle progression (Beier et al., 1999). The skeletal

phenotype observed in ATF-2 mutant animals is likely indicative of the importance of ATF-2 signaling. However, the number of potentially regulated genes involved in skeletal growth suggests that the observed phenotype is likely the result of numerous disruptions in normal skeletal development.

Pocket Proteins and Cell Cycle Control

The retinoblastoma family of proteins consists of pRb as well as the closely related p107 and p130 proteins. These proteins function as cell cycle regulators through the binding of E2F transcription factors and modulating transcription of S-phase related genes. The proteins share significant similarities within the protein domains that comprise the "pocket region" consisting of two A and B boxes. pRb shares little similarity with p107/p130 outside of the pocket domain, while p107 and p130 share a highly conserved spacer region residing between the two A and B boxes. The pocket region is critical for binding and regulation of E2F transcription factors (for review see Nevins et al., 1997).

Studies have demonstrated the cell cycle regulatory function of the pocket proteins by inducing G1 phase growth arrest following over-expression of all three proteins (Dyson, 1998). It has been shown that pRb regulates the G1 to S phase transition of the cell cycle by blocking S-phase entry, promoting terminal differentiation (Weinberg et al., 1995). The interactions between E2F and the pocket proteins play a central role in cell cycle progression and DNA replication by regulating expression of E2F responsive genes (Macaluso et al., 2005). p107 and p130 also regulate cell cycle progression through interactions with cyclin A/cdk2 and cyclin E cdk2 complexes (Mulligan and Jacks, 1998). Viral oncoproteins allow unregulated cell cycle progression by displacing cellular proteins such as E2Fs (for review see Lee and Cho, 2002). E2Fs regulate the timing and expression of many genes important for cell cycle regulation and

progression such as cyclins A, E and D1, and cdk2 (for review see Nevins, 1998). The pocket proteins show preferential binding for specific E2Fs with pRb able to bind to E2F1-5, while p107/p130 interact with E2F 4 and 5 (for review see Dimova nd Dyson, 2005). The pocket protein-E2F paradigm lies at the heart of cell cycle regulation and progression, along with further complications in cell cycle exit and cellular differentiation.

Studies have implicated the pocket proteins in development of the limb and endochondral growth. Cobrinik et al (1996) first demonstrated that p107 and p130 had overlapping roles in limb development. Moreover, p107 and p130 were shown to regulate chondrocyte proliferation. Embryos that were nullizygous for both p107 and p130 ($p107^{-/-}$; $p130^{-/-}$) displayed a higher proliferative index as demonstrated through bromo-deoxyurindine (BrdU) incorporation. Although $p107^{-/-}$; $p130^{-/-}$ embryos displayed elevated chondrocyte proliferation, they exhibited shortened limbs. Chondrocytes lacking p107/p130 cell cycle control do eventually cease proliferation, likely due to other growth regulators such as pRb and cyclin-dependent kinase inhibitors (CKIs) such as p16, p21, and p27, which function to inhibit cell cycle progression by blocking the activity of CDKs.

Subsequent studies further elucidated the functions of these proteins by illustrating distinct and overlapping roles of p107 and p130 in cartilage development. During the condensation of mesenchyme, p107 is required for cell cycle withdrawal, while p130 is not expressed at this time (Rossi et al., 2002). Growth plate chondrocytes devoid of p107 expression cause severe defects. During mesenchymal condensation, p130 can compensate for p107 loss by aiding in cell cycle withdrawal and hypertrophic differentiation (Rossi et al., 2002). Simultaneous deletion of p107, and p130 blocked hypertrophic differentiation and expression of the transcription factor *Runx2* the function of which is important in chondrocyte differentiation

(Rossi et al., 2002). pRb has yet to be implicated in cell cycle withdrawal during chondrocyte differentiation. Antiproliferative effects of FGFs in chondrocytes are mediated through the rapid dephosphorylation of p107, with a more delayed dephosphorylation of pRb and p130 (Laplantine et al., 2002; Dailey et al., 2003). It has also been shown that p107 is absolutely necessary for FGF-induced growth arrest, whereas p130 contributes to cell cycle arrest, while involvement of pRb in FGF-growth arrest has not been identified (Laplantine et al., 2002; Dailey et al., 2003). These results indicate the importance of p107/p130 in cell cycle control, but also raise the question of what role, if any, pRb may play in cartilage development.

Each of the pocket proteins contains putative CREs within their promoter regions. These promoter elements are binding sites for a multitude of transcription factors, including ATF-2. ATF-2 has been shown previously to regulate pRb promoter activity (Zacksenhaus et al., 1993). More recently, studies have shown that mutation of ATF-2 in mice leads to decreased expression of pRb (Luvalle et al., 2003).

In conclusion, development of the vertebrate skeleton is a complex, yet highly ordered, controlled process. The exquisite control is demonstrated by the phenotypic manifestations observed following genetic disruption of genes involved in the process of endochondral ossification. Ablation of ATF-2 is one example of the type of genetic disruption that alters the delicate balance within the growth plate. Loss of ATF-2 signaling influences numerous cellular processes vital for chondrocyte proliferation and differentiation. The purpose of the studies within this dissertation is to further the understanding of the regulatory role that ATF-2 plays in endochondral ossification. The studies contained herein also examine a plausible role of pRb plays in endochodral development.

CHAPTER 2
ACTIVATING TRANSCRIPTION FACTOR-2 AFFECTS SKELETAL GROWTH BY MODULATING PRB GENE EXPRESSION

Introduction

Growth of skeletal long bones occurs through the process of endochondral ossification. Endochondral bone formation is inititated with the condensation and chondrogenic differentiation of mesenchymal cells. Condensed mesenchymal cells are induced to undergo chondrogenesis by several regulatory pathways including those of transforming growth factor-β (TGF-β), parathyroid hormone relater peptide (PTHrP), hedgehog family proteins, as well as various other transcription factors. The Sox family of transcription factors regulate the formation of chondrogenic condensations and cartilage by controlling the expression of type II collagen and aggrecan, two cartilage specific markers (Bell et al., 1997; Lefebvre et al., 1998; Sekiya et al., 2000).

Endochondral bone growth is dependent upon the coordinated regulation of proliferation and differentiation of chondrocytes to form the cartilage template. Chondrocytes are found in a quiescent state in the resting zone of the growth plate where they are stimulated to proliferate. The proliferation of chondrocytes is maintained in part by both Indian Hedgehog (Ihh) action and the PTHrP gradient. After several cycles of proliferation, chondrocytes exit the cell cycle coincident with the reduction of the PTHrP gradient and begin to increase in cellular volume (hypertrophy), forming the pre-hypertrophic, or maturation, region. The expansion of hypertrophic chondrocytes contributes significantly to the length of the developing bone. Hypertrophic chondrocytes are terminally differentiated and commence expression of type X collagen. Within the late hypertrophic region, the cartilage matrix undergoes both mineralization

and vascularization. The mineralized cartilage matrix is replaced with bone matrix. The most mature hypertrophic chondrocytes undergo apoptosis, leaving behind a trabecular bone matrix.

Activating transcription factor-2 (ATF-2) is a member of the ATF/CREB (cAMP Response Element Binding protein) family of transcription factors, characterized by a basic region and a leucine zipper DNA-binding domain. This family of transcription factors binds cAMP-response elements (CREs) within target gene promoter regions in either homo- or heterodimers. ATF-2 has been shown to regulate promoter activity of genes that are important for cartilage and bone growth. Data from our laboratory has shown that ATF-2 is essential for modulating the activity of the cell cycle related genes cyclin D1 (Beier et al., 1999) and cyclin A (Beier et al., 2000) within growth plate chondrocytes. Gene expression of both cyclin A and cyclin D1 are targets of ATF-2 trans-activation, while loss of ATF-2 action in mutant ATF-2 chondrocytes leads to a decrease in gene expression. Addition of an exogenous ATF-2 expression plasmid restores promoter activity, further supporting the role of ATF-2 in regulating the expression of cyclins A and D1 in chondrocytes. The promoter regions of several other genes contain CRE motifs, but have yet to be examined for ATF-2 regulation. Additional genes, such as osteopontin, osteocalcin, alkaline phosphatase, and the Rb family that contain CREs may also be controlled by ATF-2. Within the growth plate, ATF-2 is expressed in the resting and proliferating zones, yet is excluded from the hypertrophic region. The importance of ATF-2 in skeletal development was demonstrated with the production of ATF-2 deficient mice (Reimold et al., 1996). ATF-2 deficient mice (ATF-2 m/m) express low amounts of a splice variant of ATF-2 that rescues ~50% of transgenic animals from neonatal lethality. ATF-2 - deficient mice are smaller than wild-type littermates at birth, and those that survive past weaning develop a hypochondroplasia-like dwarfism characterized by abnormal epiphysis and curvature of the

spine. Chondrocyte proliferation is also reduced as determined by in vivo [^{3}H]-thymidine incorporation (Reimhold, 1996). True ATF-2 null mice die at birth due to respiratory difficulties (Maekawa et al., 1999).

The retinoblastoma protein family, or pocket proteins, is comprised of retinoblastoma (pRb) and pRb-related proteins, p107 and p130. These proteins regulate the G1 to S phase transition by sequestering the E2F family (E2F1 to E2F5) of transcription factors necessary for cell cycle progression (for review see Cobrinik, 2005). pRb is found within resting (G0) cells in a hypo-phosphorylated state that allows for binding and sequestration of E2Fs. E2F proteins are released from pRb sequestration following phosphorylation by cyclin D-cyclin dependent kinase (cdk) 4 and 6 complexes in early G1 and cyclin E/cdk 2 complexes later in G1. After E2Fs are released, the cell enters S-phase and is committed to progress through the cell cycle. The dynamic relationship between the pocket proteins and E2Fs is essential in regulating cell proliferation and, in turn, differentiation. The pocket proteins differ with respect to their cellular expression patterns and in their interactions with E2Fs. pRb shows selective interactions with E2F1-3 while p107 and p130 bind more readily to E2F 4-5 (for review see Cobrinik 2005).

Disruption of E2F-pocket protein dynamics inhibits normal skeletal bone growth by disrupting the endochondral ossification process. Chondrocytic differentiation of ATDC5 cells, a chondro-progenitor cell line (Atsumi et al., 1990), is inhibited by ectopic expression of E2F1 as evidenced by inhibition of expression of the cartilage markers type II collagen, type X collagen and aggrecan (Scheijen et al., 2003). Fibroblast growth factor (FGF) signaling inhibits longitudinal bone growth by specifically targeting p107 and p130 to induce chondrocyte cell cycle arrest. p107 and p130 have also been implicated in limb development (Cobrinik et al., 1996) and in the regulation of chondrocyte proliferation and differentiation (Rossi et al., 2002).

Our lab has shown that protein levels of pRb are reduced in ATF-2 m/m chondrocytes (Luvalle et al., 2003) suggesting that pRb expression is dependent on ATF-2 function. Basal pRb promoter activity is regulated through ATF-2 binding of the CRE motif (Zacksenhaus, et al., 1993). The promoter regions of p107 and p130 also contain CREs that may be targets of ATF-2 regulation. The phenotype of ATF-2 m/m mice may be the result of cumulative effects of various target genes including the pRb family.

Here we further investigate the abnormal development of ATF-2 deficient mice by examining the relationship between ATF-2 and pRb. We show that the normal spatial and temporal expression of each pocket protein within the growth plates of wild type animals and their altered expression in ATF-2 mutants. Expression of pRb mRNA is reduced in ATF-2 m/m chondrocytes compared to wild type due to the reduction of pRb promoter activity. We also show that that pRb expression is upregulated in ATDC5 undergoing chondrogenic differentiation. ATF-2 deficient mice also exhibit reductions in collagen type X expression as compared to wild type counterparts. We also show that ATF- 2 mutant mice display increases cellular proliferation as compared to their wild type littermates, both *in vivo* and *in vitro*. Taken together these data show that the loss of ATF-2 activity results in the decreased expression of pRb, and this decrease contributes to chondrocyte cell cycle deregulation and differentiation affecting hypertrophy, consequently resulting in appendicular dwarfism.

Materials and Methods

Mice and genotyping

Mice harboring either wild type or inactivated alleles of the ATF-2 gene were genotyped as previously described (Reimold et al., 1996). Genomic DNA was isolated from the distal

portion of the tail by standard phenol-chloroform extraction and genotype was determined by polymerase chain reaction (PCR).

Cell Culture

ATDC5 chondrogenic cells were propagated in a 50:50 mixture of DMEM/F12 media supplemented with 5% fetal bovine serum, 100 μg/ml penicillin and 100 μg/ml streptomycin. For differentiation experiments, ATDC5cells at confluence were given complete media further supplemented with insulin-transferrin-selenium (ITS) reagent. Media was replaced every other day for the indicated time periods. Primary mouse chondrocytes were isolated from the ventral ribcage as previously described (Lefevbre et al., 1994). Briefly, ventral ribcages were excised from neonate pups less than 2 days old. Tissues were digested in pronase, followed by digestion with collagenase D (3 mg/ml) in complete DMEM (DMEM + 10% FBS, 100 μg penicillin, 100 μg streptomycin). Cells were then grown in suspension over 1.5% agarose in phosphate buffered saline (PBS) coated plates for 3 days to ensure chondrogenic phenotype. Cell aggregates were digested in collagenase and plated in monolayer culture on sterile plastic dishes at 37°C in a 5% CO_2 atmosphere in complete DMEM.

Immunhistochemistry

Knee joints from newborn, 1-week-old , and 3-week-old mice were dissected and fixed in 4% paraformaldehyde in PBS (w/v) for 24 hours at 4°C. Tissues were processed though xylenes, dehydrated through graded alcohols and embedded in paraffin wax. Tissue sections were cut at 5 μm and attached to SuperFrost Plus (Fisher) microscope slides. For immunohistochemisty, slides were deparaffinized in Citrasolve (Fisher), and rehydrated through graded alcohols to distilled water. Microwave antigen retrieval was performed using Antigen Unmasking Solution according to manufacturer's recommendations (Vector). The Vector Elite ABC Staining kit was

used for immunohistochemical localization. Antibodies and working concentrations were as follows: rabbit anti-pRb (Delta bioLabs, 0.2 μg/ml); rabbit anti-p107 (Delta Biolabs, 0.2 μg/ml) rabbit anti-p130 (ABCam, 0.2 μg/ml); rabbit anti-Ki67 (ABCam, 0.1μg/ml). As a negative control, normal rabbit IgG at the same concentration was used in place of primary antibody. Immunoflourescence for type X collagen was performed using a rabbit polyclonal antibody (gift from Danny Chan, Department of Biochemistry, The University of Hong Kong, Pokfulam, Hong Kong, China). Antigen unmasking was accomplished though incubation with 0.8% hylaranidase in PBS for 30 minutes prior to incubation with the primary antibody (1:2000). Alexa 488 (Molecular Probes, Invitrogen) goat anti-rabbit was used as a secondary antibody (1:1000). Omission of primary antibody served as a negative control. Immunofluorescence was visualized on a fluorescent microscope (DM IRBE; Leica) equipped with a digital camera. Intensity of immunofluorescence was measured using digital imaging software (IP Lab). Average intensities were determined from separate measurements of each growth plate of at least three individual animals. Bar graphs represent the mean of measured fluorescence intensity of at least 3 different animals at the indicated time points. Error bars represent the standard deviation of the mean.

RNA isolation and RT-PCR

RNA was extracted from confluent cells using the TriZol reagent according to manufacturer's suggestions. Reverse transcription of 2 μg of total cellular RNA was performed. Total cDNA was diluted 1:10 in sterile water, and 1 μl was used in subsequent PCR reactions. Quantitative real-time PCR for type X collagen mRNA expression was performed using the DyNAmo HS SYBR Green PCR kit (New England Biolabs) according to manufacturer's instructions. Real-time PCR analysis for pRb mRNA was performed using TaqMan Gene Expression Array combined with the TaqMan Universal PCR Master Mix (Applied Biosystems).

PCR results were normalized to GAPDH PCR values. Primers used for SYBR green RT-PCR are as follows (target; forward, reverse): type X collagen; 5'-TGCCCGTGTCTGCTTTTACTGTCA-3', 5'-TCAAATGGGATGGGGGCACCTACT-3'; GAPDH, 5'-CGGACTCAACGGATTTGGTCGTAT-3', 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'; β-actin, 5'-CGTGGGCCGCCCTAGGCACCA-3', 5'-TTGGCCTTAGGGTTCAGGGGGG-3'. Comparisons made between groups of SYBR green reactions were performed using the Pfaffl method (Pfaffl, 2001), and the ΔΔCt method for TaqMan Assay PCRs. The Pfaffl method takes into account different efficiencies of amplification between various primer sets in order to make accurate comparisons.

Transfections and Luciferase Assays

Primary chondrocytes were plated in a 24-well plastic culture plate at a cell density of $1.25x10^5$ in DMEM media with 10% FBS without antibiotics for 24 hours prior to transfection. Each well contained 0.2μg of reporter plasmid DNA and 0.02μg of pRLSV40 plasmid used for transfection normalization. Lipofectamine 2000 was used for transfections according to manufacturer's recommendations with few exceptions; briefly, plasmid DNA and 1 μl of transfection reagent were diluted separately in Opti-MEM serum free media. Diluted DNA and transfection reagent were combined to form complexes for 20 minutes prior to adding to cells. Cells were collected for luciferase activity 24 hours after transfection. Luciferase assays were performed as previously described (Ma et al., 2007). ATDC5 cells were transfected 24 hours prior to collection as were those for primary cultures and collected at indicated time points for luciferase activity.

Cell Cycle Analysis

ATDC5 cells were collected at the indicated times following differentiation by adding hypotonic cell lysis buffer (0.5 g Sodium Citrate, 0.1% (v/v) Triton-X100) followed by removal of cells from the culture plate with a cell scraper. Propidium iodide solution was added to a final concentration of 30 μg/ml with RNase A in order to stain DNA content. The cell suspension was then processed for flow cytometry in a FACS Calibur (BD BioSciences) flow cytometer. Cell cycle profiles were obtained using Cell Quest software. Analyses of cell cycle populations were performed using the ModFit Software package.

BrdU Incorportation

Primary chondrocytes from wild-type and ATF-2 m/m newborn mice were isolated, differentiated over agarose and plated on plastic cell culture dishes at a cell density of $1.25x10^6$ cells/ml. The next day, cells were pulse labeled with bromo-deoxyuridine (BrdU) to a final concentration of 10 μM in complete cell culture media for 4 hours. The media was removed, cells were washed with PBS, and supplemented with fresh media for 6 hours before collection for cell cycle analysis. BrdU incorporation was detected by incubation with a FITC labeled BrdU antibody (BD Biosciences) according to the manufacturer's recommendations.

Results

Several studies have documented the functions of the pRb protein family in regulating chondrocyte cell cycle arrest and differentiation (Laplantine et al., 2002). The effects of FGF signaling on chondrocyte cell cycle arrest are modulated through p107 and p130. Our laboratory, as noted earlier, has shown that loss of ATF-2 signaling leads to a reduction in pRb protein levels. Since ATF-2 expression is restricted to the resting and proliferative zones of the growth plate, we first sought to identify the spatial and temporal expression of the pRb family

members within the growth plate in wild type and ATF-2 m/m animals at 0, 1 and 3 weeks of age. Expression of pRb was localized to the resting and proliferative region in newborn wild type proximal tibial sections (Figure 2-1a). In contrast, pRb expression was observed in the maturation and early hypertrophic regions in ATF-2 m/m proximal tibia (Figure 2-1b). Expression of p107 was not detected in wild type animals (Figure 2-1c), however p107 staining was observed in tibial growth plate sections from newborn ATF-2 m/m animals (Figure 2-1d). Staining was localized to the late proliferative region. Expression of p130 was observed throughout the growth plate in both wild type and ATF-2 m/m tissues (Figure 2-1e,f). ATF-2 m/m sections displayed more intense p130 staining as opposed to wild type tissue sections.

By 1-weekof age, pocket protein expression was increased and more uniform (Figure 2-2). pRb expression was localized to the late proliferative zone and pervaded throughout most of the hypertrophic region in wild type proximal tibia sections (Figure 2-2a). Expression of pRb was more restricted to the late proliferative and early hypertrophic regions of ATF-2 m/m growth plates (Figure 2-2b). There was some observed pRb immunostaining in the resting zones of both wild type and ATF-2 m/m sections (Figure 2-2a,b). p107 expression was localized to the late proliferative and early hypertrophic zones in sections of wild type growth plate tissues (Figure 2-2c). p107 expression was observed in the late proliferative region, with sparse staining in the hypertrophic zone in ATF-2 m/m sections (Figure 2-2d). Staining for p130 expression was observed throughout the proliferative and hypertrophic zones in both wild type and ATF-2 m/m sections (Figure 2-2 e,f). At 3 weeks, pRb expression was detectable in wild type growth plates while expression in ATF-2 m/m tissues was diminished (Figure 2-3a,b). Expression of p107 was reduced in wild type and ATF-2 m/m tissues at 3 weeks of age (Figure 2-3c,d). p130 expression

was observed predominantly in the proliferative region (Figure 2-3e,f) with some staining observed in resting zones (Figure 2-3e,f).

We performed immunoblot experiments with protein from cultured primary chondrocytes to correlate the observed differences seen in immunohistochemical localization of the pRb protein family. Reduction of pRb protein expression has been demonstrated previously in ATF-2 m/m chondrocytes (pRb blot reprinted with permission LuValle et al., 2003). Expression of p107 was up-regulated in response to ATF-2 deficiency. Expression of p130 was unchnaged in ATF-2 m/m animals. Actin was used as a loading control (Figure 2-4).

ATF-2 recognizes CRE elements located in target gene promoters and subsequently alters gene expression. Given our results from immunohistochemistry and western blot experiments, we sought to determine the changes, if any, in mRNA expression in response to loss of ATF-2. Primary chondrocytes were isolated and cultured *in vitro* as described previously (Lefevbre et al., 1994). Real-time PCR TaqMan Expression Assays were used to determine quantitative differences in pRb mRNA expression between wild type and ATF-2 m/m primary cells. Total RNA was isolated using the TriZol reagent, and cDNA was reverse-transcribed in combination with an oligo-d(T) primer. ATF-2 m/m chondrocytes demonstrated a 40% reduction in pRb expression compared to cells isolated from wild type littermates (Figure 2-5A). Luciferase assays were used to examine the regulatory nature of ATF-2 in the pRb promoter region in order to correlate observed differences with the known function of ATF-2 as a transcription factor. Primary chondrocytes from wild type, heterozygous and ATF-2 m/m animals were co-transfected with plasmid DNA corresponding to a 1.3 kb fragment of the mouse pRb promoter cloned into the pGV-B luciferase reporter plasmid (Delehouzee et al., 2005). A *Renilla* expression plasmid (pRLSV40) was used to normalize data for transfection efficiency. Wild

type chondrocytes had the highest promoter activity, while ATF-2 m/m chondrocytes displayed an approximate 30% reduction in pRb promoter activity (Figure 2-5B).

To examine the role of pRb in chondrocyte development, we utilized the ATDC5 chondrogenic cell line to monitor pRb expression in response to differentiation. ATDC5 cells were grown to near confluence in DMEM/F12 (50:50) media with serum and antibiotics. Insulin-Transferrin-Selenium (ITS, Sigma) reagent was added after reaching confluence. Insulin at this concentration has been shown to induce differentiation of the cells to a more chondrocytic phenotype (Shukunami et al., 1996). Cells were then collected at various time points for mRNA expression. pRb mRNA expression was quantified using the TaqMan Expression Array as described (Pfaffl, 2001) After the cells were differentiated for 8 days there was a 4-fold increase in pRb mRNA expression compared to cells at day 0. A further increase was seen in cells at day 11, and sustained levels were seen at day 14 (Figure 2-6A, closed bars). Expression of type X collagen was low from day 0 through day 4. After day 4, type X expression levels increased until day 14 when there was an observed 6-fold increase in mRNA expression from cells collected at day 0 (Figure 2-6A, open bars). pRb promoter activity in ATDC5 cells differentiated with ITS was coincident with observed expression changes over time (Figure 2-6B). After 8 days post ITS treatment promoter activity increased 10-fold above the activity observed at the time of ITS treatment initiation (Figure 2-6B, compare D0 to D8). After day 8, promoter activity increased further and remained elevated through day 14. These data suggest that pRb expression was up-regulated in response to exogenous treatment of cells with insulin which induces chondrogenic differentiation of ATDC5 cells.

Cell cycle exit is a critical determinant in cellular differentiation. The role of pRb in regulating cell cycle progression has been well established. pRb family members p107 and p130

mediate FGF induced cell cycle arrest in chondrocytes in response to FGF signaling (Laplantine et al., 2002). Since the expression of pRb is up regulated in ATDC5 cells undergoing differentiation, we sought to correlate pRb and type X collagen expression with cell cycle exit. ATDC5 cells were collected in hypotonic lysis buffer and stained with propidium iodide for flow cytometric analysis. Supplementing ATDC5 cell culture with insulin resulted in chondrogenic cell differentiation (Shukunami et al., 1996). ATDC5 cells displayed a normal asynchronous cell cycle profile at the time of ITS supplementation (Day 0, Figure 2-7). After 24 hours (Day 1) of ITS treatment, the number of cells in S-phase increased. Cell cycle profiles of cell cultures collected at subsequent time points showed a steady decrease in the number of cells in S- and G2/M-phases. The decrease in cellular growth was coincident with the increase in the number of cells in G1/G0, indicative of cell cycle exit.

Type X collagen is a marker of terminal differentiation in chondrocytes (Kielty et al., 1985; Reichenberger et al., 1991). Type X collagen expression is restricted to the hypertrophic region within the growth plate. Immunofluorescence was used to examine the role that ATF-2 loss plays in chondrocyte differentiation. Tissues from 0-, 1- and 3-week-old wild type and ATF-2 m/m animals were fixed, processed, embedded in paraffin, and further processed for immunofluorescence. Type X collagen expression was evident in the hypertrophic region in all tissues examined. Tibial growth plate sections from wild type and ATF-2 mutant animals displayed equal staining for type X collagen at 0 weeks of age (Figure 2-8A panels a,b). Type X collagen staining was much more evident in wild type versus ATF-2 m/ m animals at 1-weekof age (Figure 2-8A panels c,d). Type X collagen staining in tibial growth plate section from 3-week-old animals was more intense in wild type as compared to ATF-2 m/m animals (Figure 2-8A panels d,e). The observed differences in type X collagen staining fluorescence intensity were

measured in each growth plate in order to provide a quantitative comparison between groups. The fluorescence intensity corresponding to type X collagen was significantly different between wild type and mutant animals at 1 and 3 weeks of age (Figure 2-8B, $P<0.005$). Growth plate sections from wild type animals demonstrated higher type X collagen expression than ATF-2 mutants at 1-weekand 3 weeks of age. These data indicate that ATF-2 mutation does affect the expression of type X collagen reflecting a plausible role in the differentiation of chondrocytes. ATF-2 affects chondrocyte differentiation indirectly through regulation of the expression of one or multiple target genes.

The regulatory function of pRb in controlling the cell cycle occurs at the G1 to S phase transition. The reduction of pRb expression observed in ATF-2 m/m animals could result in the loss of cell cycle control. We examined the proliferation of chondrocytes within the growth plate by immunostaining for Ki67, a well-known marker for proliferative cells. There was a clear increase in the number of Ki67 positive cells in ATF-2 m/m animals compared to wild-type at the 0 week time point (Figure 2-9a,b). Chondrocytes within the late proliferative and hypertrophic regions were negative for Ki67 indicative of cell cycle exit. Ki67 staining was more prevalent in wild type chondrocytes at 1-weekof age than in ATF-2 m/m littermates (Figure 2-9c,d). Finally, at 3 weeks of age, Ki67 staining was still observed in ATF-2 m/m tissues, while not detected in wild type tissues (Figure 2-9e,f).

We also tested the cell cycle behavior of primary chondrocytes by BrdU labeling. Primary chondrocytes were labeled with BrdU for 4 hours in order to determine the population of cell actively undergoing DNA synthesis. We determined a difference in G1 to S phase transition between wild type and ATF-2 m/m chondrocytes, a checkpoint controlled by pRb. Incorporation of BrdU was increased in cells from ATF-2 m/m animals (Figure 2-10). The

percentage of cells within the S-phase fraction of the cell cycle was increased in ATF-2 m/m cells. Theses results, taken together with the immunostaining for Ki67, indicate that ATF-2 loss results in aberrant cell cycle control mainly at the G1 to S transition.

Discussion

Endochondral ossification is dependent on the coordinated regulation of chondrocyte proliferation and differentiation. Previous results from our lab have demonstrated that ATF-2 acts as a global regulator of chondrocyte proliferation through regulation of the cyclin D1 (Beier et al., 1999) and cyclin A (Beier et al., 2000) promoters. We have, more recently, implicated ATF-2 in controlling apoptosis of hypertrophic chondrocytes via regulation of the bcl-2 promoter (Ma et al., 2007). Other potential target genes of ATF-2 include the pocket proteins pRb, p107 and p130. The necessary regulation of cell cycle exit and terminal differentiation of chondrocytes renders the pocket proteins ideal candidates for ATF-2 regulation. Pocket protein function involves regulation of cell cycle progression, therefore centrally modulating cellular proliferation and differentiation processes. Regulation of pocket protein expression is a plausible mechanism for ATF-2 function in chondrocytes.

Previous data from our laboratory have shown that pRb protein levels were decreased in response to ATF-2 loss (LuValle et al., 2003). Interestingly, p107 protein levels were increased in ATF-2 mutant chondrocytes while p130 remained stable. The increase in p107 levels may reflect a response to diminished levels of pRb and a compensatory mechanism whereby the increased p107 levels could interact and sequester E2F transcription factors necessary for cell cycle progression. Spatially, pRb is expressed in the resting and early proliferative region of the growth plate. However, ATF-2 deficient mice express pRb in the maturation and hypertrophic regions. The increase in pRb expression combined with the altered, more downstream spatial

expression inATF-2 m/m animals may be indicative of the important role pRb plays in regulating cell cycle exit and ultimately chondrocyte differentiation. It also suggests that the ATF-2 m/m phenotype results from not only the reduced number of proliferative cells (Reimold et al., 1996) but also a possible disruption in the normal differentiation program, specifically control of cell cycle exit. pRb plays a central role in cell cycle regulation, but also functions in terminal differentiation of numerous cell types (Cobrinik et al., 1996; Zacksenhaus et al., 1996; Walsh, 1997; Thomas et al., 2001; Fajas et al., 2002). Results from western blots indicate that p107 expression is increased in ATF-2 m/m chondrocytes while p130 levels are unaffected (Figure 2-4). Our immunohistochemical data replicate this phenomenon, demonstrating that spatial expression of p107 in the ATF-2 m/m growth plate is localized to the maturation region.

ATF-2 may control the normal differentiation of chondrocytes indirectly, by influencing the expression of pRb. There is a clear increase in pRb expression in the ATF-2 m/m growth plate that does not correlate with results from immunoblotting experiments (Figures 2-1-3 compared to Figure 2-4). However, chondrocytes within the resting zone in wild type growth plates express higher levels of pRb than those of ATF-2 m/m chondrocytes. This difference could account for the discrepancy between *in vitro* (immunoblot) and *in vivo* (immunohistochemistry) results where pRb expression is reduced in primary ATF-2 mutant chondrocytes. This phenomenon is not mirrored *in vivo* by ATF-2 mutant chondrocytes where pRb expression is not dramatically reduced. The most striking result may be the spatial change in pRb expression. pRb is expressed in the resting zone and in the late proliferative zone in wild type chondrocytes, but in ATF-2 m/m chondrocytes pRb expression is observed in the maturation zone at higher levels than in wild type, as well as throughout the hypertrophic region. There is also a clear reduction in the number of cells expressing pRb in the resting zone. The

reduction in pRb expression may be the result of sub-optimal activation of the pRb promoter following loss of ATF-2 activation. Results from promoter studies (Figure 2-5B) support this finding. Likewise, ATF-2 has been shown to be necessary for optimal pRb promoter activity (Zacksenhaus et al., 1993). The alteration of pRb expression may allow cells to enter the cell cycle earlier and exit earlier, leading to premature growth plate closure and culminating in the observed dwarfed phenotype. Our results indicate that this may be the case. Increased Ki67 immunostaining in ATF-2 m/m growth plates combined with the increase in BrdU incorporation in primary chondrocytes from ATF-2 m/m animals indicate a loss of cell cycle control which may contribute to the observed phenotype. Another contributing factor to the dwarfed phenotype of ATF-2 m/m animals is the increase of p107 expression. This increase, combined with steady levels of p130 observed in ATF-2 m/m animals, may result in a shorter period of chondrocyte proliferation, resulting in premature cell cycle exit and growth retardation.

Chondrocyte growth arrest has been largely associated with p107 and p130, with little mention regarding pRb. We were able to examine pRb expression in differentiating cells by using the ATDC5 chondrogenic cell line. We show here that pRb expression is tightly correlated with expression of type X collagen, a marker for chondrocyte differentiation. Furthermore, ATDC5 cells treated with insulin have been shown to produce increased amounts of proteoglycans, another chondrocyte differentitation marker (Phornphutkul et al., 2006). ATDC5 cells express type X collagen and pRb at higher levels following prolonged insulin treatment. These data imply that pRb plays a definitive role in chondrocyte differentiation *in vitro*.

Cell cycle exit is a hallmark of terminal differentiation. The accumulation of cells in G1/G0 of the cell cycle is a sufficient indication of removal from the cell cycle. As the number of cells in G1/G0 increase, there is a decrease in the number of cells in S- and G2/M phases of

the cell cycle. Differentiation of ATDC5 cells stimulates expression of pRb and type X collagen while simultaneously yielding an increase of cells exiting the cell cycle. These results suggest that pRb not only regulates cell cycle exit, but also plays a role in determining the normal chondrocyte differentiation program.

The temporal pattern of pRb expression in the distinct regions of the growth plate directly effect growth plate development. The altered spatial/temporal expression of pRb in response to loss of ATF-2 signaling could induce cells to exit the cell cycle prematurely. Exit from the cell cycle would likely trigger advanced terminal differentiation, ultimately leading to closure of the growth plate and a dwarfed phenotype. The reduced expression of pRb in ATF-2 mutant cells lead to delayed differentiation. The combination of reduced proliferation, delayed differentiation, and increased apoptosis in later stage hypertrophic chondrocytes is likely to contribute additively to the observed dwarfed phenotype of the ATF-2 m/m mice.

The unique ability of endochondral cartilage to undergo maturation prior to hypertrophy may delineate the role of pRb in bone development. Expression of pRb throughout the maturation region suggests a plausible role of pRb in this region. Cartilage specific deletion of pRb would best demonstrate the role of pRb in the endochondral process aside from fetal limb development (Cobrinik et al., 1996). Our lab is currently undertaking these projects to better understand the function of pRb in the growth plate.

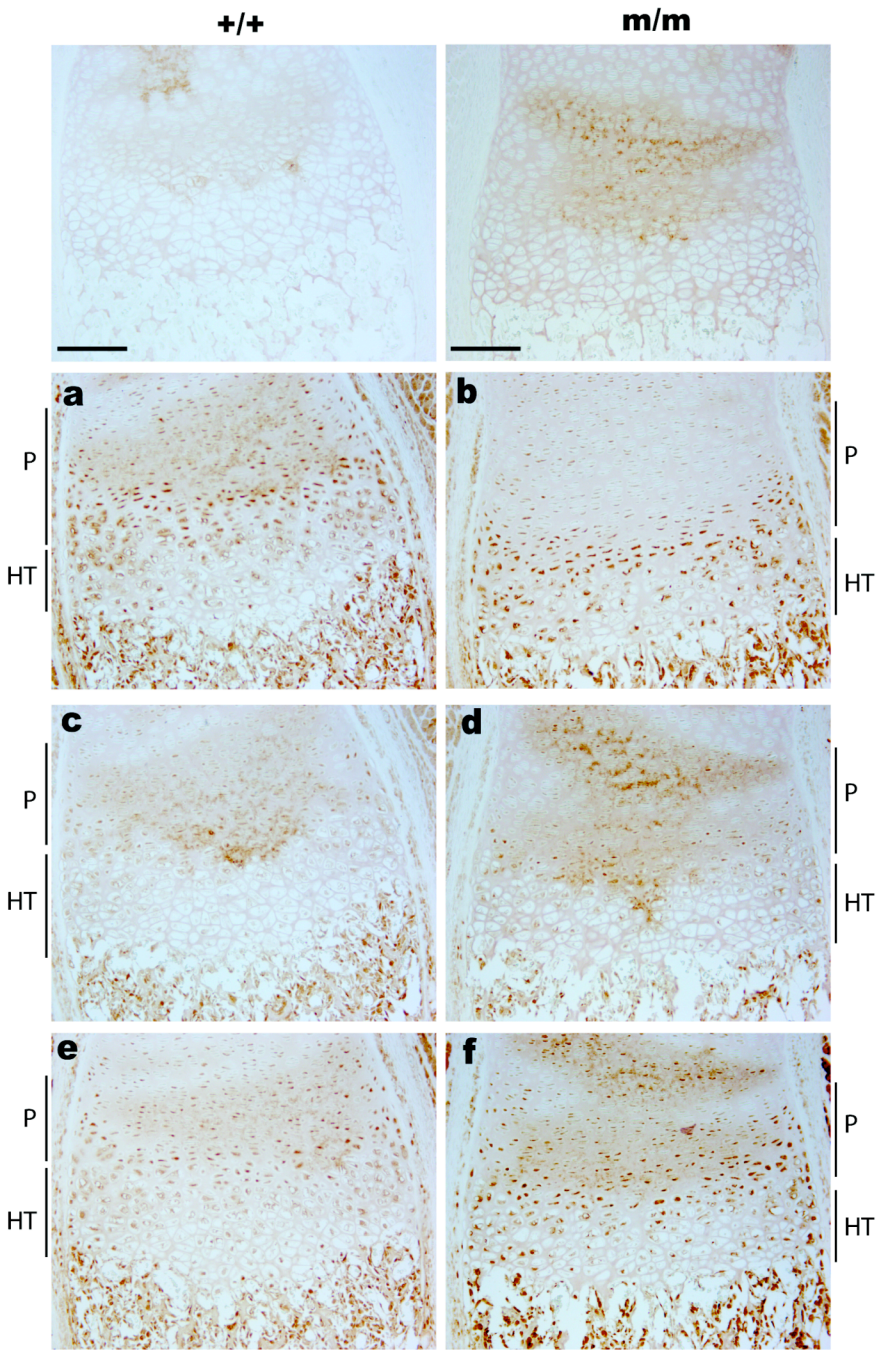

Figure 2-1. Immunohistochemical localization of pocket proteins in newborn growth plates. Knee joints from wild type (+/+) and ATF-2 mutant (m/m) newborn mice were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 μm for immunohistochemistry. Primary antibodies against pRb (a,b), p107 (c,d), and p130 (e,f) were used as noted in Material and Methods. Control sections were incubated with non-specific IgG from the same host species as primary antibodies. Micrographs were taken at 20X magnification using a Leica DM2000 microscope equipped with a Leica DFC420C digital camera.

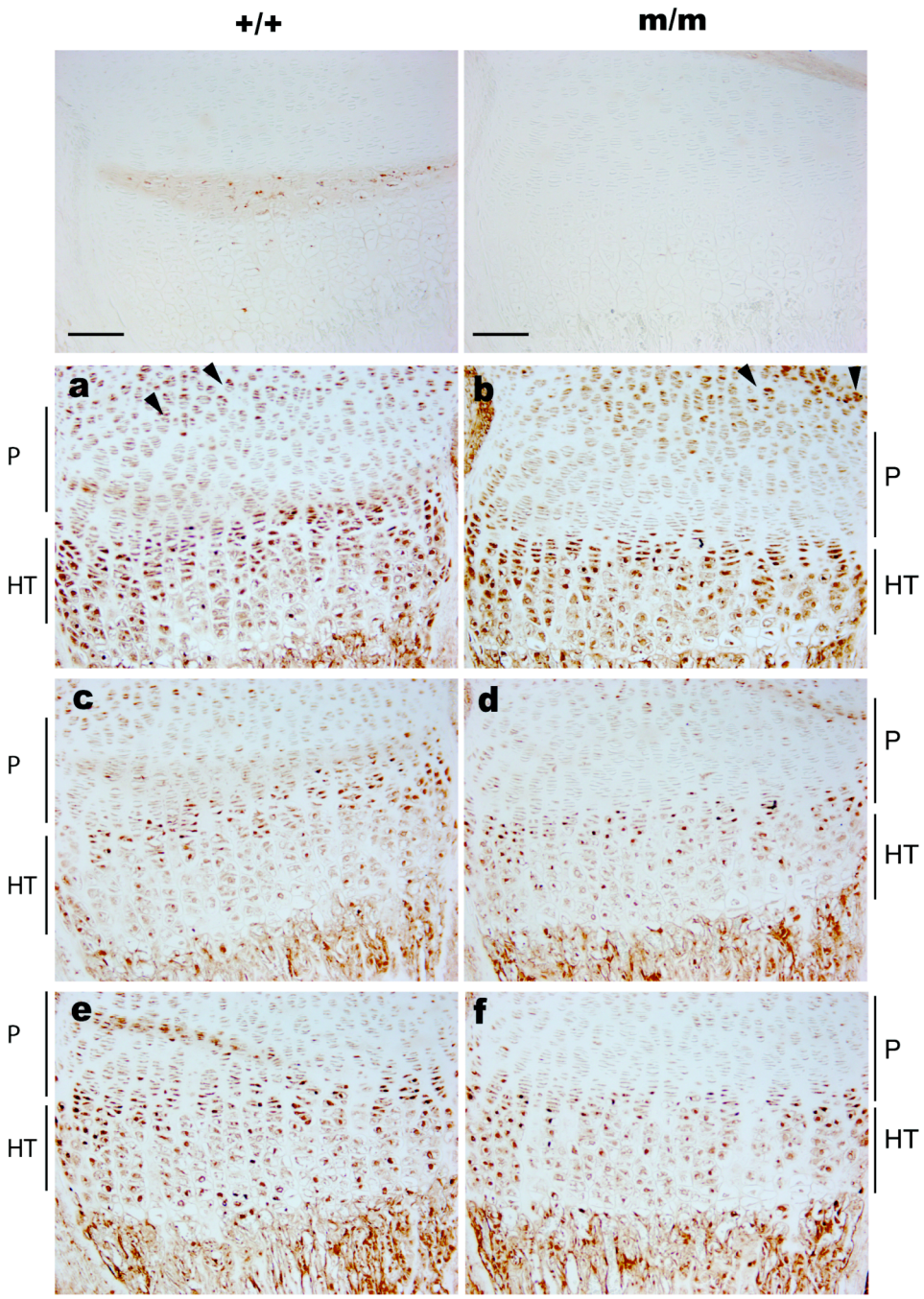

Figure 2-2. Immunolocalization of pocket proteins in 1-week old growth plates. Knee joints from 1-week old wild type (+/+) and ATF-2 mutant (m/m) mice were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 μm for immunohistochemistry. Primary antibodies against pRb (a,b), p107 (c,d), and p130 (e,f) were used as noted in Material and Methods. Control sections were incubated with non-specific IgG from the same host species as primary antibodies. Micrographs were taken at 20X magnification using a Leica DM2000 microscope equipped with a Leica DFC420C digital camera.

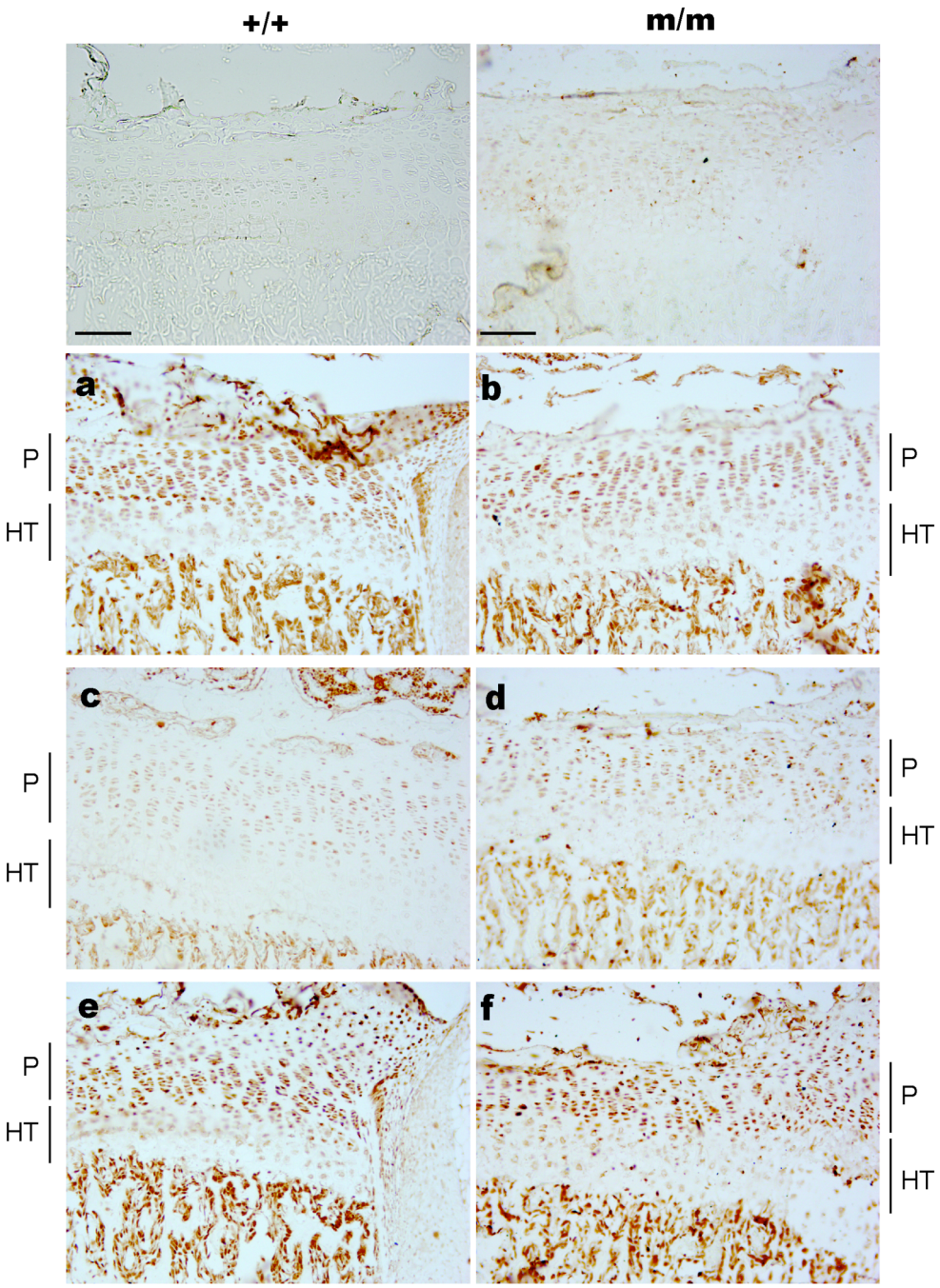

Figure 2-3. Immunohistochemical localization of pocket proteins in 3 week-old growth plates. Knee joints from wild type (+/+) and ATF-2 mutant (m/m) 3 week-old mice were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 μm for immunohistochemistry. Primary antibodies against pRb (a,b), p107 (c,d), and p130 (e,f) were used as noted in Material and Methods. Control sections were incubated with non-specific IgG from the same host species as primary antibodies. Micrographs were taken at 20X magnification using a Leica DM2000 microscope equipped with a Leica DFC420C digital camera.

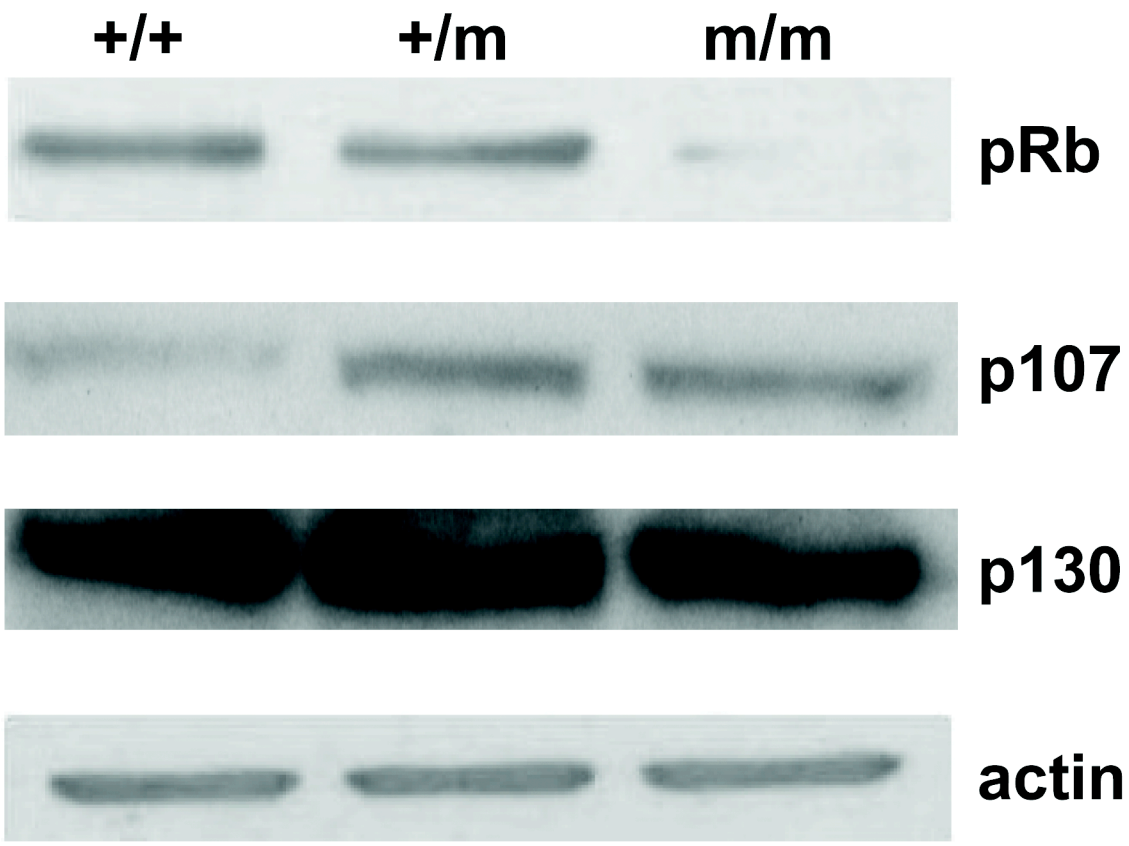

Figure 2-4. Expression of pocket proteins is altered in response to ATF-2 mutation. Total protein from primary chondrocytes isolated from wild type (+/+), heterozygous (+/-), and ATF-2 mutant (m/m) mice were analyzed by Western blot. Equal loading was demonstrated by probing with an antibody against actin.

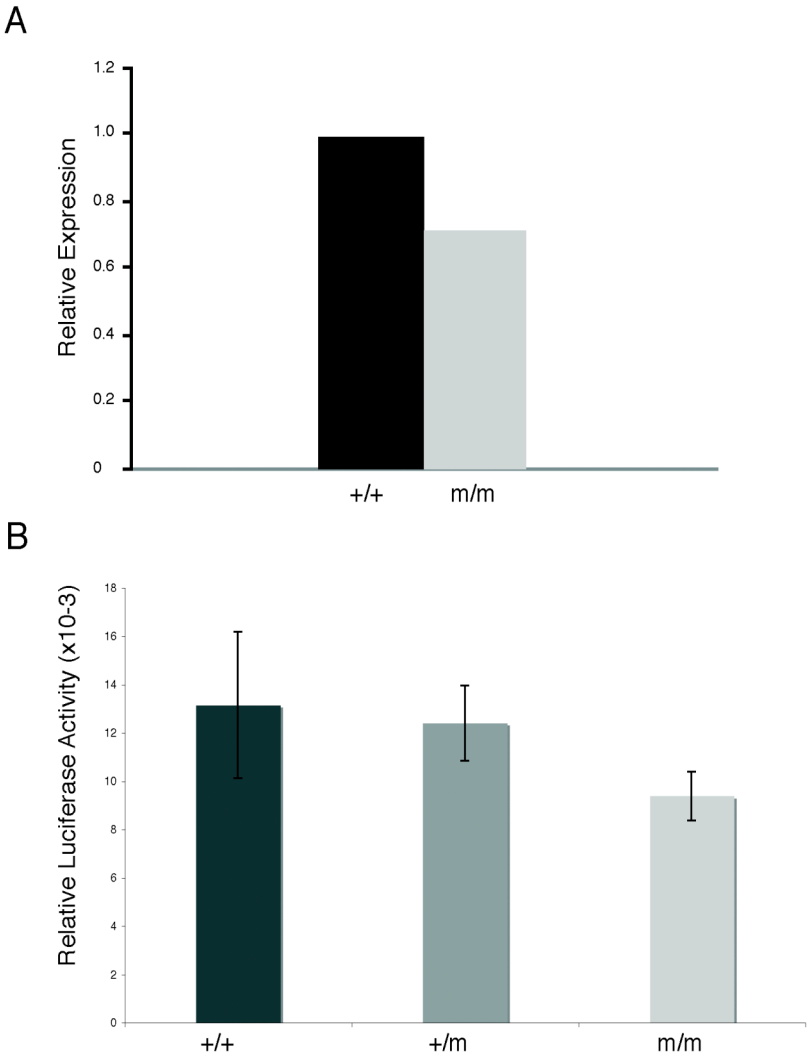

Figure 2-5. Levels of pRb mRNA are reduced in ATF-2 mutant chondrocytes as opposed to wild type. A) Total RNA was extracted from primary chondrocytes isolated from wild type (+/+) and ATF-2 mutant (m/m) mice. 2 μg of total RNA was reversed transcribed using Superscript II reverse transcriptase with an oligo-d(T) primer. Real-time PCR was performed using TaqMan Gene Expression Array kit for mouse pRb on an ABI7000 machine. Values were normalized to β-actin mRNA levels and represent means from 3 different animals run in triplicate. Comparisons between groups were made using the ΔΔCt method. B) pRb promoter activity in primary chondrocytes. A 1.3 kb portion of the pRb promoter preceding a luciferase reporter construct was transfected into primary chondrocytes isolated from wild type (+/+), heterozygous (+/m) and ATF-2 mutant (m/m) animals. Bar graphs represent the mean normalized luciferase activity of triplicate samples from three different animals for each genotype. Error bars represent SEM. pRb promoter activity in ATF-2 mutant (m/m) chondrocytes is roughly 30% decreased as compared to activity in wild type (+/+) chondrocytes.

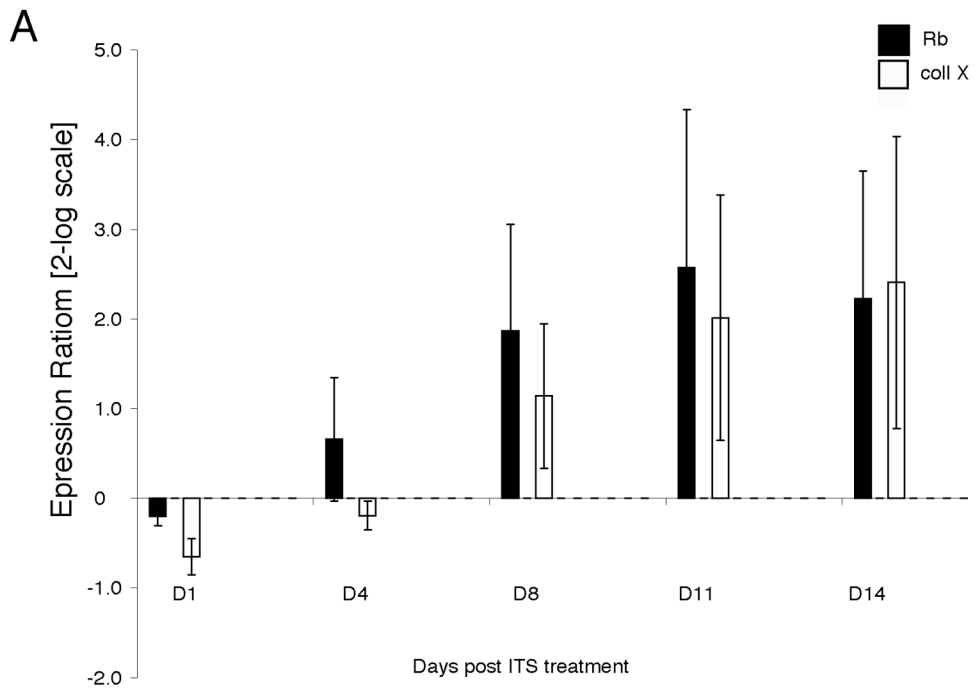

Figure 2-6. Expression of pRb mRNA is increased coincident with chondrgenic differentiation. A) ATDC5 cells were treated with insulin-transferrin-selenium reagent (ITS) to induce chondrgenic differentiation. Total RNA was extracted at various timepoints (day 0, D0; day 1, D1; day 4, D4; day 8, D8; day 11, D11; day 14, D14) after treatment initiation. Total RNA was extracted and reverse transcribed as previously described. Real time PCR for pRb was performed as previously described. Real time PCR for type X collagen was performed using Dynamo DS Sybr green qPCR kit (New England Biolabs) with primers for type X collagen and normalized to β-actin. Type X collagen mRNA expression comparisons were determined using the Pfaffl method (Pfaffl, 2001), accounting for unequal PCR efficiencies between primer sets. RT-PCR was performed in triplicate on cDNA from at least 3 different animals. B) pRb promoter activity in differentiated ATDC5 cells. Differentiated ATDC5 cells were transfected 24 hours prior to collection with the pRb promoter-luciferase construct. Cell were collected and assayed for luciferase activity on days indicated (D0, day 0= initiation of ITS treatment). Luciferase activity was normalized to Renilla luciferase activity for transfection efficiency. Bar graphs represent the mean normalized luciferase activity of triplicate samples from three independent experiments. Error bars represent SEM. pRb promoter activity is increased as differentiation progresses, mirroring the observed increased expression of pRb mRNA.

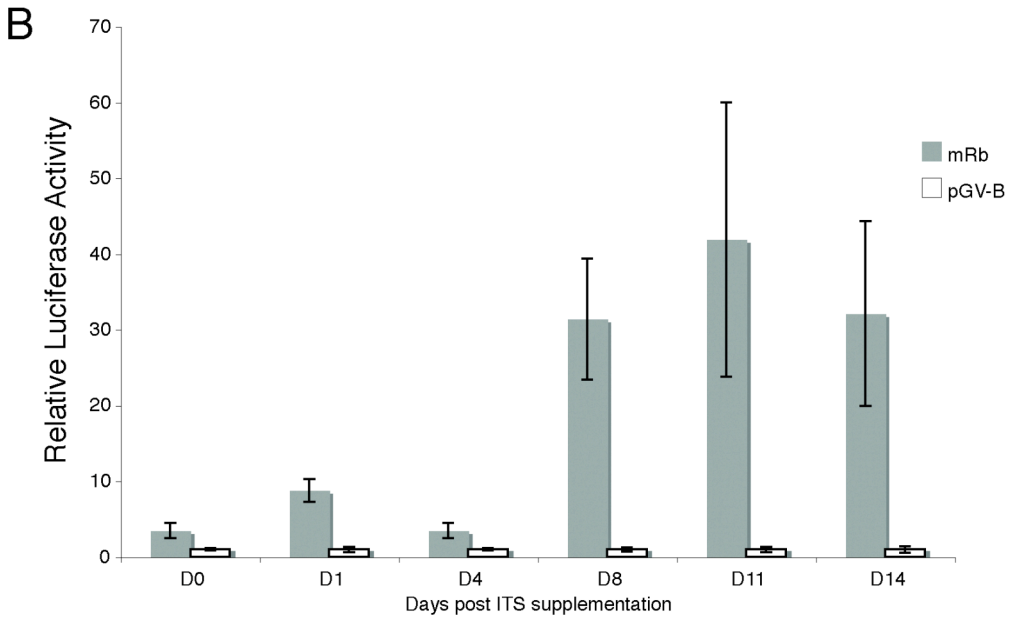

Figure 2-6. Continued

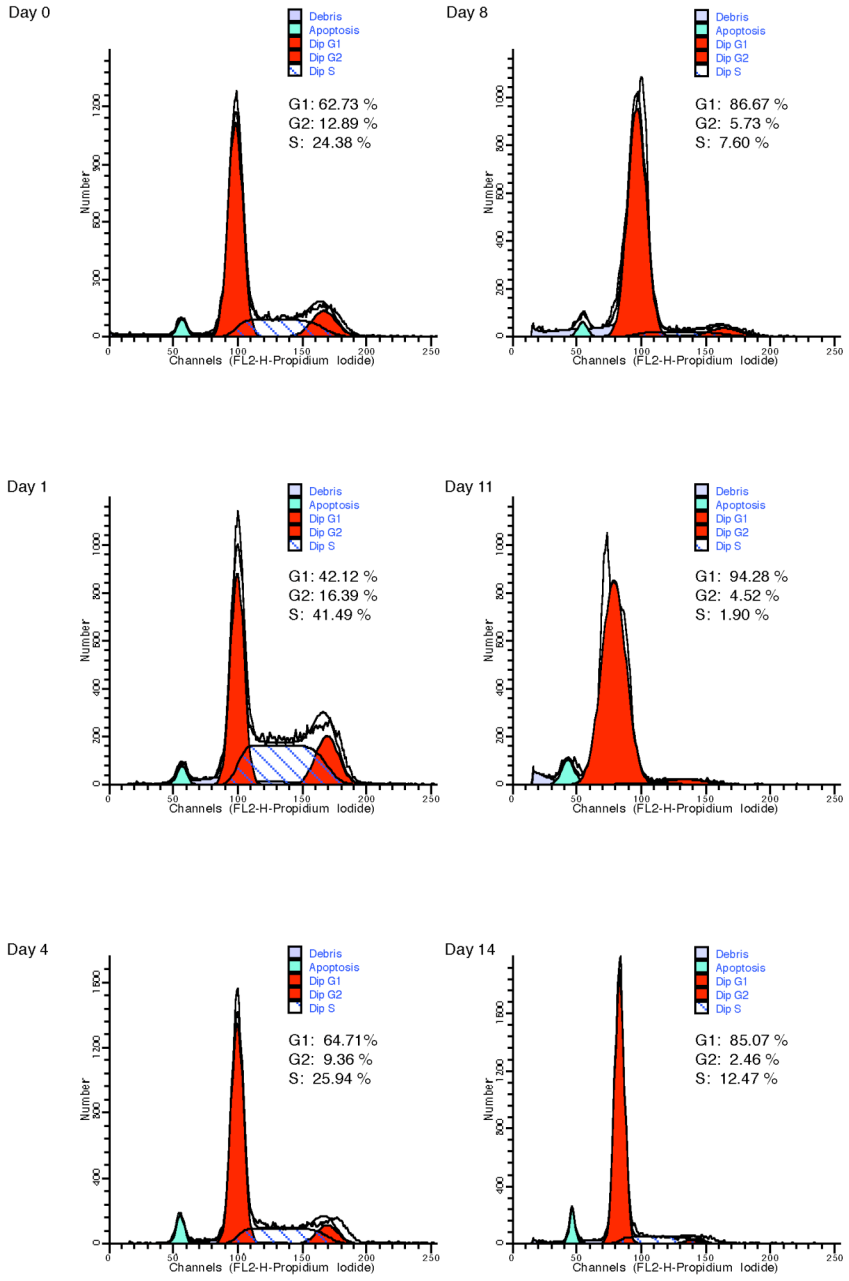

Figure 2-7. Cell cycle analysis of ATDC5 cells undergoing chondrgenic differentiation. ATDC5 cells were cultured and treated with ITS reagent to stimulate chondrogenic differentiation. At indicated timepoints, cells were collected in hypotonic lysis buffer with propidium iodide and processed for flow cytometry. DNA content was measured to determine the cell cycle profile. At least 30,000 cell nuclei were analyzed for each collected time point. Cell cycle profiles were analyzed using the ModFit software package. Accumulation of cells in G1/G0 occurs over time is indicative of cell cycle exit. At the time of ITS initiation (D0), 62% of cells were found in G1/G0. After 24 hours of ITS treatment, 42% of cells were in G1/G0. The number of cells in G1 then increases over time to a maximum of 94% at D11. The sub-G1 peak represents the sub-population of cells undergoing apoptosis.

A

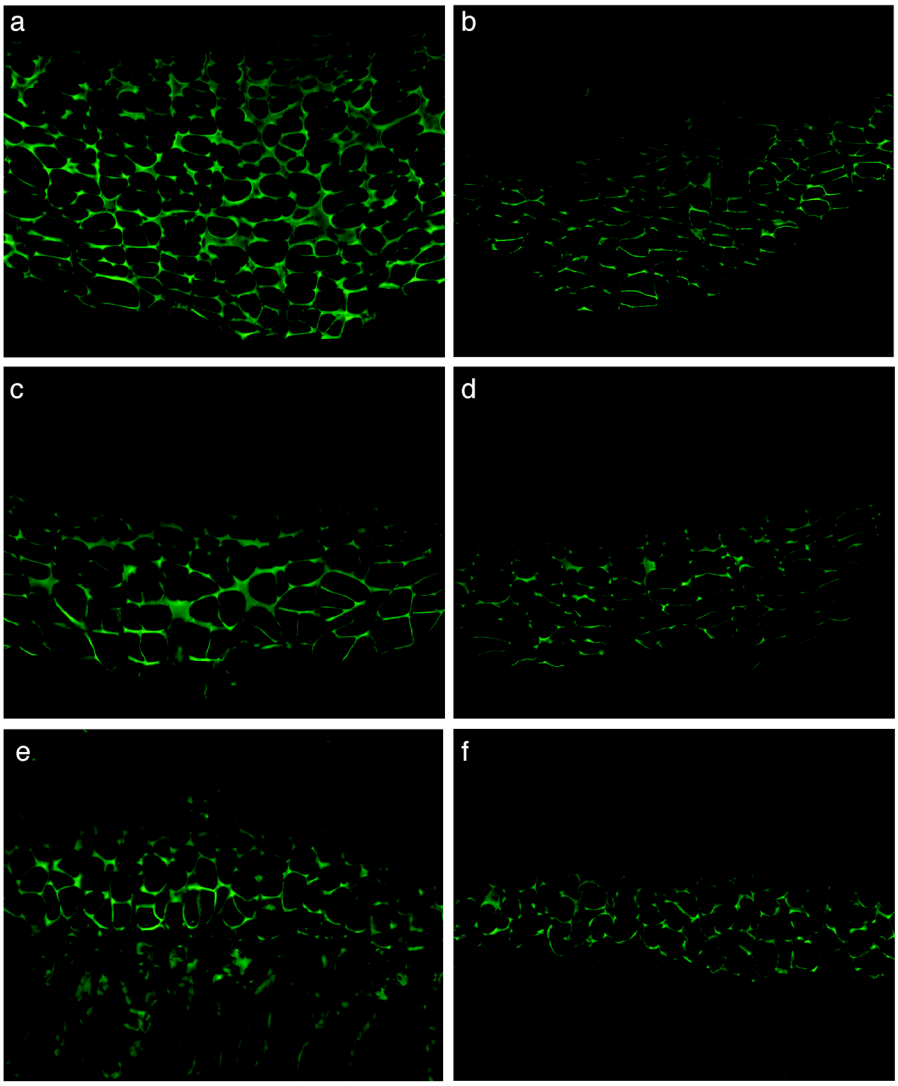

Figure 2-8. Type X collagen immunostaining in wild type (+/+) and ATF-2 mutant (m/m) hypertrophic chondrocytes. A) Growth plates from wild type and ATF-2 m/m animals were dissected, processed and sectioned for immunohistochemistry as previously described. Tibial growth plates from newborn (a,b), 1-week (c,d) and 3-week (e,f) old mice were used. Staining was restricted, as expected, to the hypertrophic region. B) Intensity of fluorescence from immunostaining was measured using IP Lab digital imaging software. Mean intensity per unit area was measured from at least three different animals per time point and genotype. Statistical comparisons were made using Student's t-test. Bars represent mean intensities $\pm$ the standard deviation of the respective mean. Asterisk (*) indicates significant difference ($P<0.05$) between wild type and mutant growth plates at the specific age.

B

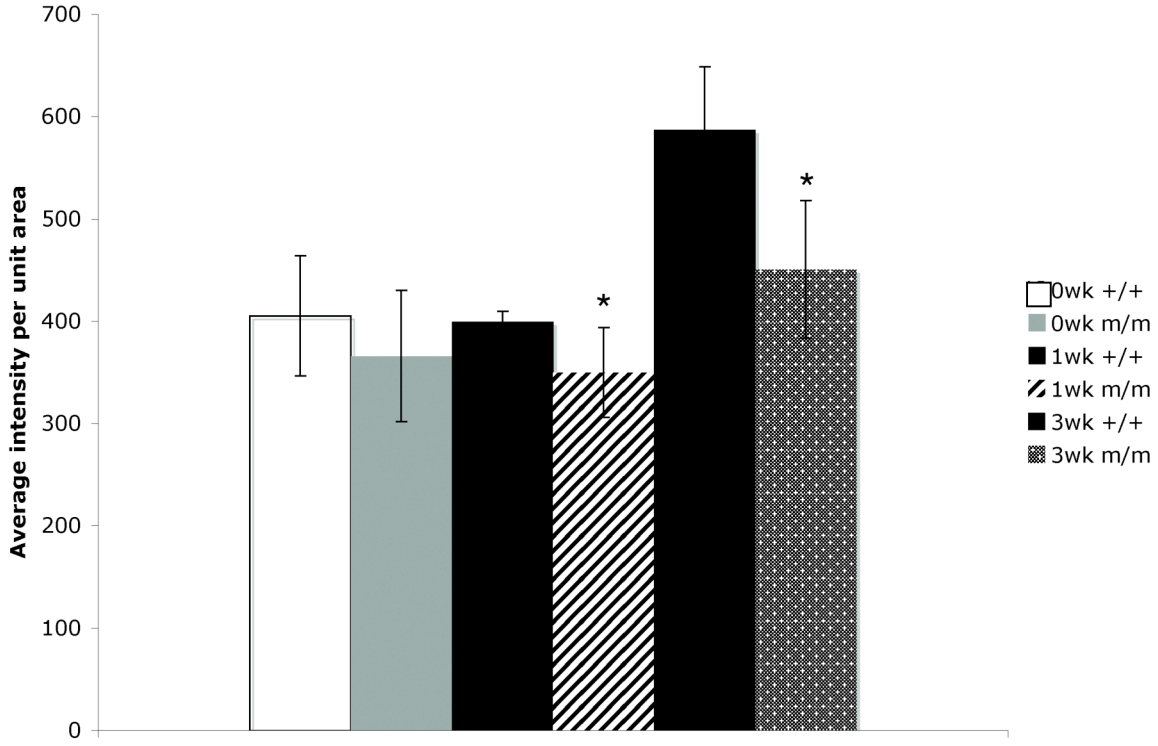

Figure 2-8. Continued

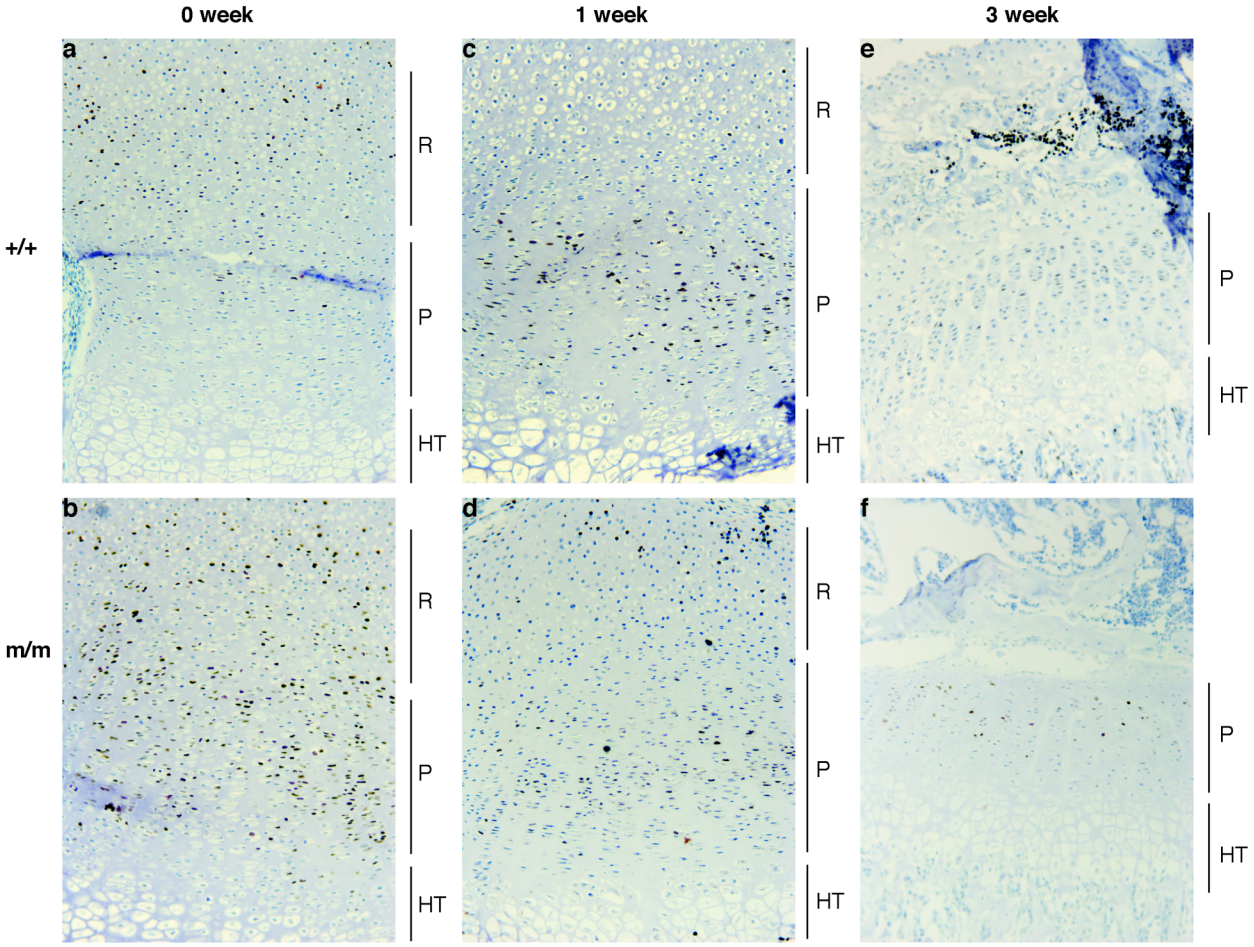

Figure 2-9. Immunostainingfor Ki67 in wild type (+/+) and ATF-2 mutant (m/m) animals. Growth plates from wild type and ATF-2 m/m animals were dissected, processed and sectioned for immunohistochemistry as previously described. Tibial growth plates from newborn (a,b), 1-week (c,d) and 3-week (e,f) old mice were used. Hematoxylin was used as a counterstain. Ki67 staining was observed within chondrocytes actively undergoing cell cycle progression.

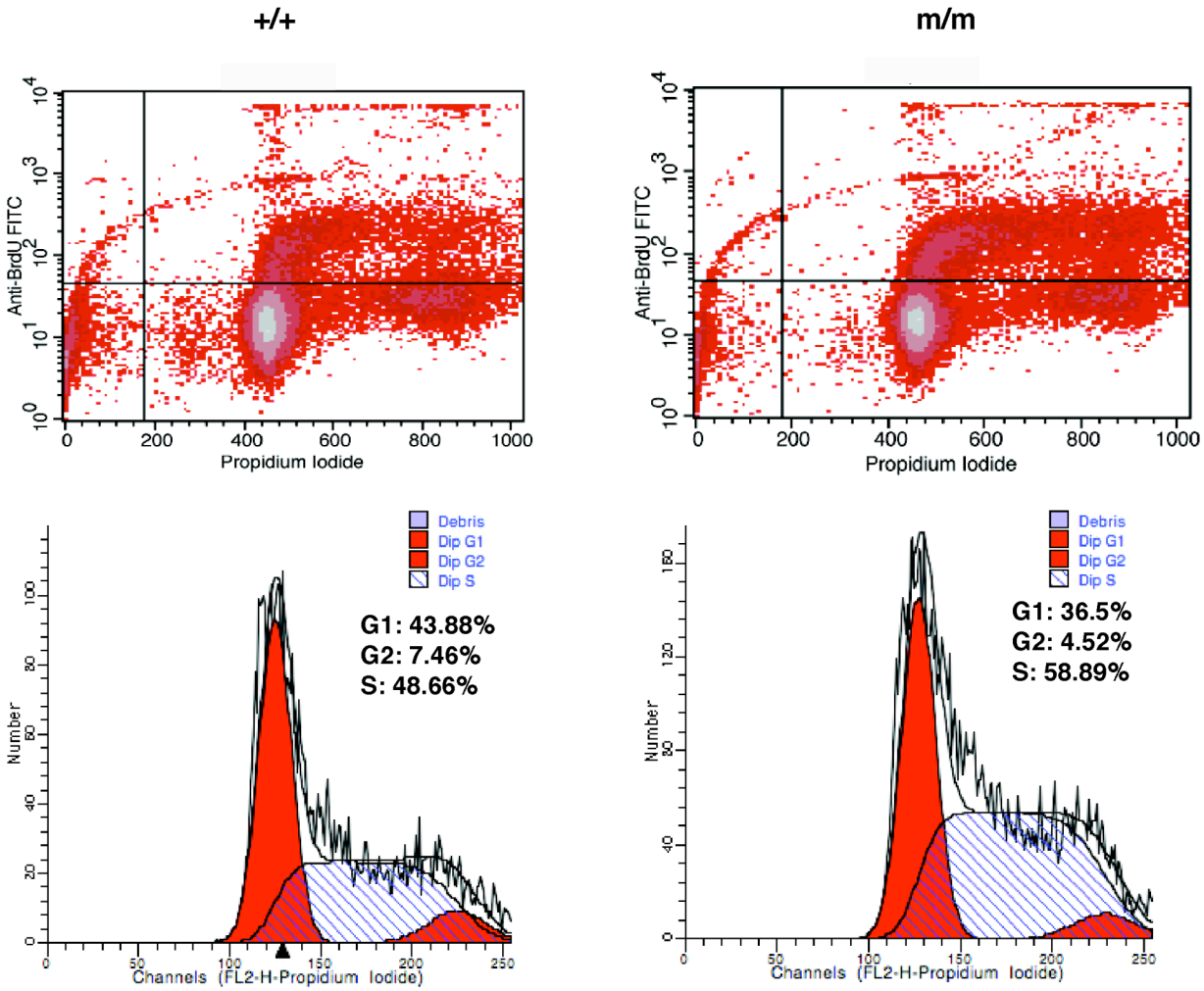

Figure 2-10. Incorporation of BrdU in wild type (+/+) and ATF-2 mutant (m/m) primary chondrocytes. Primary chondrocytes from wild type and ATF-2 mutant animals were isolated and plated in plastic cell culture plates. Cells were labeled with 10 μM BrdU for 4 hours in complete DMEM media. BrdU containing media was then removed, followed by washing with PBS and replacement with fresh media. Cells were allowed to recuperate for 6 hours before being collected and processed for flow cytometric analysis of cell cycle status, and BrdU incorporation. The number of BrdU positive cells was increased in ATF-2 m/m chondrocytes. Cell cycle analysis of the fraction of cells that were BrdU positive shows an increase in the S phase fraction.

CHAPTER 3
CONDITIONAL DELETION OF RETINOBLASTOMA IN CHONDROCYTES

Introduction

Endochondral development of skeletal long bones is dependent upon the proliferation and differentiation of chondrocytes. Ordered control of proliferation is necessary to ensure and adequate number of cells are present to promote longitudinal growth. As noted previously, several signaling mechanisms are essential in maintaining chondrocyte proliferation including the Ihh-PTHrP negative feedback loop, FGFs and TGF-β pathways. Various cellular proteins are also vital in controlling proliferation, such as cyclin D1 (Beier et al., 1999), ATF-2 (Luvalle et al., 2003) and E2Fs. Differentiation of chondrocytes is dependent on additional signaling mechanisms (for review see Adams et al., 2007). Cell cycle exit and hypertrophy of chondrocytes are two hallmarks of chondrocyte terminal differentiation. Disruption of any of the signaling mechanisms or activity of vital cellular proteins severely inhibits longitudinal bone growth.

The retinoblastoma (pRb) family of proteins include pRb, p107 and p130, which function as cell cycle regulators. These proteins regulate cell cycle progression by binding and sequestration of E2F transcription factors that are necessary for S phase progression (for review see Dimova and Dyson, 2005). Phosphorylation of these proteins modulate their ability to bind E2Fs. In the hypo-phosphorylated state, the proteins are capable of interactions with E2Fs thus inhibiting cell cycle progression. Following mitogenic stimulation, cyclins bind to their respective cyclin dependent kinases (CDKs) allowing for phosphorylation of the pocket proteins. Hyperphosphorylation of the proteins weakens its affinity for E2Fs, allowing for free E2F transcription factors to bind responsive promoters and induce transcription of vital S-phase related genes. Pocket protein family members can also inhibit E2F activity by recruiting

chromatin modifying enzymes to E2F sites thereby making DNA inaccessible to transcription factors (Brehm et al., 1998; Stiegler et al., 1998). The pocket proteins differ with respect to their expression during the cell cycle and in their affinity for certain E2Fs. pRb is expressed in both quiescent and cycling cells. pRb is present during G1 in the hypo-phosphorylated, active form complexing with E2F1 and 3 predominantly (Lipinski and Jacks, 1999). p130, in quiescent cells, is mainly found bound with E2F4 acting as a transcriptional repressive complex. As cells enter the cell cycle and progress to S-phase, p107 replaces p130 as the major partner for E2F4 (Nevins, 1998).

Inactivation of the pocket proteins leads to loss of cell cycle control in several cellular systems (for review see Giacinti and Giordano, 2006 and Genovese et al., 2006). Numerous cancers harbor genetic alterations leading to inactivation of pRb function. Loss of pRb function leads to abrogated restriction checkpoint control allowing for continuous cell cycle progression.

The pRb family has been shown to regulate terminal cell cycle exit through two distinct mechanisms. The first is a direct result of their function in regulating E2Fs and the second is unique to cellular differentiation and the interaction of pRb family members with transcriptional repressors. As cells enter terminal mitosis, p107 levels decrease whereas p130 levels increase resulting in a predominance of p130/E2F4 complexes (for review see Lipinski and Jacks, 1999). This switch is simultaneous with a decrease in free E2Fs to very low levels in fully differentiated cells. pRb activity during differentiation is much more complex and is cell type dependent. Its levels are not subject to the dramatic differences in expression as observed in p107 and p130. pRb levels can increase slightly as seen in muscle (Condorelli et al., 1995; for review see De Falco et al., 2006), remain stable as in adipocytes (Rampalli et al., 1998) or decrease as observed in most neuronal differentiation systems (Callaghan et al., 1999).

The control of proliferation and differentiation of chondrocytes is vital in maintaining normal endochondral bone development. Cell cycle related genes, such as the pRb family, have been shown to play important roles in chondrocyte proliferation and differentiation. Embryos that are devoid of p107 and p130 display perinatal lethality and pronounced defects in endochondral bone development due to abnormal cell cycle exit (Cobrinik et al., 1996). pRb has not been linked to defects in endochondral ossification. Indirectly, pRb has been shown to regulate the activity of the transcription factor Cbfa1 which has important roles in hypertrophic differentiation (Inada et al., 1999; Kim et al., 1999; Takeda et al., 2001; Ueta et al., 2001). It is possible that pRb regulates chondrocyte maturation and differentiation through regulation of Cbfa1, or other genes important for differentiation. pRb has also been shown to regulate the activity of ATF-2 (Kim et al., 1992), an important transcription factor in developing chondrocytes. E2F1 is another transcription factor that is regulated by pRb. Overexpression of E2F1 in chondrocytes results in severe defects in endochondral ossification by inhibiting differentiation (Scheijen et al., 2003). Given the role that pRb plays in regulating E2F1 activity and its role in terminal cell cycle exit required for differentiation (for review see Lipinski and Jacks, 1999), it is probable that pRb plays an integral role in chondrocyte differentiation.

A conditional knockout mouse was generated in order to disseminate the role of pRb in controlling chondrocyte proliferation and differentiation. Mice harboring a floxed pRb (Rb-loxP) allele were crossed with mice expressing Cre recombinase under the control of the type II collagen (Col2Cre) promoter. These pairings resulted in conditional functional deletion through the excision of exon 19 of the pRb gene within developing chondrocytes. Conditonal deletion of pRb allows for examination of the role pRb plays in endochondral development.

Material and Methods

Mice and Genotyping

Genetically engineered mice containing two loxP (Rb fl/fl) sites flanking exon 19 of the retinoblastoma gene as described previously (Vooijs et al., 1998) were obtained from the National Cancer Institute. Mice were housed in a specific pathogen free environment and cared for according to IACUC guidelines. Mice harboring the gene encoding the bacterial Cre recombinase under the control of the type II collagen promoter (Col2Cre +/+) were transferred from the University of Texas (a gift from Gerard Karsenty). These mice express the transgene in chondrocytes as described previously by Ovchinnikov and collegues (2000). Mice were genotyped by polymerase chain reaction (PCR). Lysis of liver tissue was performed in 200 μl of 50 mM KCL, 10 mM Tris (pH 8.0), 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% NP-40 (v/v) and 0.45% Tween-20 (v/v) and 2 μl of 20 mg/ml proteinase K. Samples were incubated at 65°C for 2 hours, followed by incubation at 95°C to inactivate proteinase K. An appropriate amount (0.5-2 μl) of DNA was used in subsequent PCR reactions. Primers used for genotyping are as follows: for Rb loxP: RbfloxFW 5'-GGCGTGTGCCATCAATG-3' and RbGen701 5'-ATCTACCTCCCTTGCCCTGT-3'; for Col2Cre: CreFW 5'-GAGTGATGAGGTTCGCAAGAAC-3' and CreRV 5'-TCGCCATCTTCCAGCAGG-3'.

Histology

Hind limbs from Rb fl/fl and Col2Cre +/-; Rb fl/fl mice were dissected from newborn mice and fixed for 24 hours in 4% paraformaldehyde or embedded in tissue OCT (TissueTek) for frozen sectioning. Sections were then transferred from paraformaldehyde to phosphate buffered saline (PBS) and processed for paraffin embedding at the University of Florida College of Medicine Molecular Pathology Core Lab. Paraffin sections were cut at 6 μm and attached to

Fisher SuperFrost Plus slides. Hematoxylin and eosin staining was performed by the Molecular Core Lab using an automated staining apparatus.

Skeletal Preparations

Newborn, 1-week, and 3-week old mice were sacrificed by CO_2 asphyxiation according to IACUC guidelines. Mouse carcasses were skinned and eviscerated. Liver tissue was collected for genotyping. Excess skeletal muscle and fat were removed. Skeletons were then immersed in Alcian Blue staining solution (0.1% Alcian Blue 8X, 90% EtOH, 10% Acetic Acid) for 1-2 days. Alcian blue stains for cartilaginous elements of the skeleton. Skeletons were then rehydrated through graded alcohols as follows: 2X 100% EtOH for 1 hr, 2X 90% EtOH for 1hr, 2 X 70% EtOH for 1 hr, 1X H_2O 1 hr. Following rehydration, skeletal preps were rinsed in NaCl solution (0.15 M NaCl), and subsewuently digested with 1% trypsin (Gibco-BRL) in NaCl solution for 3 hours to overnight depending on the size of the skeleton. Remaining skeletal muscle and excess tissue was then removed, followed by incubation with alizarin red stain in 1% KOH solution for 24-48 hours. Stained skeletons were then processed through a series of 1% KOH: glycerol solutions for long-term storage (3 parts 1% KOH, 1 part glycerol; 2 parts 1% KOH, 2 parts glycerol; 1 part 1% KOH, 3 parts glycerol).

Von Kossa Staining

Frozen embedded tissues were sectioned at 6 μm and attached to SuperFrost Plus microscope slides. Sections were fixed in ice cold 70% methanol for 10 minutes then allowed to air dry. Fixed sections were incubated in 1% silver nitrate solution (w/v) under UV-irradiation for 10 minutes. Slides were then rinsed in distilled water followed by removal of excess silver ions by incubation in 5% sodium thiosulfate (w/v) for 5 minutes. Slides were then rinsed in distilled water and counterstained with Nuclear Fast Red staining solution (VectorLabs) for 3

minutes. Stained slides were then dehydrated through an ethanol gradient and mounted for microscopic evaluation.

Immunohistochemistry

Sections from paraffin embedded tissues were cut at 6 μm and attached to charged microslope slides. Sections were processed for immunohistochemistry by dewaxing and rehydration through graded alcohols to distilled water. Microwave antigen retrieval was performed by heating in Antigen Unmasking Solution (VectorLabs) buffer for 20 minutes. Endogenous peroxide activity was blocked with 3% H_2O_2 in water for 5 minutes. Antibody incubation and detection of antibody binding was performed using the VectaStain Elite kit (Vector Labs) according to manufacturer's recommendations. Diaminobenzidine was used as a peroxidase substrate for the colorimetric reaction. Digital images were taken using a Leica DFC 720 model camera and associated capture software.

Results

Conditional knockout animals were produced in order to investigate the importance of pRb in chondrocyte proliferation and differentiation,. Mice expressing Cre recombinase under the control of the type II collagen promoter were bred to mice with loxP site flanking exon 19 of the pRb gene. Excision of exon 19 yielded a truncated pRb protein resulting in a functional deletion equivalent to a null allele (Vooijs et al., 1998; Vooijs and Berns, 1999). Double heterozygous individual males were produced and backcrossed to RbloxP homozygous females in order to maximize the number of knockout animals. Mice were genotyped by PCR for homozygosity for the presence of loxP sites and positive expression of the Cre enzyme. Conditional knockout animals were observed at normally expected ratios, indicating that deletion of pRb within chondrocytes is not embryonic lethal. Conditional pRb negative neonates were

smaller in size compared to their wild type (Cre -/-; pRb fl/fl) littermates (Figure 3-1A). At one week of age, pRb negative animals were still smaller than Cre -/-; pRb fl/fl littermates (Figure 3-1B). However, by 3 weeks of age, Cre +/-; pRb fl/fl conditional knockouts were observed to be of equal size compared to Cre -/-; pRb fl/fl littermates (Figure 3-1C).

Skeletal staining preparations were utilized to visualize the calcification and cartilage formation within the bones. Newborn littermates displayed reduced body size in response to pRb mutation. Comparison of bone length of the humerus from Cre -/-; pRb fl/fl animals and Cre +/-; pRb fl/fl conditional knockouts demonstrates the size difference (Figure 3-2A). All normal elements of the skeleton were present, however conditional mutants were smaller in size compared to their wild-type littermates (Figure 3-2 A-D). Normal formation and calcification of the ribs and vertebrae were evident (Figure 3-2 C,D). Skeletal staining of one-week old animals revealed no difference in the amount of calcified bone within the long bone (Figure 3-3 A,B). However, within the secondary ossification centers, alizarin red staining was less prominent in Cre +/-; pRb fl/fl conditional mutants than that seen in Cre -/-; pRb fl/fl animals (Figure 3-3 A,B arrows). The overall size of the bones was smaller in conditional mutants, but not as dramatic as seen in the neonates.

The histological architecture of the developing bone was examined by staining with hematoxylin and eosin. Cre -/-; pRb fl/fl and Cre +/-; pRb fl/fl animals were sacrificed and tissues were fixed and embedded in paraffin wax. Sections from the proximal tibia were examined for histology. The characteristic zones of the growth plate were present in Cre -/-; pRb fl/fl animals and sufficiently normal (Figure 3-4A). Cre +/-; pRb fl/fl growth plate sections displayed some deviations from normal (Figure 3-4B). Resting zone chondrocytes within Cre +/-; pRb fl/fl animals were smaller in cellular volume. Proliferative chondrocytes develop

characteristic columns in the normal tissues. However, the columns were less organized in the conditional mutants (Figure 3-4B). There is a clear pre-hypertrophic region in the Cre -/-; pRb fl/fl growth plate and the hypertrophic region is well defined. Tissue sections from Cre +/-; pRb fl/fl animals displayed little to no pre-hypertrophic region and a slightly disorganized and smaller hypertrophic region (Figure 3-4B). Histological examination of growth plates from 1-week-old Cre -/-; pRb fl/fl (Figure 3-4C) and Cre +/-; pRb fl/fl (Figure 3-4D) mice revealed little difference in response to pRb deletion. Interestingly, there was an observed reorganization of the growth plate in Cre +/-; pRb fl/fl tissues (compare Figure 3-4 B to C,D). At 1-weekof age, the growth plate had developed the characteristic columns of proliferating cells. This re-organization resulted in a growth plate displaying no visible differences from that of wild-type (Cre -/-; pRb fl/fl) animals.

Von Kossa staining was used to further investigate calcification of the developing bone. Whole skeletal staining revealed a reduction in the amount of calcified bone observed in Cre +/-; pRb fl/fl animals as compared to their wild type litter mates. Tissue sections from the proximal tibia were incubated with 1 % silver nitrate and exposed to UV light for 5 minutes. This procedure displaces calcium within the bone for the silver ions allowing for observation of calcified bone. Tissues from Cre -/-; pRb fl/fl animals displayed normal amounts of calcified bone equivalent to that observed in Cre +/-; pRb fl/fl tissues (Figure 3-5 A, B). To confirm or refute the observed difference in calcium staining in the secondary ossification centers seen in skeletal preparations, tissues from 1 week-old mice were processed for von Kossa staining also (Figure 3-5 C, D). Bone mineralization was not effected by pRb deletion in 1-week tissues as observed by von Kossa staining. Areas of secondary ossification were not observed to be calcified at a delayed rate as suspected from skeletal preparations. Differences among individual

animals or the precise area of sectioning may account for the lack of noticeable mineralization of secondary ossification centers.

The inherent cellular function of pRb is to regulate the transition from the G1 phase of the cell cycle to S phase. Deletion or genetic alteration of normal pRb function leads to abnormal cell cycle progression and is often associated with uncontrolled cell proliferation. Immunohistochemical localization of cellular proliferation markers were used to ascertain the effect of the pRb conditional deletion on chondrocyte growth. Sections of proximal tibia from Cre -/-; pRb fl/fl (Figure 3-6 A, B, C) and Cre +/-; pRb fl/fl (Figure 6D, E) were stained for the cellular proliferation markers, Ki67 and PCNA. Staining for Ki67 in Cre -/-; pRb fl/fl tissues was observed in early proliferative cells of the resting zone and throughout the characteristic columns of expanding chondrocytes (Figure 3-6B). Notably, staining for Ki67 is not observed in the pre-hypertrophic or hypertrophic regions. The lack of immunoreactivity is expected given that Ki67 is a cellular nuclear antigen present within cells actively engaged in the cell cycle. Chondrocytes within these areas cells have exited the cell cycle, and thus do not express Ki67. Ki67 expression in Cre +/-; pRb fl/fl tissues was observed in cells throughout the resting zone and proliferative region (Figure 3-6D). Staining was less prevalent in Cre -/-; pRb fl/fl tissues as compared to Cre +/-; pRb fl/fl tissues (compare Figure 3-6B to D). PCNA is most closely associated with cellular DNA synthesis, another hallmark of proliferating cells. Immunostaining for PCNA in Cre -/-; pRb fl/fl tissues were observed in chondrocytes in the resting, proliferative and to a lesser extent in the pre-hypertrophic and hypertrophic regions (Figure 3-6C). Expression of PCNA was also observed in the resting, proliferative, pre-hypertrophic and hypertrophic regions in Cre +/-; pRb fl/fl tissues (Figure 3-6E). Conditional deletion of pRb allows for the increase of cellular proliferation observed through the expression of proliferative

markers. Chondrocytes from Cre +/-; pRb fl/fl tissues express proliferative markers at higher levels and at earlier spatiotemporal points than in chondrocytes from Cre -/-; pRb fl/fl tissues. The sustained expression of Ki67 in chondrocytes in the pre-hypertrophic and hypertrophic regions further signifies the alteration in chondrocyte proliferation resulting from pRb deletion.

Discussion

Normal endochondral bone formation is dependent on adequate chondrocyte proliferation and subsequent terminal differentiation. Alteration of chondrocyte proliferation by genetic mutations in many cell cycle regulatory pathways results in abnormal development, such as the cyclin D1 and $p21^{Cip1/Waf1}$ genes (for review see Beier, 2005). The importance of cell cycle control is not only manifested in the proliferation of chondrocytes, but also in terminal cell cycle exit and hypertrophic differentiation. Several cell cycle-related proteins have been shown to be important in regulating cell cycle progression and terminal exit.

The retinoblastoma family of proteins is a group of proteins central to cell cycle control. Family members pRb, p107 and p130 are key cell cycle regulators that bind and sequester E2F transcription factors necessary for DNA synthesis. p107 and p130 have been shown previously to play important roles in growth plate development (Cobrinik et al., 1996; Rossi et al., 2002; Laplantine et al., 2002) through the regulation of chondrocyte growth arrest and hypertrophic differentiation. Previous results from our lab have implicated pRb in chondrocyte growth in response to ATF-2 loss (Chapter 2, Luvalle et al., 2003).

In order to assess the contribution that pRb plays in chondrocyte proliferation and differentiation, a conditional pRb deletion mutant was utilized. Conditional mutant animals displayed no gross phenotypic abnormalities besides the reduced size at birth (Figure 3-1A, and Figure 3-2). The difference in body size observed in newborn mice was overcome as the

conditional mutants grew to adulthood (Figure 3-1C). These findings suggest that pRb may be necessary for optimal chondrocyte growth in utero, but likely has a diminished post-natal role.

Members of the retinoblastoma protein family display affinity for specific members of the E2F family of transcription factors. pRb has been shown to bind preferentially to E2F 1-3a, whereas p107 and p130 bind to E2F 4-5 (for review see Macaluso et al., 2006). Evidence also exists that supports a compensation mechanism whereby p107 expression is up-regulated in response to pRb mutation or deletion (Lee et al., 1996; Jiang and Zacksenhaus, 2002; Donovan et al., 2006). Constitutive overexpression of E2F1, the preferential binding partner for pRb, results in dwarfism and skeletal abnormalities (Scheijin et al., 2003). The lack of similar defects in pRb conditional mutants as observed with E2F overexpression suggests a plausible role for a compensatory mechanism. It is possible that p107 or p130 can counteract the loss of pRb function through the binding and sequestration of E2Fs normally bound by pRb.

It is possible that pRb plays a very insignificant role in controlling chondrocyte proliferation in general. Chondrocyte growth arrest is induced by FGF signaling primarily through p107 and p130, but not pRb (Rossi et al., 2002; Laplantine et al., 2002). Disruption of p107 and p130 together prevent hypertrophic differentiation of growth plate chondrocytes whereas pRb does not appear to play a significant role (Rossi et al., 2002). The observed phenotype of pRb conditional mutant animals suggests a role for pRb upstream of chondrocyte cell cycle exit. Increased expression of proliferation markers Ki67 and PCNA in conditional mutants demonstrates that pRb functions by regulating cellular proliferation in early postnatal animals. However, the observed re-organization of growth plate chondrocytes at 1-week postnatal age indicates the insignificance of pRb in normal endochondral development. The re-organization of the growth plate may signify an increased role of p107 in response to pRb

deletion. As observed in response to ATF-2 loss, p107 may be up-regulated with the loss of pRb.

Another plausible explanation for the seemingly transient role pRb plays in endochondral development are the roles that pRb plays in other cellular processes. In addition to its role in cell cycle control, pRb is a transcription factor that can regulate transcription of target genes through regulation of respective promoter regions (Markey et al., 2002). pRb may regulate the expression and/or activity of putative growth factors required for endochondral development during late embryonic and early post natal development. pRb may directly or indirectly control the expression of p107 and/or p130 through modulating their respective promoters. Loss of ATF-2 signaling causes a reduction of pRb levels, with a coincident increase in p107 levels (Chapter 2, Luvalle et al., 2003).

The findings described here provide evidence that pRb is not required for normal endochondral bone development. However, there exists a possible role for pRb in early postnatal growth that is overcome by more important signaling mechanisms. Likely, it is a compensatory mechanism by p107 and the roles of p107 and p130, more than those of pRb, that insure normal endochodral development in pRb conditional mutants.

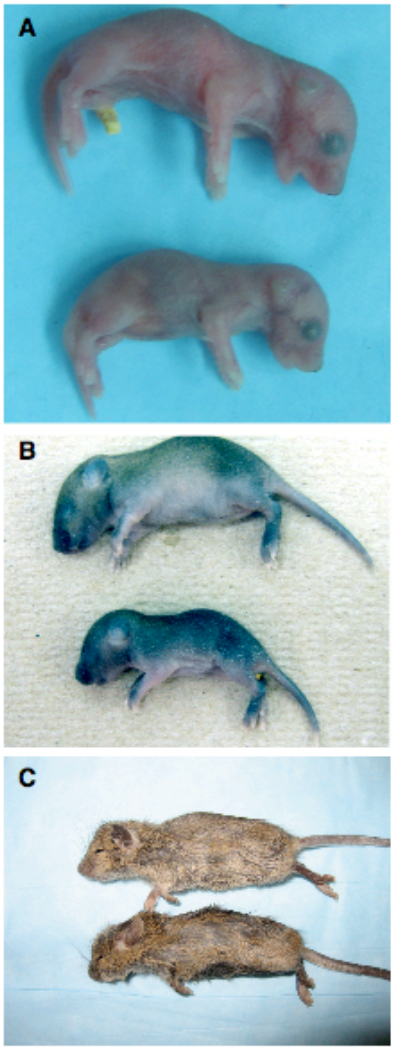

Figure 3-1. Comparison of normal and condtional mutant animals. (A) Conditional mutants were smaller at birth but displayed no gross phenotypic skeletal abnormalities in response to pRb loss. B) At 1-week of age, conditional mutants were still slightly smaller than wild type littermates. (C) By 3 weeks of age, there was no observed difference in body size between wild type and conditional mutants.

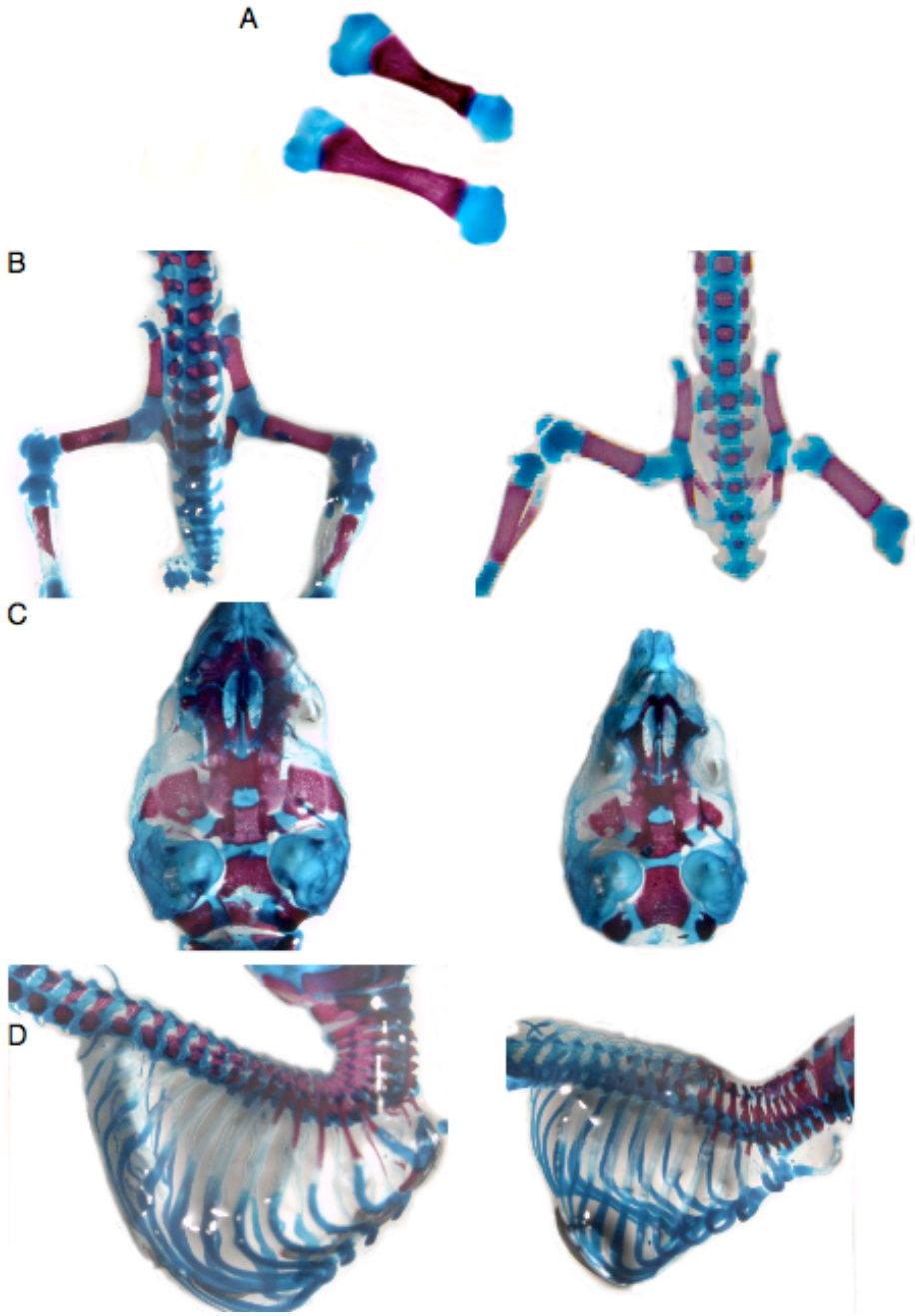

Figure 3-2. Skeletal staining of newborn wild type and pRb conditional deletion mutants. (A) Humerus bones from Cre -/-; pRb fl/fl and Cre +/-; pRb fl/fl animals demonstrate the observed size difference among littermates. (B) The hips and vertebral column of littermates showed no difference in axial skeleton development other than a reduced size. (C) Staining of the cranial bones showed no difference in craniofacial development aside from the size difference. (D) Normal development and mineralization of the ribs and vertebrae were observed wild type and pRb conditional mutants.

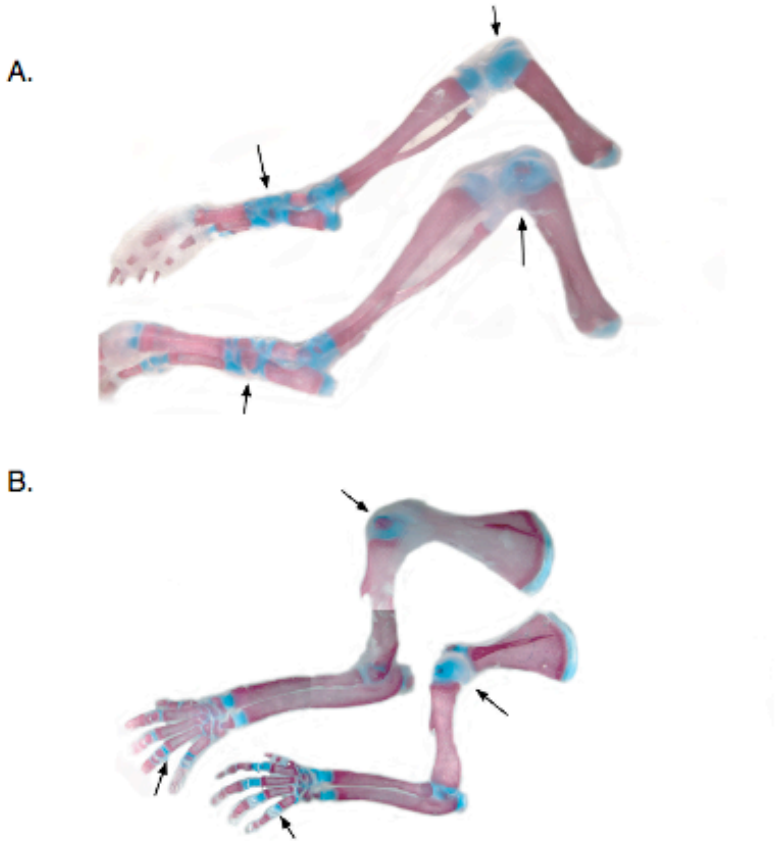

Figure 3-3. Skeletal staining of 1-week-old pRb conditional knockout and wild type mice. (A) Hindlimbs from conditonal knockout mice (upper) displayed slightly smaller overall length of the limb as compared to wildtype littermates (lower). Secondary ossification centers (arrows) were more prominent in normal tissues. (B) Forelimbs from mice with the pRb conditional deletion (lower) displayed reduction in size and delayed ossification as compared to wild-type littermates (upper). Arrows again indicate centers of secondary ossification.

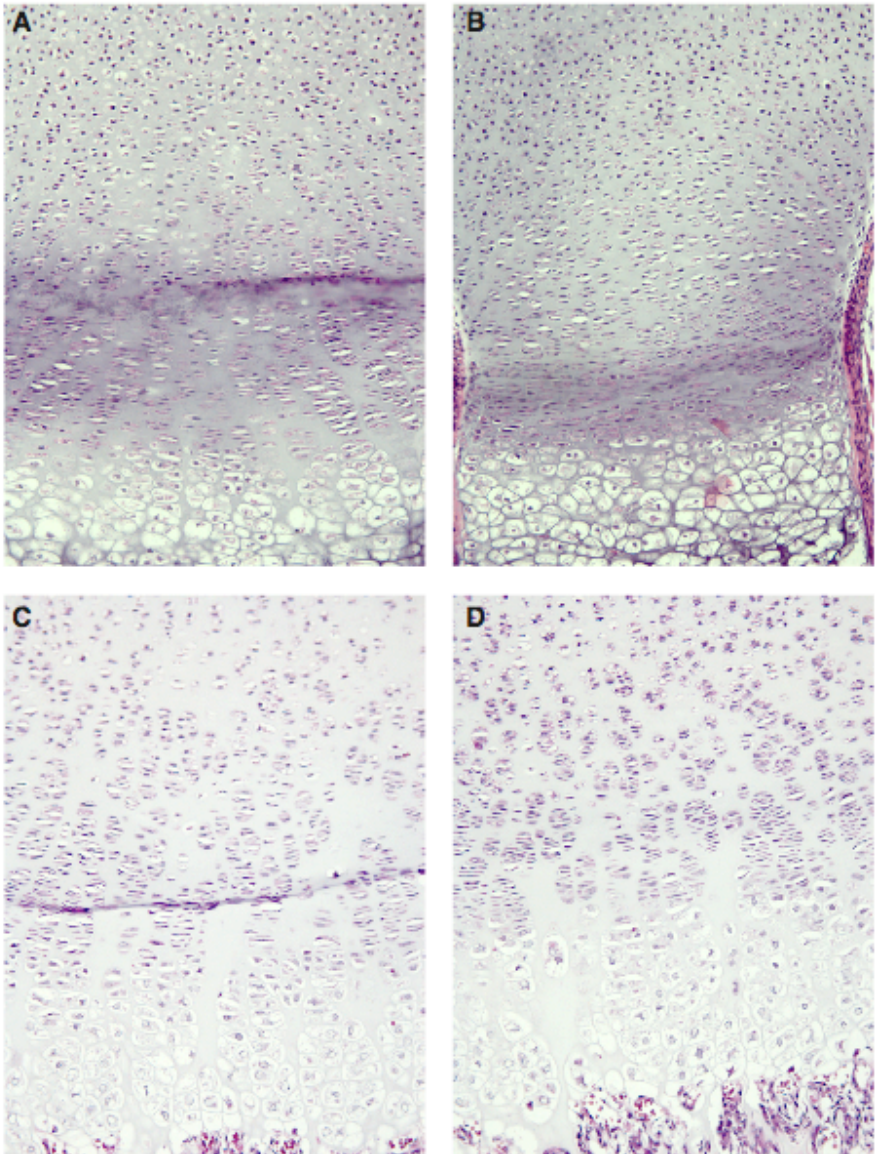

Figure 3-4. Histological examination of tibial growth plates of wild type and conditional mutants. Tissue section from proximal tibial growth plates were sectioned and stained with hematoxylin and eosin. Tissues from newborn (A, B) and 1-week-old (C, D) mice are presented. Cre-/-; pRb fl/fl mice diplsayed the normal architecture of the growth with requisite columns of proliferating chondrocytes and a clear defined hypertrophic region (A). Cre +/-; pRb fl/fl mice do not display the normal columnar formation and show increased numbers of cells (B). At 1-weekof age, Cre -/-; pRb fl/fl animals show normal growth plate development (C). Cre +/-; pRb fl/fl animals also show normal growth plate development indicating a re-organization of the growth plate (D).

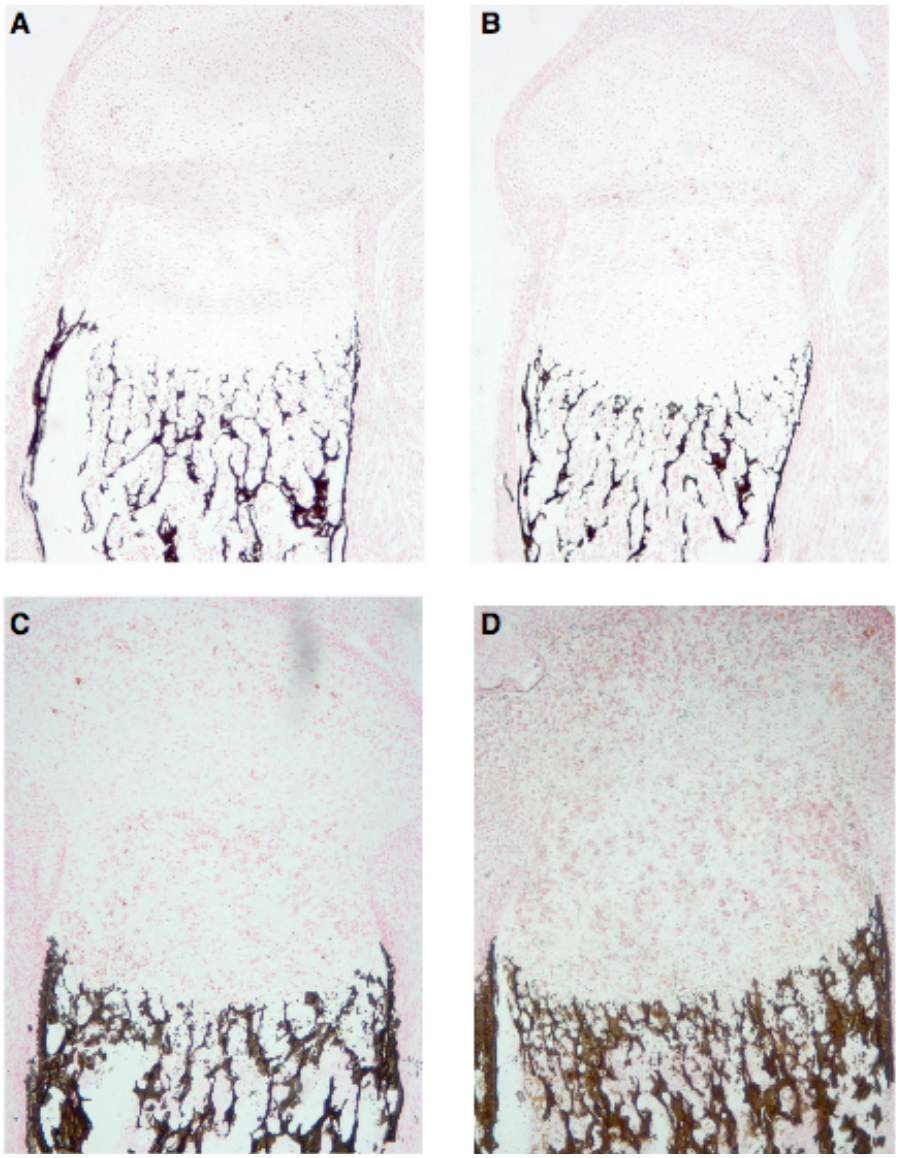

Figure 3-5. Von Kossa staining of growth plate of wild type (A, C) and conditional mutants (B, D). Frozen sections of proximal tibial growth plates were analyzed for mineralization by von Kossa staining. (A) Newborn tissue section from Cre -/-; pRb fl/fl animals showed normal calcification of bone. (B) Newborn Cre +/-; pRb fl/fl conditional mutant bone displayed similar mineralization as their wild type littermates. (C) Tissues from 1-week-old Cre -/-; pRb fl/fl mice showed normal bone mineralization. (D) Cre +/-; pRb fl/fl tissues displayed normal bone mineralization similar to that observed from wild type littermates (compare C, D).

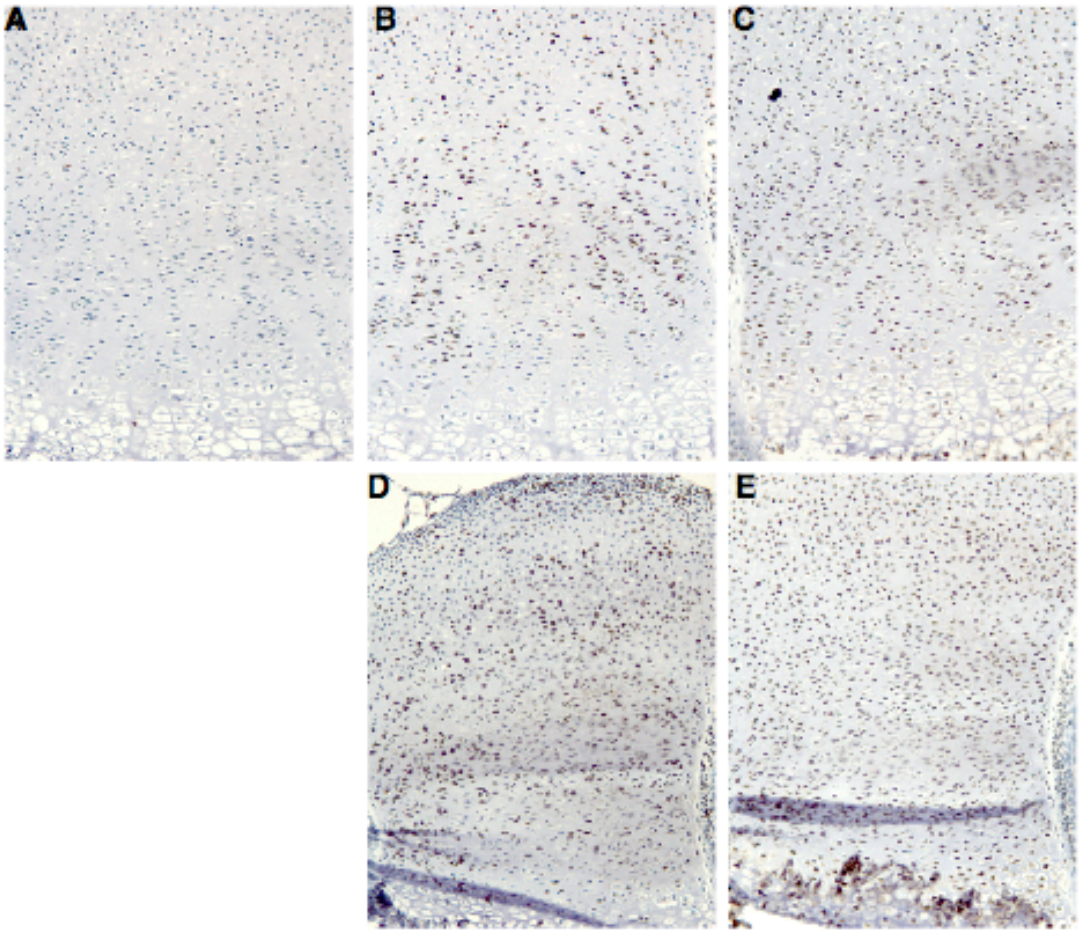

Figure 3-6. Immunolocalization of proliferation markers Ki67 and PCNA in wild-type and pRb conditional mutants. Proximal tibial sections from newborn Cre -/-; pRb fl/fl (A, B, C) and Cre +/-; pRb fl/fl (D, E) mice were processed for immunohistochemistry and counterstained with hematoxylin. (A) Control section of newborn wild type growth plate. (B) Tissue section from Cre -/-; pRb fl/fl animals immunostained for Ki67. (C) Tissue section from Cre -/-; pRb fl/fl animals immunostained for PCNA. (D) Immunostaining for Ki67 in Cre +/-; pRb fl/fl conditional mutants. An increase in Ki67 immunoreactiivity is observed in conditional mutants over wild type littermaters (compare B and D). (E) Immunostaining for PCNA in Cre +/-; pRb fl/fl conditional mutant animals. Immunoreactivity for PCNA is similar between wild type and conditional mutants (compare C and E).

CHAPTER 4
CONCLUSIONS

Overview of Findings

At the time these studies began, the role of ATF-2 in endochondral ossification was primarily viewed as a modulator of expression of genes involved in cell cycle progression. Most of the research on the role of ATF-2 in bone and cartilage development focused around promoter activation of genes involved in chondrocyte proliferation. The research presented here seeks to examine the role that ATF-2 plays in regulating the expression of cell cycle regulators, pRb, p107 and p130. Moreover, an understanding of the interplay between ATF-2 and the pocket proteins in chondrocyte proliferation and differentiation, as well as the role of pRb in regulation of chondrocyte cell cycle progression is justified in terms of developing a better understanding of how ATF-2 controls growth plate development and the role of pRb in skeletal growth.

Building on the knowledge of the function of ATF-2 in control of promoter activity by binding the CRE located in target gene promoters, we examined the expression of possible target genes pRb, p107 and p130. Given the cellular function of these proteins as cell cycle regulators and the alteration of pRb expression in response to ATF-2 loss, we confirmed the importance of pRb in chondrocyte differentiation using an *in vitro* chondrogenic system (Chapter 2). Chondrogenic differentiation of ATDC5 cells induced cell cycle exit. This was temporally associated with increased expression of pRb and type X collagen mRNA, and supported a role for pRb in chondrocyte differentiation (Chapter 2). Building upon the observed role of pRb in chondrocyte differentiation *in vitro*, we sought to characterize its role *in vivo*. Conditional deletion of pRb in chondrocytes did not produce gross skeletal abnormalities, and skeletal growth was affected transiently, at best (Chapter 3). A reduced size was observed at birth, but was overcome by 3 weeks of age. Examination of the histology and immunolocalization of

proliferation marker Ki67 and PCNA revealed increased chondrocyte proliferation in conditional mutants (Chapter 3). The difference in histology observed between conditional pRb mutants and normal animals was resolved by 1-weekof age. These studies suggest that the role of pRb within growth plate development is insignificant as compared to that of p107 and p130.

Questions and Future Studies

As is the case with any scientific research, when results are obtained, more questions arise and future directions are explored. Expression of ATF-2 is predominantly localized to the resting and proliferating zones of the growth plate suggesting its function is concentrated in those areas. However, loss of ATF-2 function yields effects on the growth plate at distal sites from those having ATF-2 expression (Chapter 2; Ma et al., 2007). ATF-2 is expressed in the resting and proliferating regions of the growth plate, yet ATF-2 loss results in alteration in the hpyertrophic zone, a region lacking ATF-2 expression. The reduction in type X collagen expression and overall size of the hypertrophic region observed in ATF-2 deficient animals suggests a role for ATF-2 in controlling the differentiation of growth plate chondrocytes. It is still unknown how ATF-2 regulates this process. It is possible that through the altered expression of the pocket proteins that chondrocytes exit the cell cycle earlier. Premature chondrocyte cell cycle growth arrest combined with the decreased expression of bcl-2 in ATF-2 deficient animals (Ma et al., 2007) may lead to increased apoptosis within the hypertrophic region, ultimately resulting in a reduction in hypertrophic region size. The reduced expression of type X collagen observed in ATF-2 deficient animals (Chapter 2) may be an indirect result of the reduced size of the hypertrophic region.

The increase in p107 levels in response to ATF-2 deficiency is another unresolved topic. Expression of p107 is increased in response to pRb loss (Lee et al., 1996; Jiang and

Zacksenhaus, 2002; Donovan et al., 2006). The p107 promoter region contains a putative CRE that may be a target for ATF-2 regulation. The reduction of pRb levels may result in a transcriptionally activated p107 promoter. pRb can repress transcription of genes by forming a repressor complex on target promters. It is possible that the p107 promoter is a target of pRb repression. Lengthy promoter deletion as well as chromatin immunopreciptation studies would be necessary to determine if pRb can regulate p107 promoter activity in chondrocytes. Secondary to this would be the elucidation of the plausible compensatory mechanism that may exist between pRb and p107. Overexpression of E2F1 results in skeletal defects (Scheijin et al., 1003) suggesting that p107 cannot compensate for pRb while pRb is still expressed. It would be interesting to determine if E2F1 overexpression results in similar anomalies in pRb conditional mutants, or if p107 is highly up-regulated and compensates for pRb loss. Similarly, it would be beneficial to examine which E2F family members interact with p107 in ATF-2 deficient chondrocytes. These experiments would demonstrate how p107 may compensate for the reduction in pRb expression. It would also be informative to investigate the role of p130 in compensating for pRb loss.

Another unresolved issue is the discrepancy between the observed decrease in chondrocyte proliferation (Reimold et al., 1996) and the loss of pRb expression in ATF-2 mutant animals. The two results seem contradictory. Also, our results from pRb conditional mutants (Chapter 3) suggest that there is a transient increase in proliferation seen in newborn growth plate chondrocytes. Again, the importance of p107 in chondrocyte proliferation is demonstrated. The increase in p107 expression seen in ATF-2 mutant animals could compensate for pRb loss, and provide increased cell cycle control and possibly premature growth arrest. Contributing to this could be the role that ATF-2 plays in regulating the expression of cyclin D1 (Beier et al.,

1999). Reduced cyclin D1 expression may influence the phosphorylation status of p107 which in turn would allow for a hypophosphorylated state whereby p107 can bind E2Fs and contribute more greatly to chondrocyte growth arrest. In the case of pRb conditional mutants, chondrocytes still express a truncated form of the protein. It is possible that the truncated protein still confers some properties that may influence p107 expression. Studies investigating the occupancy of the p107 promoter in the pRb conditional mutants would be informative to determine changes in transcriptional activity.

In conclusion, the studies described in this dissertation have identified the regulatory role ATF-2 plays on the expression of the pocket proteins and provided evidence for the importance of pRb in regulating chondrocyte cell cycle progression. These findings will affect future efforts of scientists investigating cartilage development and growth plate biology.

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BIOGRAPHICAL SKETCH

Dustin Shawn Vale-Cruz was born in Providence, Rhode Island, to Lani Vale-Cruz and Daniel Miguel Jr. He attended Bishop Hendricken High School in Warwick, RI. He received a Bachelor of Science degreein animal science from the University of Rhode Island where he met his wife, Kristi. After graduation, he enrolled in graduate school at the University of Florida (UF) in the department of Animal Science. He received his Masters of Science degree in animal science in 2001. He worked for 2 years prior to returning to the UF to pursue his doctoral degree in the Interdisciplinary Program for Biomedical Research in 2003. In summer 2004, he joined the Anatomy and Cell Biology Department under the supervision of Dr. Phyllis LuValle. In 2005, Dustin and Kristi celebrated the birth of their daughter, Avery Ilene.