Delineating the molecular mechanisms regulating chondrogenesis

by

Konstantina Karamboulas

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Abstract

Sox9, (SRY-type HMG box), has been shown to play a critical role throughout chondrogenesis. Haploinsufficiency of *Sox9* in humans leads to a skeletal malformation syndrome known as campomelic dysplasia. To understand the regulation of *Sox9* during chondrogenesis, the developing mouse limb was used to identify and characterize regulatory regions within the *Sox9* promoter. Luciferase-based reporter assays in mouse revealed a proximal promoter spanning – 2 kb from the transcriptional start site, while mobility shift assays demonstrated that a CCAAT motif is involved in the transactivation of *Sox9*. Moreover, luciferase-based reporter assays revealed a proximal promoter spanning – 4 kb in *Fugu rubripes*, and potential regulatory regions spanning the remainder of the promoter. Comparison of mammalian *Sox9* upstream intergenic sequences to that of *Fugu* has identified 5 conserved regions that are contained within 18 kb of upstream *Fugu* sequence. Analysis of the transcriptional activity of these sequences has led to the identification of regulatory elements within the *Sox9* promoter.

Several studies also provide evidence of a role for wingless (WNT) and bone morphogenetic (BMP) signaling molecules in the regulation of chondrogenesis. TCF/LEF-*LacZ* reporter mice show activated canonical WNT signaling distributed throughout the embryonic age (E) 9.5 forelimb. At later stages, *LacZ* expression becomes confined to distal regions of the limb bud. Previous studies have demonstrated that canonical WNTs inhibit chondrogenesis. Our studies demonstrate that treatment of cultures derived from E11.5 proximal limb buds with the canonical WNT, WNT3a, inhibits chondrogenesis. However, treatment of cultures derived from E9.5 and distal E11.5 limb buds with WNT3a stimulates chondrogenesis. Quantitative PCR (qPCR) also demonstrates that WNT3a modulates a number of genes expressed throughout chondrogenesis. To gain insights into BMP function in the early limb, we have characterized BMP action in sub-populations of cells from the E10.5 limb. Surprisingly, BMPs were found to

inhibit cartilage formation in immature cells, while promoting cartilage formation in more mature cells. Transcriptional profiling coupled with qPCR and time course analyses revealed that the extent of induction of *Gatas* by BMPs was associated with its stimulatory versus inhibitory activity. Further, SOX9 activity was inhibited following over-expression of *Gatas*.

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Co-Authorship Statement

CHAPTER II: Identification of GATA Factors as Targets of BMP Signaling in Early Limb Mesenchyme & Chapter III: Canonical WNT Signaling Exhibits Stage-dependent Activities in Skeletogenesis

All of the following were carried out by Konstantina Karamboulas and T. Michael Underhill: identification and design of research program, performing the research, data analyses and manuscript preparation.

CHAPTER IV: Comparative Functional Analysis of the Mouse and *Fugu Sox9* Promoters. All of the following were carried out by Konstantina Karamboulas and T. Michael Underhill: identification and design of research program, performing the research, data analyses and manuscript preparation.

A former MSc. student, Leslie Hodge, carried out the Electrophoretic mobility shift assays (EMSAs).

CHAPTER I: Introduction

1.1 Significance

Cartilage has many important functions in both the developing embryo and the mature adult. During development, cartilage provides the foundation for most of the adult skeleton whereby a cartilaginous template is initially laid down and subsequently mineralized into bone. In adults, remnants of this embryonic cartilage are found at the articular surfaces of bone. Abnormal cartilage development leads to a wide range of birth defects including chondrodysplasias, some types of dwarfisms, and craniofacial and skeletal abnormalities (Mundlos and Olsen, 1997). In adults, degeneration of articular cartilage results in arthritis. Unfortunately cartilage has limited regenerative capacity, such that even relatively minor joint injuries cause permanent damage to cartilage, culminating in arthritis. Osteoarthritis, or degenerative joint disease, is one of the most common forms of arthritis that affects approximately 10% of the population in Canada and this rises to more than 85% for those past the age of 65.

Our studies are aimed at identifying the molecular mechanisms that regulate cartilage formation. It is anticipated that this knowledge will provide the background necessary for the development of novel therapeutics to stimulate cartilage repair for the treatment of joint diseases such as osteoarthritis.

1.2 Vertebrate limb development

The formation of the vertebrate skeleton relies on two developmental processes, intramembranous ossification and endochondral ossification. Intramembranous ossification occurs by the direct ossification of embryonic mesenchymal tissue and ultimately gives rise to a small subset of bones, including some craniofacial and flat bones, especially those found in the skull, scapula and hip (Couly et al., 1993; Hall and Miyake, 1992). However, most of the vertebrate skeleton (axial and appendicular) develops through endochondral ossification, whereby a cartilaginous template is initially formed for subsequent mineralization and

replacement by bone (Figure 1.1) (Olsen et al., 2000). Cartilage formation, or chondrogenesis, involves a series of events including the condensation of mesenchymal chondroprogenitor cells. Indeed, the condensation of prechondrogenic mesenchymal cells represents the earliest stages of skeletal patterning and are the forebearers of mature skeletal elements (Hall and Miyake, 1992; Hall and Miyake, 1995; Hall and Miyake, 2000). Following condensation, chondroprogenitors within the condensation differentiate into chondroblasts, which initiate the synthesis of a matrix rich in type II collagen (*Col2a1*).

Following chondrogenesis, endochondral ossification ensues with the terminal differentiation of chondrocytes to the hypertrophic phenotype, cartilage matrix calcification, vascular invasion and ossification (Ballock and O'Keefe, 2003; Colnot and Helms, 2001; Ferguson et al., 1998). This process is initiated when cells in the central region of the cartilage analgen differentiate into large, hypertrophic chondrocytes. Adjacent to this zone of hypertrophic cartilage, cells proliferate along the longitudinal axis of the bone which results in the formation of “columns” of chondrocytes, before cells exit the cell-cycle and gradually differentiate into hypertrophic chondrocytes. These zones of proliferating, pre-hypertrophic and hypertrophic chondrocytes form the cartilage growth plate which controls the longitudinal growth of endochondral bones (Ballock and O'Keefe, 2003). Subsequently, hypertrophic cartilage is mineralized, invaded by blood vessels and degraded by osteoclasts, while hypertrophic chondrocytes undergo apoptosis. The remnants of this calcified matrix serve as a template for osteogenesis.

Numerous factors have been identified and have been shown to regulate the different stages of chondrogenesis and subsequent endochondral bone development; however, the intracellular mechanisms involved are not clearly understood (DeLise et al., 2000).

1.3 Chondrogenesis

During embryogenesis, the vertebrate limb is derived from the dual contribution of the

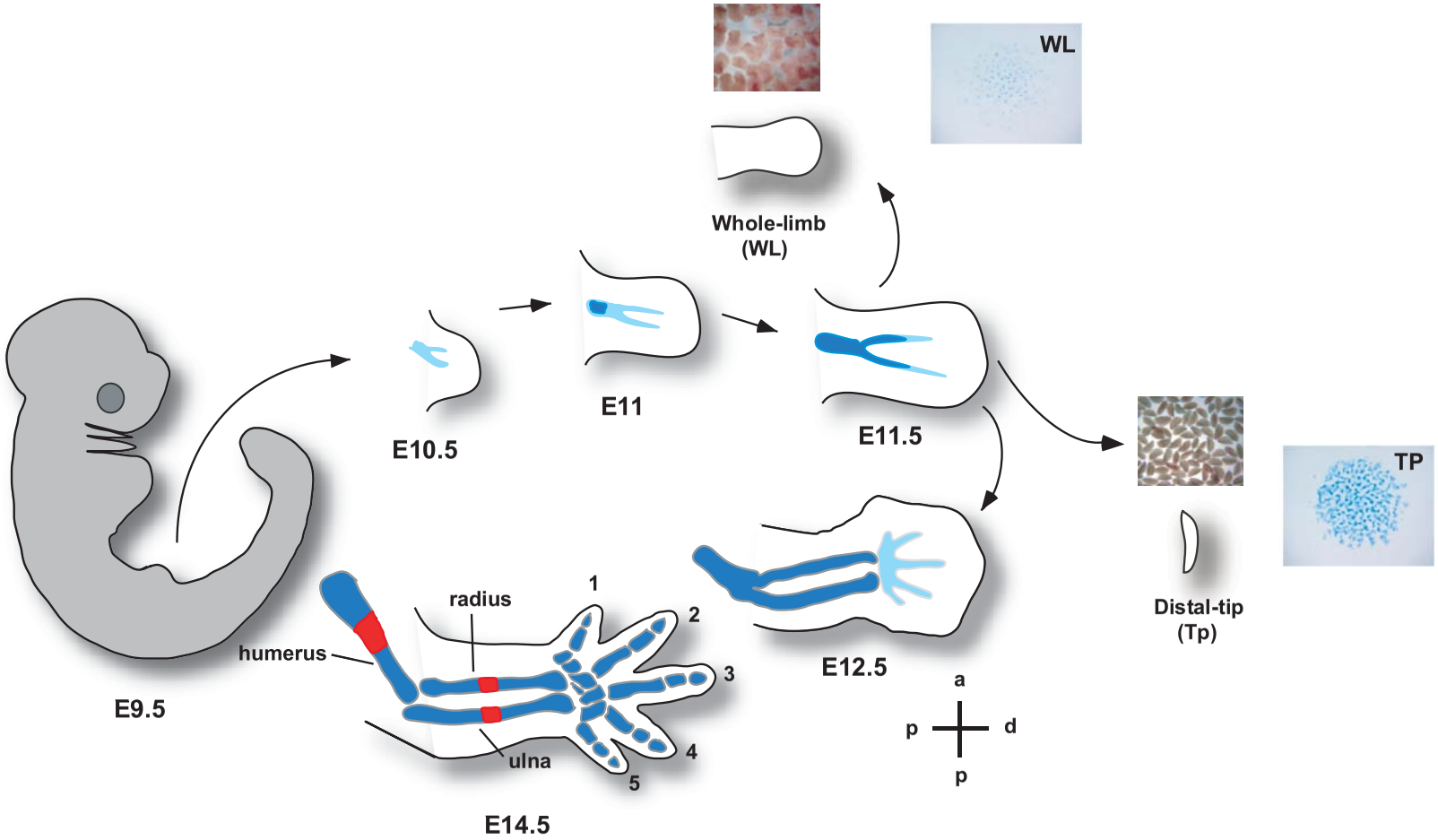

Figure 1.1: Overview of murine skeletal development and derivation of primary limb mesenchymal cultures. Cultures are established by removing and dissociating the limb mesenchyme of E9.5 to E11.5 mouse limb buds. Four-day old primary mesenchymal limb cultures derived from both the whole-limb (WL) and distal-tip (Tp) with alcian blue-stained cartilage nodules are shown on the right. In the schematic, condensations are shown in light blue, cartilage in dark blue and bone in red. Adapted from Tickle and Eichele, Ann. Rev. Cell Biol.. 1994; 10:121.

lateral plate mesoderm and the somitic mesoderm (Chevallier et al., 1977; Christ et al., 1977). Cells that contribute to the skeletal elements of the embryonic limb are derived from the lateral plate mesoderm. Specifically, through the differential proliferation of the flank, specific regions of the lateral plate mesoderm form buds at presumptive limb levels (Searls and Janners, 1971). The homeobox (*Hox*) transcription factors *HoxA* and *HoxD* have been shown to provide spatial cues during development of many embryonic structures in vertebrates, including those that allocate limb fields. Specific combinations of *Hox* genes are expressed at different levels of the embryonic trunk, thereby conferring positional identity of the limb buds along the anterior-posterior axis of the developing embryo (reviewed in (Zakany and Duboule, 2007)). Shortly after the forelimb and hindlimb fields have been specified at precise locations along the embryonic flank, cells from the lateral edges of nearby somites (myotomes) migrate into the limb and ultimately give rise to all adult limb skeletal muscle (Chevallier et al., 1977; Christ et al., 1977; Ordahl and Le Douarin, 1992).

Cells within the lateral plate mesoderm undergo extensive cell proliferation resulting in the formation of the limb bud. The early limb bud consists of a mass of mesenchymal cells encompassed by an ectodermal "jacket". The distal tip forms a specialized epithelial structure called the apical ectodermal ridge (AER) running along its anterior-posterior axis at the interface of dorsal and ventral territories (Fallon and Kelley, 1977; Saunders, 1998). Subjacent to the AER, prechondrogenic mesenchymal cells remain in a proliferative, undifferentiated state. These prechondrogenic mesenchymal cells produce an extracellular matrix rich in hyaluronan, collagen type I (*Col I)* and collagen type IIA (*Col2a1)* (Figure 1.2) (Sandell et al., 1994). Establishment of the chondrogenic template in the limb occurs in a proximal-distal direction, whereby cells in proximal regions of the limb bud form densely packed cellular aggregates prior to more distal cells (Olsen et al., 2000; Summerbell, 1974). In this manner, cartilage differentiation is coordinated along the proximal-distal axis in the limb, such that the humerus

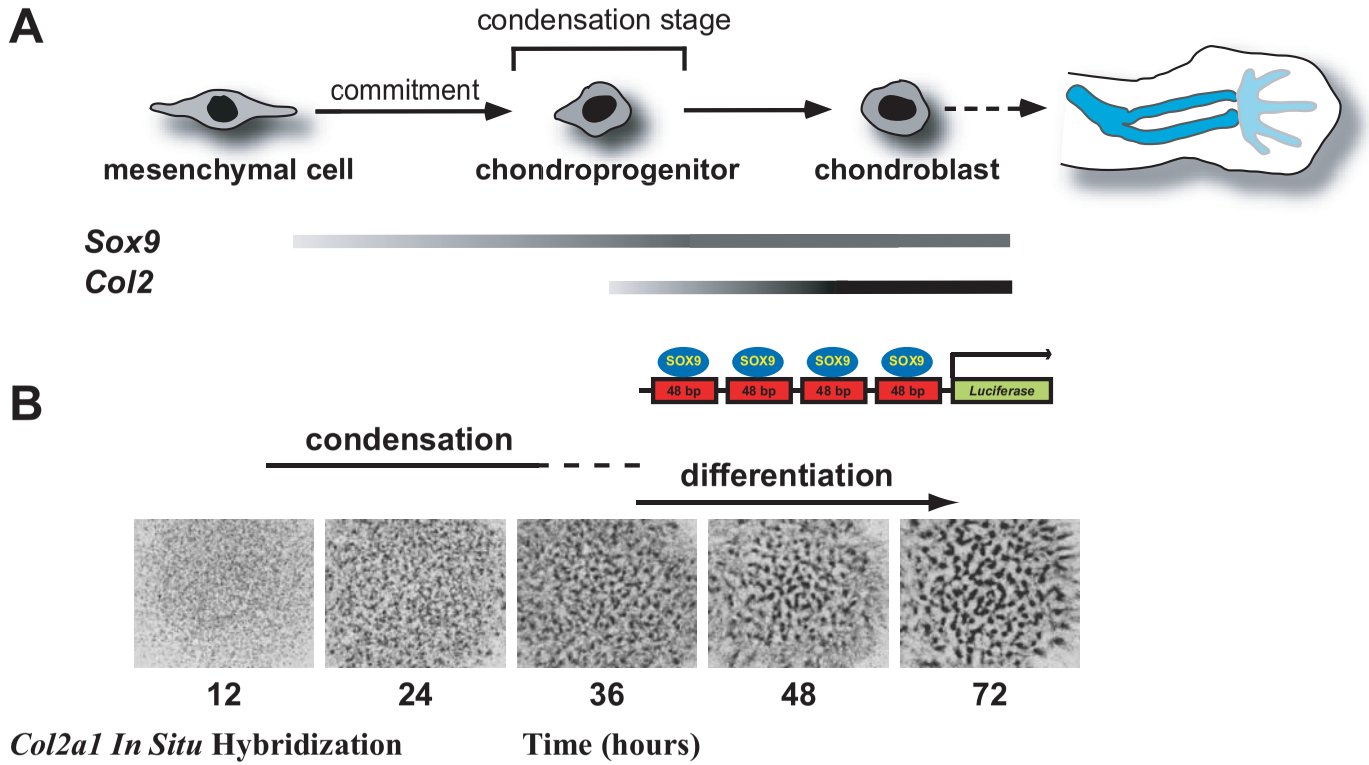

Figure 1.2: Schematic representation of chondrogenesis in the developing vertebrate limb. A. Chondrogenesis involves commitment of mesenchymal cells to the chondrocytic lineage, condensation of precartilaginous mesenchyme and differentiation of chondroblasts into chondrocytes, which ultimately leads to the formation of cartilage and endochondral bone. Upon formation of precartilaginous condensations, cells differentiate into chondroblasts which begin elaborating a cartilaginous matrix rich in collagen II (*Col2*). *Col2* contains a 48 base pair enhancer element within its first intron that binds the transcription factor SOX9. *Sox9* is expressed in mesenchymal cells, pre-chondrocytes and differentiated chondrocytes. To efficiently evaluate the function of gene products within the chondrogenic program, a SOX9-responsive reporter gene is used that contains four re-iterated SOX9 binding sites (4x48 bp) upstream of a minimal collagen promoter coupled to a *luciferase* gene. B. *In situ* hybridization of *Col2a1* in primary limb mesenchymal cultures reveals an increase in *Col2a1* expression throughout the chondrogenic program (12 h - 72 h).

forms first, followed by the radius and ulna, and lastly the digits (Tabin and Wolpert, 2007). This differentiation gradient predicts that more distal cells will be at an earlier chondrogenic stage.

The appearance of precartilaginous condensations represents the earliest stage of limb patterning when the shape, size, position and number of skeletal elements are initially established (Hall and Miyake, 1992; Hall and Miyake, 1995; Hall and Miyake, 2000). This process of precartilaginous mesenchymal condensation formation is dependent on signals initiated by secreted factors, cell-cell and cell-matrix interactions and is associated with increased cell adhesion, formation of gap junctions and changes in cytoskeletal arrangement. Specifically, the initiation of condensation formation is associated with increased hyaluronidase activity that allows for close cell-cell interactions. The establishment of close cell-cell interactions is potentially involved in triggering one or more signal transduction pathways that initiates chondrogenic differentiation (Toole et al., 1972). The cell adhesion molecules, neural cadherin (N-cadherin), and neural cell adhesion molecule (N-CAM) are expressed in the condensing mesenchyme and are both implicated in this process (Oberlender and Tuan, 1994b; Tavella et al., 1994). Disruption of N-cadherin or N-CAM function inhibits or alters chondrogenesis both *in vitro* and *in vivo*, further supporting a role for these cell adhesion molecules in mediating the chondrogenic program (Oberlender and Tuan, 1994a; Widelitz et al., 1993). In addition to cell-cell interactions, cell-matrix interactions also appear to play an important role in chondrogenesis. For example, fibronectin is an extracellular matrix molecule that exhibits increased expression upon formation of mesenchymal condensations (Kulyk et al., 1989).

Mesenchymal condensations are easily visualized using standard light microscopy as closely packed mesenchymal cells in chondrogenic regions of the developing limb bud. In addition, condensing prechondrogenic mesenchymal cells express cell surface molecules that bind the lectin peanut (*Arachis hypogaea*) agglutinin (PNA) which can be used histochemically

to identify cellular condensations during skeletal development. PNA is a lectin composed of four identical subunits which preferentially binds to a carbohydrate sequence present in many soluble and membrane-associated glycoproteins and glycolipids. Contribution or exclusion of cells from precartilaginous condensations and cartilage nodules can be evaluated using specific PNA stains, including rhodamine-labeled PNA (Aulthouse and Solursh, 1987; Gotz et al., 1991).

Many transcription factors which play essential roles in precartilaginous condensation formation have been identified, including members of the paired box gene (PAX) protein family. *Pax* genes are expressed early during embryogenesis in a temporally and spatially restricted manner. *Pax1* and *Pax9* are transcriptional activators, with a paired box DNA-binding domain whose expression is similarly activated in a number of tissues, including the paraxial mesoderm-derived sclerotomes and limb buds, during mouse development (Deutsch et al., 1988; Neubuser et al., 1995; Peters et al., 1998; Peters et al., 1999; Timmons et al., 1994; Wallin et al., 1994). In the developing limb, *Pax9* is expressed in the anterior mesenchyme, whereas *Pax1* expression is restricted to more proximal regions of the limb bud. The more proximal expression of *Pax1* correlates with its earlier onset as proximal limb structures develop prior to more distal structures (Neubuser et al., 1995). Furthermore, $Pax1^{-/-}Pax9^{-/-}$ mice virtually lack a vertebral column and cells fail to proliferate, condense properly and maintain the expression of cartilage markers, SRY-box containing gene 9 (*Sox9*) and *Col2a1* (Peters et al., 1999; Wallin et al., 1994). Additionally, skeletal preparations reveal that *Pax9* mutants develop preaxial digit duplications in the forelimbs and hindlimbs. These studies show that in the absence of *Pax9*, additional mesenchyme is formed in the anterior limb region suggesting that *Pax9* regulates pattern formation of the anterior skeletogenic mesenchyme (Peters et al., 1998). Moreover, in the vertebrate limb, establishment of the anterior-posterior axis is controlled by a group of cells located in the posterior limb mesenchyme known as the zone of polarizing activity (ZPA) (Tickle and Eichele, 1994). *Sonic hedgehog* (*Shh*) is a key molecule secreted by the ZPA which induces

the expression of *Pax1* and *Pax9* (Rodrigo et al., 2003). *Shh* has been shown to play a key role in patterning of the limb, in that, misregulation of *Shh* results in severe limb abnormalities (Hill, 2007; Towers et al., 2008).

The *Runx* family of DNA-binding transcription factors regulates cell fate determination in a number of tissues and has been shown to play an essential role in the differentiation of osteoblasts. Specifically, the runt-domain transcription factor, *Runx2*, is required for osteoblast differentiation since studies have shown that deletion of *Runx2* in mice results in a complete loss of bone (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997). *Runx2* (also known as core binding factor, *Cbfa1* and osteoblast-specific transcription factor 2, *Osf2*) is also expressed in prechondrogenic mesenchymal progenitors (Inada et al., 1999; Otto et al., 1997) and hypertrophic chondrocytes (Inada et al., 1999). Specifically, embryonic expression is present in osteochondroprogenitor cells during mesenchymal condensations as early as embryonic day 10, before overt chondrocyte differentiation or osteoblast differentiation (Ducy et al., 1997; Smith et al., 2005). Hence, a strong stage-dependent inhibition of *Runx2* must occur before cell commitment to the chondrocytic lineage. Furthermore, *Runx2*$^{-/-}$ mice also exhibit a reduction in the proliferation of chondroblasts and a disturbance in chondrocyte maturation in some skeletal elements (Inada et al., 1999); however, the precise role of *Runx2* in early stages of chondrogenesis still remains unclear.

Along with many other genes, these transcription factors control the diversity of shapes and sizes of the skeletal elements. However, although a number of genes have been implicated in regulating the formation of precartilaginous condensations, the molecular mechanisms governing this process still remain unclear.

Following precartilaginous condensation formation, cells differentiate into chondroblasts which begin elaborating a cartilaginous extracellular matrix (ECM) rich in collagens II (*Col2a1)*, IX (*Col9a1, Col9a2* and *Col9a3),* XI (*Col11a1, Coll11a2), aggrecan* (*Acan)* and other

proteoglycans. The matrix is highly hydrated providing compression and resistance (Lefebvre and Smits, 2005). Furthermore, aggrecan is a large aggregating proteoglycan that is abundantly and almost exclusively expressed in cartilage. This combination of aggrecan, collagen and proteoglycans instills cartilage with its mechanical properties. Chondrocytes become entrapped in this ECM and acquire a characteristic ovoid morphology. The cartilage ECM significantly modulates chondrocyte differentiation and activity and is essential for the biomechanical properties of the tissue (Kirn-Safran et al., 2004; So et al., 2001).

Following further differentiation and hypertrophy, there is a significant increase in collagen type X (*Col10a1*) expression; however, there is a marked decrease in the expression of *Col2a1*. *Col2a1* is the most abundant extracellular protein made by chondrocytes which starts to be expressed immediately following mesenchymal condensation formation and thus represents an early marker of chondrocyte differentiation (Figure 1.2) (Ng et al., 1993; Vuorio and de Crombrugghe, 1990). *Col2a1* is important in maintaining the integrity of cartilaginous structures, and disruptions of its synthesis due to mutations in the *Col2a1* gene have been linked to human chondrodysplasias (Ritvaniemi et al., 1995). Furthermore, transgenic mice display abnormal skeletal formation upon mutation of type II collagen genes (Vandenberg et al., 1991). *Col2a1* is also expressed in some non-chondrogenic tissues during embryonic development, including the notochord, heart, epidermis and brain (Cheah et al., 1991). However, expression in these tissues is low and the precise role of *Col2a1* in these tissues is not clearly understood.

In attempts to identify cis-regulating elements of *Col2a1*, a 48 base pair (bp) enhancer element within the first intron of *Col2a1* was identified and was shown to be sufficient to drive cartilage-specific expression of a *lacZ* reporter gene in transgenic mice (Zhou et al., 1998). Furthermore, reiteration of this 48 bp enhancer element, strongly increased promoter activity in transfected rat chondrosarcoma (RCS) cells and mouse primary chondrocytes but not in C3H10T1/2 fibroblasts (Lefebvre et al., 1996). The transcription factor, *Sox9*, binds to a site

within this 48 bp element which is essential for enhancer activity in chondrocytes (Bell et al., 1997; Lefebvre et al., 1997; Ng et al., 1997). This site is highly homologous to the consensus binding site for other SOX and HMG domain proteins (Lefebvre et al., 1997). However, in contrast to the strong activation observed with SOX9, other SOX family members such as SOX4 and SOX5 were unable to activate the *Col2a1* enhancer (Lefebvre et al., 1997).

1.4 Importance of *Sox9*

Sox9 belongs to a large family of transcription factors which are characterized by a high-mobility group (HMG) DNA-binding domain similar to that of the master testis-determining gene, *Sry* (Lefebvre et al., 1997; Ng et al., 1997; Wright et al., 1995) (sex-determining region of the Y chromosome) and has been shown to play a critical role in the chondrogenic program (Bell et al., 1997; Lefebvre et al., 1997; Ng et al., 1997). Comparable with other SOX (SRY-like HMG box) and HMG containing proteins (Connor et al., 1994; Love et al., 1995; Werner et al., 1995), *Sox9* binds to DNA within the minor groove of the double helix (Lefebvre et al., 1997). During mouse embryogenesis, *Sox9* is expressed in all cartilage primordia and cartilages, and in other tissues such as the central nervous system, heart, pancreas and urogenital systems (Ng et al., 1997; Wright et al., 1995; Zhao et al., 1997). Furthermore, during skeletal development, *Sox9* is expressed in mesenchymal chondroprogenitors and differentiated chondrocytes but not in hypertrophic chondrocytes (Figure 1.2) (Ng et al., 1997; Wright et al., 1995). *In situ* hybridization during embryogenesis further reveals that the expression pattern of *Sox9* slightly precedes but greatly resembles that of *Col2a1* (Zhao et al., 1997).

Furthermore, it was shown in experiments in mouse chimeras using *Sox9* $^{-/-}$ embryonic stem (ES) cells that *Sox9*-null cells are excluded from the developing cartilages. A few *Sox9* $^{-/-}$ cells remained in the periphery of condensations, but these cells did not express chondrogenic markers such as *Col2a1* or *Aggrecan1* (Bi et al., 1999). Subsequent studies in which *Sox9* was conditionally removed from limb mesenchymal cells or in mesenchymal cells following

condensation has demonstrated a requirement for *Sox9* both in precartilaginous condensation formation and chondrocyte differentiation (Akiyama et al., 2002). Moreover, forced expression of *Sox9* in non-chondrogenic cells leads to the expression of chondrocytic genes (Bell et al., 1997; Healy et al., 1999). *Sox9* is therefore considered to be both necessary and in some instances sufficient for cartilage formation, and thus has been termed a master regulator of the chondrogenic program (reviewed in (Akiyama, 2008).

Other *Sox* genes, including (long-form) *L-Sox5* and *Sox6*, are co-expressed with *Sox9* in the developing limb which together are necessary for cartilage formation (Lefebvre et al., 1997; Lefebvre et al., 1998; Smits et al., 2001). *L-Sox5* and *Sox6* are activated in chondroprogenitor cells and chondroblasts in all developing cartilage elements of the mouse embryo and have a high degree of sequence identity with each other, but have no sequence homology with *Sox9* except in the HMG box (Lefebvre et al., 1998). Unlike *Sox9*, *L-Sox5* and *Sox6* do not contain either a trans-activation or trans-repression domain and may thus act only to facilitate the organization of transcriptional complexes. Homozygous *Sox5* null mutants and *Sox6* null mutant mice are born with relatively mild skeletal anomalies; however, *Sox5-Sox6* double null mutants die *in utero* with very severe defects in cartilage formation (Smits et al., 2001). More specifically, chondroprogenitor cells develop normal precartilaginous condensations; however, these cells fail to undergo proper chondroblast differentiation. This severe impairment of chondroblast differentiation occurs despite the normal expression of *Sox9*, suggesting that *Sox9* requires *Sox5* and *Sox6* for overt chondrocyte differentiation. *L-Sox5* and *Sox6* also participate in the direct activation of *Col2a1*, since they have been shown to bind and activate *Col2a1* enhancer constructs (Lefebvre et al., 1998). Additionally, a number of cartilage matrix genes were down-regulated in $Sox5^{-/-}/Sox6^{-/-}$ embryos.

Studies initially revealed the importance of *Sox9* in chondrogenesis with the identification of heterozygous mutations in, or translocations around *Sox9*, in human patients

with campomelic dysplasia (CD), a severe form of chondrodysplasia that is often associated with XY sex reversal (Foster et al., 1994; Wagner et al., 1994). CD is characterized by skeletal defects, such as dwarfism, bowing of the femora and tibiae and pelvic malformations, along with non-skeletal defects including sex-reversal and kidney, heart and central nervous system anomalies (Houston et al., 1983; Mansour et al., 1995). CD is dominantly inherited and is usually lethal soon after birth due to respiratory distress. However, the severity of the disorder is variable, in that, some patients survive until adulthood. Collectively these findings demonstrate that *Sox9* plays a central role throughout early skeletogenesis, and we propose to define the networks that function upstream to regulate its expression.

1.5 *Sox9* promoter structure

In several of the reported CD cases, translocations and inversions occur with breakpoints outside of the open reading frame (ORF) of *Sox9*. Some of these breakpoints mapped to locations at least 50 kb and further from the transcriptional start site of *Sox9* (Foster et al., 1994; Kwok et al., 1995; Wagner et al., 1994). Another study revealed the presence of breakpoints in CD patients spanning 140-950 kb proximal to the transcriptional start site, by using bacterial-artificial chromosome (BAC) and P1-artificial-chromosome clones that contained 1.2 Mb upstream sequence of *Sox9* (Pfeifer et al., 1999). Although these cases were less severe than missense or nonsense mutations, each breakpoint is believed to result in the disruption of the spatial and temporal expression of *Sox9*. These observations further demonstrate a central role of *Sox9* in chondrogenesis and also highlight the complex nature of its regulation.

Other studies have demonstrated the complex regulation of *Sox9* by generating transgenic mice carrying human *Sox9-LacZ* yeast artificial chromosomes (YACs) (Wunderle et al., 1998). In these studies, upstream sequences of *Sox9* were manipulated similar to those observed in CD cases and *Sox9-LacZ* expression patterns were observed. These studies revealed the presence of several skeletal-specific regulatory elements scattered 200 to 350 kb upstream of the *Sox9*

transcriptional start site. Mouse embryos carrying a 350 kb YAC (-275/+75 kb) showed a significant decrease in *Sox9* gene expression levels, especially in tissues that undergo chondrogenesis. Analysis of these YAC-containing transgenic mice revealed the presence of tissue-specific regulatory elements located within the 350 kb upstream sequence of *Sox9* (Wunderle et al., 1998). In particular, they suggested that proper skeletal expression of *Sox9* relies on distal elements within the 200 and 350 kb upstream region, as well as more proximal elements (Wunderle et al., 1998).

Delineation of potential regulatory elements in the *Sox9* promoter and identification of molecular factors that bind to these elements would provide valuable information about the tissue- and temporal-specific expression of *Sox9*. To identify regulatory elements upstream of *Sox9*, a comparative genomic analysis was preformed between the human (*Homo sapiens)*, mouse (*Mus musculus*) and pufferfish (*Fugu rubripes*) genome. This approach has been validated in several studies and is useful for delineating regulatory elements since essential sequences required for common cellular functions should be highly conserved over 430 million years of evolution between *F. rubripes* and mammals (Aparicio et al., 1995; Miles et al., 1998). Comparative analysis of large intergenic sequences upstream of *Sox9* in human, mouse and *F. rubripes* has revealed the presence of five short, highly conserved elements (E1-E5) within the *Sox9* promoter (Figure 1.3) (Bagheri-Fam et al., 2001). Elements E3-E5 could be potential enhancers of *Sox9* gene expression in the limbs and vertebral column since they are located in the same region identified by the YAC transgenic data, 200 to 350 kb 5' to the *Sox9* transcriptional start site. Additionally, in published CD cases, 8 out of 10 breakpoints separated E3-E5 from the transcriptional start site of *Sox9* (Wunderle et al., 1998). Nonetheless, complete transcriptional control of *Sox9* is likely to involve a combination of such distal and proximal, enhancer and silencer elements which bind both cell-specific and ubiquitous factors.

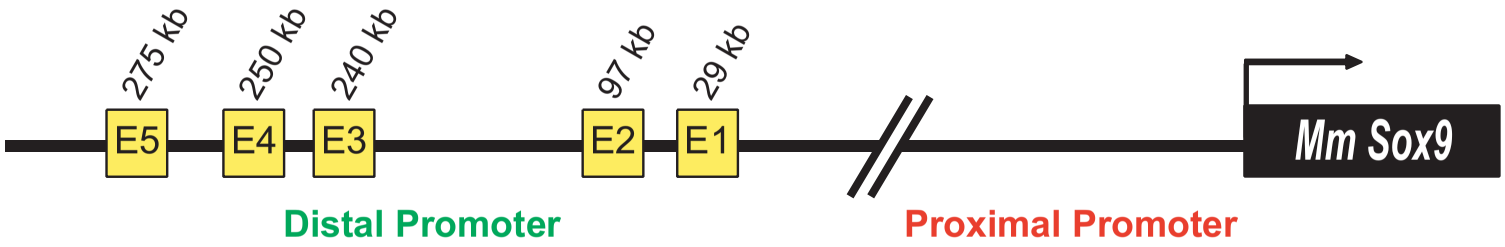

Figure 1.3: Schematic representation of the murine *Sox9* promoter. Comparative analysis of large intergenic sequences upstream of *Sox9* in human, mouse and *Fugu rubripes* has revealed the presence of five short, highly conserved elements (E1-E5) within the *Sox9* promoter (Bagheri Fam et al., 2001). The identified conserved elements (~ 100 bp), 5' to Sox9, are listed with their distances to the transcriptional start site of the murine *Sox9* gene. The most proximal portion of the *Sox9* promoter is also highly conserved between mouse and human sequences (Kanai and Koopman, 1999).

1.6 Known modulators of *Sox9*

Sox9 has been shown to play an essential role in chondroblast differentiation and has been referred to as a master regulator of chondrogenesis; however, previous studies have only identified a few factors and molecules that modulate its expression and activity. For example, SHH and several bone morphogenetic proteins (BMPs) induce and maintain *Sox9* expression (Semba et al., 2000; Yi et al., 2000; Yoon et al., 2005; Zehentner et al., 1999). Fibroblast growth factors (FGFs) have also been shown to upregulate *Sox9* mRNA expression in mouse primary chondrocytes and in mesenchymal C3H10T1/2 cells through a MAP kinase mediated pathway (Murakami et al., 2000a). Moreover, retinoid signaling has been shown to play a critical role in chondrogenesis, in that the down regulation of the retinoic acid receptor α (RARα) is essential for proper limb development (Weston et al., 2000; Yamaguchi et al., 1998). Recently, it has been shown that BMPs function in the limb, by attenuating the expression of *Aldha1a2* (involved in RA synthesis), thereby stimulating *Sox9* expression and activity, and elaborating a chondroblastic phenotype (Hoffman et al., 2006). Thus, BMP and RA signaling, appear to converge on negative regulators of *Sox9*. Conversely, the cytokines, tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β), strongly inhibit both *Sox9* gene expression and activity in primary chondrocytes (Murakami et al., 2000b; Sekiya et al., 2001) which appears to be mediated through NF-κB (Murakami et al., 2000b).

Although a number of molecules have been identified that regulate *Sox9* gene expression and activity, the precise mechanisms regulating the transcription of *Sox9* are poorly defined. In fact, there have only been a few studies that have functionally analyzed the *Sox9* promoter. One study involved a comparison of mouse and human *Sox9* proximal promoter regions along with functional analyses of the mouse *Sox9* promoter (Kanai and Koopman, 1999). These studies uncovered conserved binding sites for GATA, CREB, SOX/Sry and CBF/NF-Y transcription

factors. However, these studies used gonadal somatic cells and liver cells and did not address the role of *Sox9* promoter fragments in cells undergoing chondrogenesis.

More recent studies revealed that removal or mutation of two functional CCAAT boxes (CCAAT-1 and CCAAT-2) within the human *Sox9* proximal promoter diminishes promoter activity. CCAAT boxes are usually found between -60 and -100 bp of the transcriptional start site and multiple functional CCAAT boxes within a proximal promoter are not uncommon. The CCAAT box, bound most commonly by the heterotrimeric CCAAT-box binding factor (Nuclear Factor-Y, NFY), is one of several common elements found within the promoters of eukaryotic genes (25 % of eukaryotic promoters) (Maity and de Crombrugghe, 1998). NFY is a ubiquitous heteromeric transcription factor, composed of three subunits, NFY-α, NFY-β and NFY-γ, which are all necessary for DNA binding (Coustry et al., 1995). Although NFY is ubiquitous and constitutive, it has been shown to regulate promoter activity in a tissue-specific manner (Mantovani et al., 1992). In general, CCAAT boxes operate at many levels of transcriptional control of a number of genes and may play an essential role in regulating *Sox9* gene expression (Colter et al., 2005).

1.7 Skeletogenic patterning

Specialized regions of the developing mouse limb coordinate the development of the limb bud along three axes. Proximal-distal identity (1) is established by the AER at the distal-tip of the developing limb bud (Solursh and Reiter, 1988; Tickle et al., 1975). Anterior-posterior identity (2) is established by the zone of polarizing activity (ZPA) within the posterior limb mesenchyme (Tickle, 1981; Tickle et al., 1975). Finally, dorsal-ventral identity (3) is established by ectodermally derived signals (Geduspan and MacCabe, 1989; Tickle, 2003). Members of the FGF, BMP and WNT Wingless (WNT) protein families have all been shown to play critical roles in the coordination of signaling events along the three axes of the developing limb (Tickle, 2002).

Proximal-distal patterning

Limb outgrowth occurs in a proximal-distal direction, whereby the femur and humerus form first, followed by the formation of more distal elements such as the digits (reviewed in (Capdevila and Izpisua Belmonte, 2001; Johnson and Tabin, 1997)). This proximal-distal outgrowth and subsequent patterning of the limb bud depends on ectodermal signals, particularly those emanating from the AER (Saunders, 1998). Previous studies have suggested that the limb ectoderm exerts both stimulatory and inhibitory effects on cartilage differentiation, depending on the stage of the underlying mesoderm (Hamburger and Hamilton, 1992). Specifically, the early mesoderm requires the presence of the ectoderm for its survival and for the formation of cartilage. For example, explanted limb buds from early mouse embryos lacking the ectoderm, fail to form cartilage and die in culture (Solursh and Reiter, 1988). At later stages, this dependence is lost and instead, the formation of cartilage is inhibited by the presence of the ectoderm (Solursh and Reiter, 1988).

The prevailing model for the orgin and specification of the proximal-distal development of the vertebrate limb has been the “progress zone” (PZ) model (Summerbell et al., 1973). According to this well-established “progress zone” model of vertebrate limb development, cells acquire positional information in the “progress zone” – a region of undifferentiated, proliferating cells at the distal end of the developing limb subjacent to the AER. Within this zone, cells closest to the AER remain in a proliferative state, whereas those more proximal to the body wall differentiate, which results in the formation of the humerus first, followed by subsequent formation of the radius, ulna and digits (reviewed in (Capdevila and Izpisua Belmonte, 2001; Johnson and Tabin, 1997)). This has been shown in studies involving the removal of the AER at different stages of limb development, which results in limb truncations. Proximal to the level of truncation, limb parts develop normally, but cells immediately subjacent to the AER undergo massive cell death (Saunders, 1998; Summerbell, 1974). The exact level of truncation depends

on when exactly the AER was removed in that removal of the AER shortly after its formation results in severe truncations of the entire limb, whereas removal at progressively later stages in development allows the outgrowth of more distal elements in a progressive fashion (Saunders, 1998; Summerbell, 1974). Recent studies have questioned the validity of this "progress zone model"; however, at present there is no reason to accept alternate models of vertebrate limb development (Tabin and Wolpert, 2007).

The AER secretes a wide range of signaling molecules including FGFs, BMPs and WNTs, which are required for the coordination of growth and patterning of the developing limb (reviewed in (Capdevila and Izpisua Belmonte, 2001)). At the onset of limb outgrowth, *Fgf10* expression is restricted to the limb mesenchyme by WNT signals, and FGF10 in turn activates *Wnt3a* expression in the overlying ectoderm (Kawakami et al., 2001; Niswander, 2002). WNT3a, which acts through *β-catenin*, subsequently induces the expression of *Fgf8* in the ectoderm contributing to the formation of the AER. FGF8 also maintains the expression of *Fgf10* in the underlying mesenchyme and vice versa (Kengaku et al., 1997; Martin, 1998; Min et al., 1998). Continued secretion of FGF8 by the AER sustains limb formation throughout the process of limb development. FGFs have been shown to regulate different stages of limb development including proper AER formation and function. Loss of *Fgf10*, or of other molecules that act downstream of *Fgf10* abolishes AER formation (Min et al., 1998; Sekine et al., 1999). Conversely, AER derived *Fgf4* and *Fgf8* are required for AER function but not AER formation and maintenance (Moon and Capecchi, 2000; Sun et al., 2002). Although the roles of FGFs in the limb ectoderm and mesenchyme have been elucidated, the role of WNT/β-catenin signaling in early limb development is not as clearly understood.

Dorsal-ventral patterning of the limb bud

Upon formation of the limb bud, dorsal-ventral patterning is regulated by a number of factors emanating from the limb ectoderm. This was demonstrated in studies involving rotation

of the limb bud ectoderm 180^{o} which resulted in abnormal dorsal-ventral polarity (MacCabe et al., 1974). In this regard, *Wnt7a* which is expressed exclusively in the dorsal ectodermal compartment of the developing mouse limb was an ideal candidate for dorsal-ventral patterning. Indeed, loss of *Wnt7a* function in the mouse affects the dorsal half of the limb and results in a ventralized phenotype (Parr and McMahon, 1995). Moreover, the LIM-homeodomain transcription factor (*Lmx1*) is expressed in the dorsal mesenchyme of the developing limb and the onset of its expression is concurrent with the onset of *Wnt7a* expression in the overlying ectoderm (Riddle et al., 1995). Furthermore, *Lmx1b* expression can be induced in the ventral mesenchyme in response to ectopic *Wnt7a* in the ventral ectoderm (Riddle et al., 1995; Vogel et al., 1995).

Ventrally, the homeodomain transcription factor *Engrailed-1* (*En-1)* represses *Wnt7a* since in the absence of *En-1*, *Wnt7a* is ectopically expressed in the ventral ectoderm (Loomis et al., 1996). Conversely, ectopic expression of *En-1* throughout the limb ectoderm results in the suppression of *Wnt7a* in the dorsal ectoderm (Logan et al., 1997). Both *Wnt7a* and *En-1* are required for the proper patterning of the other limb axes. *En-1* also regulates proximal-distal patterning through the repression of AER markers (*Fgf8, Bmp-2*) in the ventral ectoderm (Crossley and Martin, 1995; Lyons et al., 1990). *Wnt7a* is also required for proper patterning along the anterior-posterior axis, in that *Wnt7a* mutants display decreased *Shh* expression in the ZPA of the developing limb (Parr and McMahon, 1995).

BMP signaling has also been implicated in dorsal-ventral patterning of the developing limb. Specifically, BMP signaling is necessary and sufficient to trigger a cascade of signaling events resulting in the upregulation of *En-1*. This serves to restrict the expression of *Wnt7a* and *Lmx1b* to the dorsal aspect of the developing limb. This differential expression of *En-1, Wnt7a* and *Lmx1b* is known to be critical for proper dorsal-ventral patterning of the limb (Ahn et al., 2001; Pizette et al., 2001). Additionally, conditional mutants of the BMP receptor, *Bmpr1a*, in

the limb mesenchyme display altered *Lmx1b* expression, which results in abnormal formation of dorsal-ventral boundaries within the developing limb (Ovchinnikov et al., 2006). Nonetheless, BMP signaling is critical for dorsal-ventral patterning of the developing mouse limb.

Anterior-posterior patterning of the limb bud

Patterning along the anterior-posterior axis of the developing limb bud is involved in the specification of digit formation. The zone of polarizing activity (ZPA), which is located in the mesenchyme of the posterior margin of the developing limb, is essential for the correct formation of a limb bud that is asymmetrical along the anterior-posterior axis with a well defined posterior and anterior side (Johnson et al., 1994; Summerbell, 1979). Surgical removal and grafting of the ZPA at more anterior sites results in digit duplication (Summerbell, 1983; Tickle et al., 1982). Additionally, those cells that are closest to the ZPA become posterior structures and more distant cells differentiate into anterior structures (Francis et al., 1994). The signaling molecule, SHH, is produced specifically by the cells of the ZPA and has been shown to play a pivotal role in anterior-posterior patterning, in that, homozygous null mutations of *Shh* results in a severe limb phenotype characterized by the loss of all digits in the forelimb (Chiang et al., 2001; Masuya et al., 1995; Pearse and Tabin, 1998).

A number of signaling molecules modulate *Shh* signaling in the developing limb, some of which serve to restrict *Shh* to the posterior limb bud (Masuya et al., 1995; Milenkovic et al., 1999). More specifically, at E9.5 in the mouse, expression of the transcription factor *Gli3* is restricted to the anterior region of the developing limb bud and potentiates the expression of the transcription factor, *Aristaless-like4* (*Alx4*) (te Welscher et al., 2002). Concurrently, *Gli3* restricts the expression of the basic-helix-loop-helix transcription factor gene *dHand* to the posterior mesenchyme where *dHand* defines the anterior-posterior boundary of *Gli3* and *Alx4* (te Welscher et al., 2002). These molecular events pre-pattern the limb mesenchyme by contributing to the initiation and correct positioning of *Shh* expressing cells (Charite et al., 2000).

Upon initiation of *Shh* expression a continuous series of events expands and/or maintains the expression of *Shh* depending on signaling from the AER. For example, FGF signaling from the AER to the ZPA is required for maintaining normal levels of *Shh*. Subsequently, SHH maintains the expression of *Fgf*s (Martin, 1998). In the *Shh* knockout *Fgf4* and *Fgf8* are down-regulated. Similarly, double conditional knockouts of *Fgf4* and *Fgf8* results in the loss of *Shh* expression, as well as extensive limb defects (Martin, 1998). Hence, *Shh* acts in a positive feedback loop with FGF signaling from the AER to allow proliferation of the distal mesenchyme and subsequent digit formation (Mariani and Martin, 2003; Niswander, 2002). Furthermore, SHH has been shown to regulate the expression *Bmp-2, -4 and -7* (Bitgood and McMahon, 1995; Roberts et al., 1995). It is thought that this regulation of *Bmps* gives rise to anterior-posterior structures possibly through *Hox* gene regulation (Pagan et al., 1996).

1.8 Signaling pathways involved in chondrogenesis

A number of signaling molecules that are involved in mesenchymal cell migration, proliferation, condensation formation and chondrocyte differentiation have been identified (DeLise et al., 2000; Hall and Miyake, 2000; Olsen et al., 2000), including members of the WNT and BMP protein families and their downstream effectors (β-catenin, SMADs).

1.8.1 WNT signaling

WNTs were initially discovered as Wingless (wg) in Drosophila and the MMTV proto-oncogene (int-1) in mammalian cells (Rijsewijk et al., 1987). There are 19 known WNTs in the mammalian system which form a family of highly conserved secreted signaling molecules that are involved in many aspects of embryonic development. Several studies provide evidence of a role for members of the WNT family of signaling molecules in the regulation of cartilage formation (Galceran et al., 1999; Yamaguchi et al., 1999).

WNTs can be classified into general classes (canonical & non-canonical), based on their ability to induce an effector protein, β-catenin (Figure 1.4) (Akiyama, 2000; Eastman and

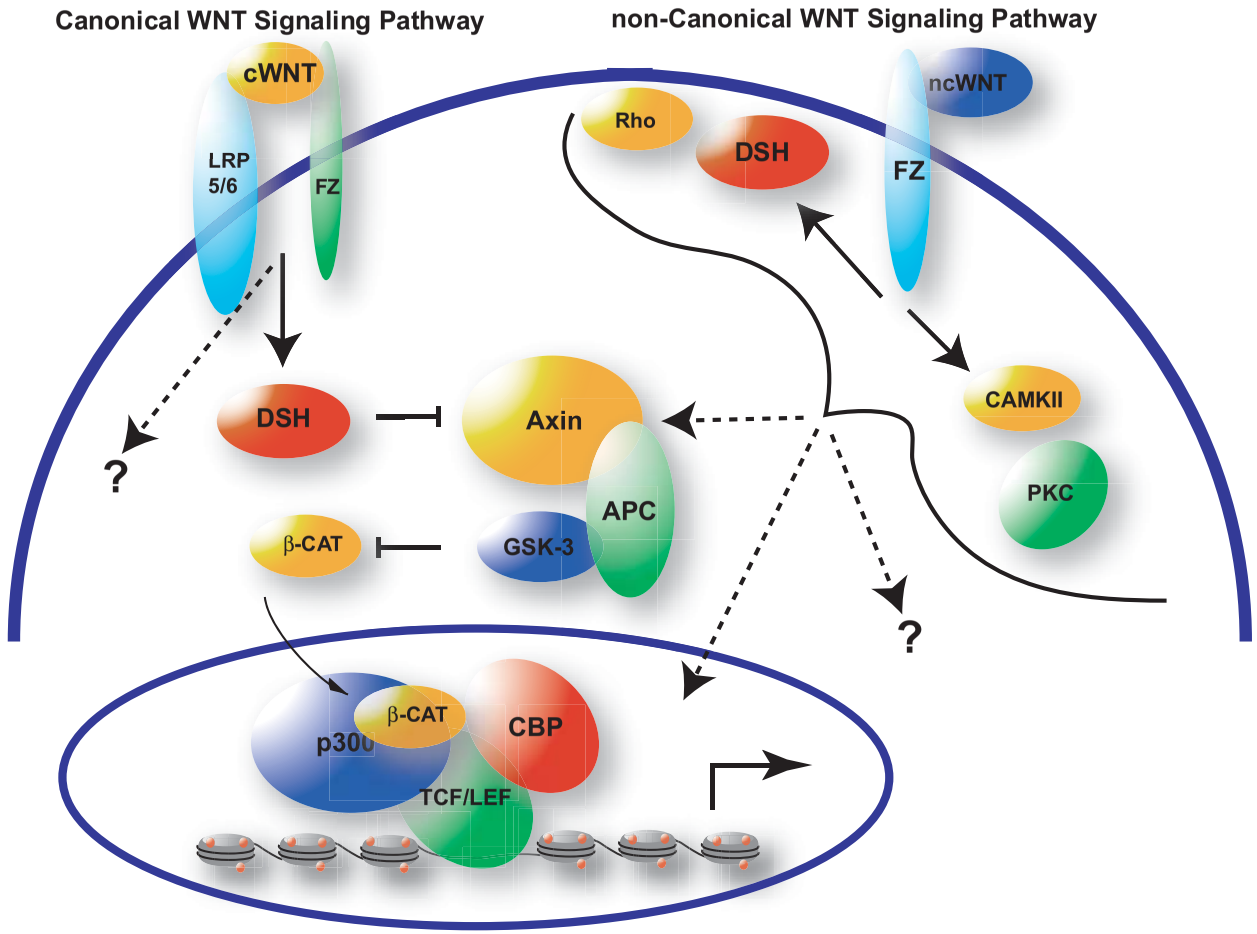

Figure 1.4: Simplified overview of canonical and non-canonical WNT signaling pathways. WNTs interact with the cell surface receptor Frizzled (FZ) and in canonical-WNT signaling, this leads to the activation of Disheveled (DSH), which inhibits APC/GSK3/Axin-targeted destruction of β-catenin (β-CAT). In the presence of WNT, β-CAT is stabilized and translocates to the nucleus where it binds to transcription factors (TCF/LEF). Non-canonical WNT signaling potentially involves multiple pathways, two of which are shown here. Non-canonical WNTs can also inhibit the activation of the canonical WNT signaling pathway.

Grosschedl, 1999). In the developing limb, both pathways are equally important, in that, mutations of either canonical WNTs or non-canonical WNTs results in severe limb malformations (Galceran et al., 1999; Yamaguchi et al., 1999).

The canonical WNT signaling pathway through which a number of WNTs (WNT-1, -3, -3a, -7a and -8) transduce their signals plays an important role in controlling cell proliferation and differentiation during embryonic development. Canonical WNTs signal through frizzled (Fz) receptors which contain seven transmembrane receptors, an extracellular cysteine rich domain (CRD) and an intracellular carboxy tail (Hsieh et al., 1999). Signaling through frizzled receptors requires low density lipoprotein (LDL) receptor related protein (LRP) co-receptors (LRP5/LRP6), which are single transmembrane proteins.

Briefly, when cells are not exposed to WNT ligand, a β-catenin destruction complex formed by proteins that include Axin, Adenomatous Polyposis Coli (APC) and glycogen synthase kinase-3 (GSK-3) keep cytoplasmic levels of β-catenin low, specifically through the phosphorylation of β-catenin by GSK-3. Phosphorylated β-catenin becomes ubiquitylated and is targeted for degradation by the proteasome (Zeng et al., 1997). As a result, TCF/LEF transcription factors can associate with Groucho proteins to mediate repression of WNT target genes (Bienz, 1998). Four members of the TCF/LEF family of transcription factors have been identified in mammals: lymphoid enhancer factor-1 (LEF1), T cell factor-1 (TCF-1), TCF-3 and TCF-4 (Korinek et al., 1998; Travis et al., 1991; van de Wetering et al., 1991; Waterman et al., 1991). Null mutations of these transcription factors (*Tcf/Lef*) causes defects in early limb development, such that $Lef^{-/-}/Tcf^{-/-}$ limb buds do not form the AER (Barrow et al., 2003; Galceran et al., 1999).

Conversely, upon binding of WNT ligand to the Fz/LRP receptor complex, a signal is transduced to the cytoplasmic phosphoprotein, Dishevelled (*Dsh*) (Noordermeer et al., 1994). It

has been postulated that *Dsh* transduces the Wnt signal into the cell through a direct binding of *Dsh* to the Fz receptor (Chen et al., 2003; Wong et al., 2003). In mammals, there are three Dsh proteins (Dsh-1, Dsh-2, Dsh-3) which contain three highly conserved domains (Wharton, 2003). *Dsh* is a key transducer of WNT signaling that operates at the plasma membrane or in the cytoplasm. Upon activation of canonical WNT signaling, *Dsh* induces the stabilization of β-catenin which allows for cytoplasmic accumulation and translocation of β-catenin to the nucleus (Logan and Nusse, 2004). In the cytoplasm, regulation of β-catenin is also mediated by a number of proteins including: Axin, APC and GSK-3, such that this destruction complex is inhibited when cells receive WNT signals, leading to a block in β-catenin phosphorylation and subsequent degradation. Stabilization of β-catenin occurs which allows it to accumulate in the nucleus where it interacts with TCF/LEF transcription factors to activate WNT target genes. Many genes have been identified that are activated by *β-catenin-Lef/Tcf* mediated transcription, including those genes involved in cell cycle progression (c-myc, cyclin D), development and tissue remodeling. Similar to other growth factors, WNT signaling can be antagonized by secreted factors. These antagonists include secreted frizzled related proteins (SFRPs), Cerebrus, Dickkopfs (DKKs) and Wnt inducible factor (WIF-1).

Although WNT pathway activity through β-catenin can be detected in a broad range of tissues both during development and in adult mice, *Wnt* genes themselves often show temporally restricted and highly localized expression patterns. This indicates that many WNT family members are involved in similar cellular activities at different sites and times of development. An example of this is the dynamic expression of *Wnt* genes in the developing limb which are differentially expressed in the ectoderm and mesenchyme of the limb bud depending on the specific stage of development. Moreover, double-knockout mice often show a more severe phenotype than would be anticipated based on defects observed in single-mutant animals,

indicating some functional redundancy. Indeed, mice deficient for canonical and non-canonical Wnt pathway components have generated insights into the role of WNT signal transduction in vertebrates. In many cases, embryonic lethality and severe developmental defects have limited analyses of knockout mice. Hence, the ability to generate conditional knockouts in a specific tissue at a specific time of interest has enabled the study of WNT signal transduction at certain stages of development. For example, conventional *Wnt3*-knockout mice fail to form a primitive streak and are unable to complete gastrulation (Liu et al., 1999). However, conditional ablation of *Wnt3* in the early limb ectoderm has revealed an essential role for *Wnt3* in limb development, in that, loss of *Wnt3* in the mouse limb ectoderm results in a wide range of limb abnormalities implicating an essential role for *Wnt3* in limb patterning and formation of the AER (Barrow et al., 2003). Similarly, mutations of *Wnt3* in humans results in tetramelia which is characterized by the complete absence of all four limbs (Niemann et al., 2004). This observation may indicate that WNT3 may be required early in the limb mesoderm to possibly restrict and maintain FGF10 expression.

Wnt3a was first identified based on its sequence similarity with *Wnt3*, as they are nearly 90% identical at the amino acid level. In mice, *Wnt3a* signaling has been implicated in the formation of the primary body axis since expression of *Wnt3a* is first detected at late primitive streak stages, starting at ~ E7.5, and continuing in the tail bud until at least ~ E9.5 (Takada et al., 1994). *Wnt3a* is also expressed in cells that generate the somites and lateral mesoderm, suggesting that *Wnt3a* signaling may normally regulate the formation of these mesodermal cell types (Takada et al., 1994). *Wnt3a* null embryonic mice lack caudal somites, have a disrupted notochord, and fail to form a tailbud (Takada et al., 1994). In addition, *Wnt3a* is expressed in the dorsal CNS; hence, mutant embryos display CNS dysmorphology and ectopic expression of dorsal CNS markers (Lee et al., 2000).

Loss of other canonical WNTs or of specific molecules that act downstream to transduce canonical WNT signaling (*Lrp6, Lef1, Tcf1*) results in severe defects in the formation of the limb (Galceran et al., 1999; Pinson et al., 2000). For example, mouse embryos lacking components of the WNT pathway, including loss of transcription factors, *Lef1* and *Tcf1*, exhibit defects in AER formation of the developing limb (Galceran et al., 1999; Pinson et al., 2000). However, because there are components of the WNT signaling pathway that are ubiquitously expressed in both the limb mesenchyme and ectoderm, it is not clear which components are in fact required for proper limb development.

Ectopic expression of the canonical WNT, *Wnt1*, in developing chick limb buds also results in skeletal malformations. These results are consistent with the possibility that canonical WNT signaling inhibits or delays chondrogenesis (Day et al., 2005; Hill et al., 2005; Rudnicki and Brown, 1997; Tufan et al., 2002). Moreover, other studies have demonstrated that overexpression of the cWNT, *Wnt1*, in micromass cultures of the limb mesenchyme dramatically suppresses chondrogenic differentiation (Rudnicki and Brown, 1997). *Wnt7a* which is expressed endogenously in the dorsal ectoderm (Akita et al., 1996; Parr et al., 1993) of the limb also has a similar inhibitory effect (Rudnicki and Brown, 1997; Stott et al., 1999). Additionally, ablation of β-catenin and thereby cWNT signaling in cultured limb mesenchyme stimulates cartilage formation (Guo et al., 2004); whereas, targeted disruption of β-catenin signaling promotes chondrogenesis, further strengthening the idea that canonical WNTs inhibit chondrogenic differentiation (Akiyama et al., 2004; Guo et al., 2004).

In contrast, non-canonical WNTs operate through the modulation of other pathways in a variety of assays, some of which may involve the stimulation of intracellular calcium (Ca^{+2}) release and activation of protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CAMKII) (Sheldahl et al., 1999). Non-canonical WNT activity has also been shown

to antagonize canonical WNT activity both in *Xenopus* embryos and mammalian cells (Ishitani et al., 2003; Mikels and Nusse, 2006; Torres et al., 1996).

The non-canonical WNT, *Wnt5a*, is the only known member of the WNT family which is expressed in the early limb mesenchyme (Parr et al., 1993). *Wnt5a* expression is first observed just after the forelimb has started to bud (E9.5) where it is localized to the ventral limb ectoderm and shortly thereafter to the early AER (Gavin et al., 1990; Parr et al., 1993; Yamaguchi et al., 1999). As the limb bud elongates distally, *Wnt5a* continues to be expressed in the AER but expression in the limb mesenchyme becomes graded, with the highest level of expression observed in the distal ectoderm of the developing AER and underlying mesenchyme (Parr et al., 1993). (Yamaguchi et al., 1999). Interestingly, the highest levels of *Wnt5a* expression are localized in the progress zone, which contains the mitotic precursors that form the different limb structures (Parr et al., 1993). By E11.5, ectodermal expression of *Wnt5a* is down-regulated and graded transcripts of *Wnt5a* become confined to the distal mesenchyme (Gavin et al., 1990; Yamaguchi et al., 1999).

The proximal-distal gradient of *Wnt5a* expression in the limb mesenchyme and ectoderm suggests that *Wnt* genes may contribute to patterning along this axis (Gavin et al., 1990). *Wnt5a* is believed to act as a positive regulator of chondrogenesis, since *Wnt5a* null mutants exhibit severely truncated limbs, with increased severity in a proximal to distal direction (Parr et al., 1993; Yamaguchi et al., 1999). The reduced size of the limb skeleton along the proximal-distal axis, together with the loss of distal structures, suggests that early deficiencies in the function of the AER or in the progress zone may account for the observed phenotype (Yamaguchi et al., 1999). It is unlikely that *Wnt5a* is required for AER activity, since the AER appears normal in *Wnt5a* mutants; however, the decreased proliferation observed in the progress zone suggests that *Wnt5a* could mediate FGF mitogenic activity in the progress zone (Yamaguchi et al., 1999).

Furthermore, *Wnt5a* antagonizes Wnt/β-catenin signaling in the distal tip of the developing limb, since recent studies demonstrate that in the absence of *Wnt5a*, increased activation of the canonical WNT (β-catenin) signaling pathway is observed in the distal underlying mesenchyme (Topol et al., 2003). The mechanism by which WNT-5a inhibits canonical WNT signaling is unclear, however it has been postulated that WNT-5a can antagonize the canonical WNT signaling pathway by promoting β-catenin degradation. Furthermore, the induction of β-catenin degradation appears to be independent of GSK-3 and does not require the activation of CAMKII or NF-AT (Topol et al., 2003). Since it has been shown both *in vivo* and *in vitro* that increased β-catenin activity leads to an inhibition of chondrogenesis (Ryu et al., 2002), it has been postulated that robust expression of *Wnt5a* in the distal underlying mesenchyme inhibits canonical WNT signaling, thereby providing conditions conducive to chondrogenesis. These results suggest a dual role of WNTs in the chondrogenic program.

1.8.2 BMP signaling

During limb outgrowth, signals that promote as well as inhibit chondrogenesis are important determinants of skeleton formation. Various members of the transforming growth factor-β (TGF-β) super family figure prominently at multiple stages within the chondrogenic program, including BMPs which exhibit potent pro-chondrogenic activity.

Several *Bmps* are expressed in both the ectoderm and mesoderm of the developing limb. In particular, *Bmp4* and *Bmp7* are expressed in the lateral mesoderm which is a major contributor to the developing limb mesenchyme, throughout stages preceding limb bud formation (Ahn et al., 2001; Francis et al., 1994). *Bmp2*, *Bmp4* and *Bmp7* are also dynamically expressed in the overlying limb ectoderm, with highest levels of expression in the developing AER (Ahn et al., 2001; Francis et al., 1994). At E9.5 in the forelimb and E10 in the hindlimb, highest levels of expression of *Bmp2*, *Bmp4* and *Bmp7* are found in the AER (Francis-West et al., 1995). In the

mesenchyme, *Bmp4* and *Bmp7* are expressed in both anterior and posterior domains, while *Bmp2* expression is mostly restricted to the posterior domain (Francis et al., 1994).

Indeed, BMP activity was first identified in demineralized bone extracts (Urist, 1965), and have subsequently been shown to induce cartilage formation both *in vivo* (Wozney et al., 1988) and *in vitro* (Chen et al., 1991; Chen et al., 1992; Wozney et al., 1988). During limb development, BMPs have been implicated in AER formation (Ahn et al., 2001; Pizette et al., 2001), AER regression (Pizette and Niswander, 1999), cartilage and bone differentiation (Karsenty and Wagner, 2002; Tsumaki and Yoshikawa, 2005) and interdigital webbing regression (Zuzarte-Luis and Hurle, 2005). Hence, BMPs play different roles at different stages of limb development. In general, mutation or disruption of BMPs or their receptors negatively impacts growth and development of the skeleton (Kingsley, 2001; Yoon et al., 2005). In previous studies, BMPs were attributed a negative role in the regulation of the AER and in distal outgrowth of the limb. However, recent studies have identified that BMP signaling may be involved in the induction of the AER at the onset of limb formation. More specifically, early in limb bud development, studies involving tissue-specific inactivation of the Bmp type 1 receptor (*Bmpr1a*) in the ventral ectoderm of mouse embryos, have shown that BMP signaling is required for both dorsal-ventral patterning and AER induction (Ahn et al., 2001; Ovchinnikov et al., 2006; Pizette et al., 2001; Pizette and Niswander, 1999; Soshnikova et al., 2003). As the AER develops, inhibition of BMP activity by the BMP extracellular antagonist, *Gremlin*, is necessary to maintain the AER and the *Fgf-Shh* feedback loop for proper patterning of the developing vertebrate limb (Khokha et al., 2003). Later in limb development, BMPs also play a role in programmed cell death of the interdigital region. More specifically, *Bmp2*, *Bmp4*, and *Bmp7* are all expressed in the interdigital regions of the developing mouse limb bud prior to and during the occurrence of apoptosis, suggesting a role for these molecules in cellular death (Chen and Zhao,

1998). This process is required to separate the digits and prevent webbing of the digits (Chen and Zhao, 1998; Zuzarte-Luis and Hurle, 2005).

The study of BMPs has been limited since *Bmp2* and *Bmp4* mutants are both lethal early in embryogenesis (Winnier et al., 1995). In addition, the BMP subfamily comprises more than 10 proteins which exhibit high degrees of homology at the amino acid level and may be functionally redundant. However, recent studies involving the deletion of *Bmp2* and *4* specifically within the limb mesenchyme results in a loss of precartilaginous condensations (Bandyopadhyay et al., 2006). Similarly, deletion of BMP receptors, *Bmpr1a* and *1b*, leads to an absence of most limb condensations and in those that do form, the cells fail to differentiate into chondrocytes (Yoon et al., 2005). In addition, misexpression of the BMP antagonist NOGGIN (Zimmerman et al., 1996) leads to an inhibition of chondrogenesis in embryonic limb mesenchyme (Pizette and Niswander, 1999; Weston et al., 2000), whereas *Noggin*-null animals present with increased cartilage (Brunet et al., 1998). This inhibition of cartilage formation with increased NOGGIN, appears to be due to reduced formation of prechondrogenic condensations suggesting again that BMPs play a critical role in initiating mesenchyme cell condensation and subsequent cartilage differentiation (Pizette and Niswander, 1999; Weston et al., 2000). Consistent with this idea, recent studies have demonstrated that BMP signaling is required for the "compaction" of chondroprogenitors whereby mesenchymal cell aggregates coalesce and form tight interactions that lead to the establishment of cartilage nodules. Thus, BMP signaling is required for initiating chondrogenic condensation; however, once chondrogenesis is initiated, cartilage differentiation can be sustained (Barna and Niswander, 2007).

Members of the TGF-β superfamily, including TGF-βs, activins, nodal and BMPs, are secreted cytokines that regulate a number of cellular processes including cellular proliferation, differentiation, migration and apoptosis (Itoh and ten Dijke, 2007). Misregulation of their signaling has been implicated in several developmental disorders and in various human diseases

including cancer, fibrosis and auto immune diseases (Massague et al., 2000). Members of the TGF-β superfamily initiate signaling by assembling receptor complexes that activate Mothers Against Decapentaplegic Homologs, or SMAD transcription factors (Figure 1.5) (Massague, 1998). Seven type I (also termed activin-receptor-like kinases, ALKs) and five type II receptors have been identified in vertebrates. Each member of the TGF-β superfamily can bind to various combinations of type I and type II receptors. More specifically, upon ligand binding type I and type II serine/threonine kinase receptors form a tetramer consisting of two pairs of type I and type II receptors. Type II receptors then phosphorylate type I receptors (Wrana et al., 1992). This phosphorylation is both essential and sufficient for TGF-β signaling (Shi and Massague, 2003). The activated type I kinase receptors propagates the signal inside the cell through the phosphorylation of receptor-regulated Smads (R-Smads: Smad1, Smad2, Smad3, Smad5 and Smad8) (ten Dijke and Hill, 2004). Smad1, Smad5 and Smad8 are R-Smads that transduce BMP signals, and Smad2 and Smad3 are R-Smads that transduce TGF-β and activin signals. Phosphorylated R-Smads then form heteromeric complexes with common-partner Smad (Co-Smad) (2), Smad4. These complexes accumulate in the nucleus, where they regulate gene expression in a cell-type-specific and ligand dose-dependent manner through interactions with transcription factors, co-activators and co-repressors.

The duration and intensity of TGF-β signaling is critically regulated in physiological conditions and is essential for signaling specificity. Negative feedback of TGF-β superfamily signaling pathways is mediated by the induction of inhibitory SMADs (I-SMADs), SMAD6 and SMAD7. I-SMADs are transcriptionally induced in response to TGF-β and BMPs in a SMAD-dependent manner; however, the regulatory mechanisms for the induction of I-Smads are not fully understood. They were originally shown to inhibit phosphorylation of R-SMADs by

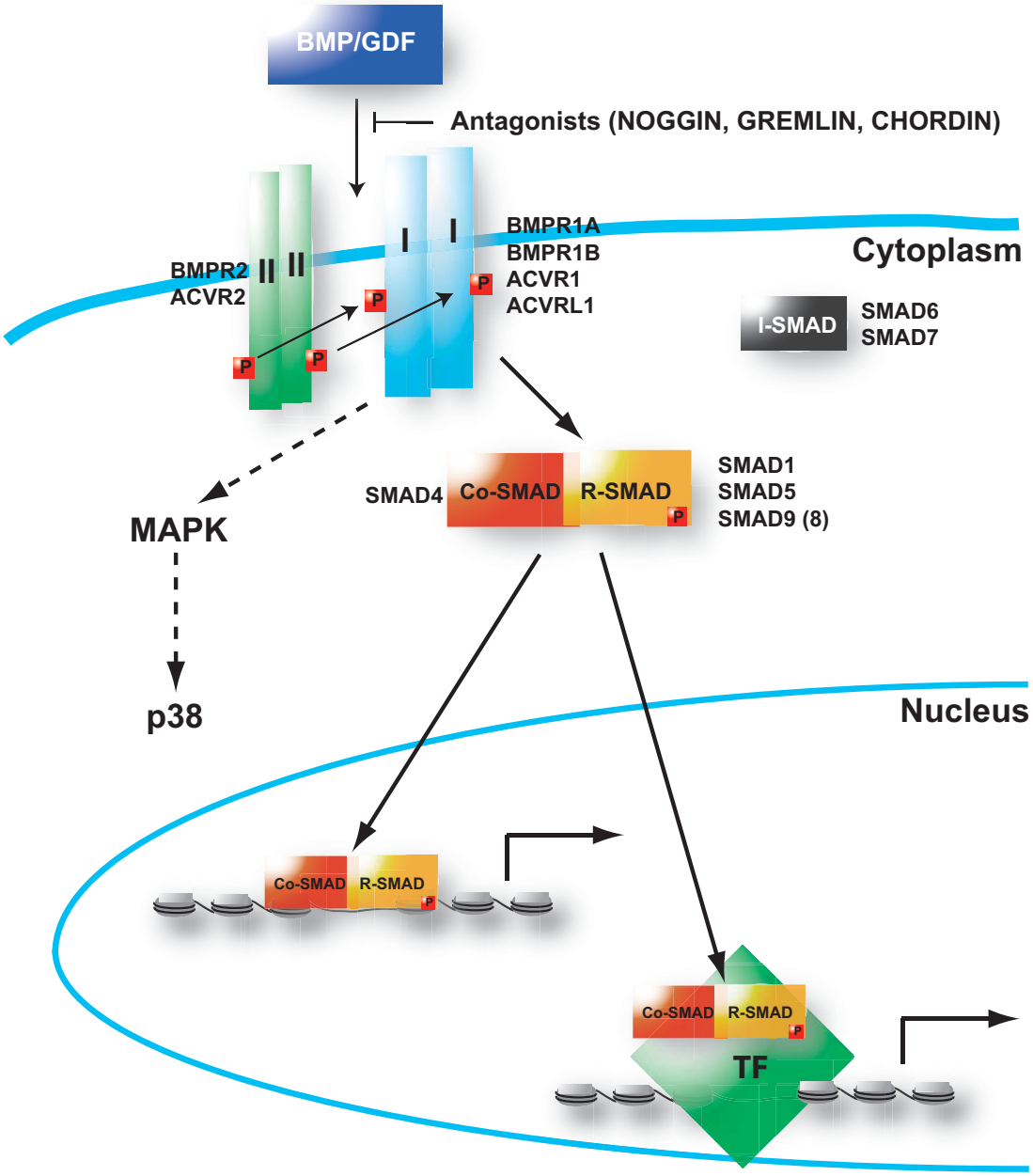

Figure 1.5: Simplified overview of the BMP signaling pathway. Bone morphogenetic proteins, members of the TGF-β superfamily of secreted signaling molecules, have important functions in a number of cellular processes. They bind to specific serine/threonine kinase receptors, which transduce the signal to the nucleus through Smad proteins. Nuclear cofactors have been identified that cooperate with Smads in regulating specific target genes depending on the cellular context. Additionally, BMP signaling is regulated at the cell surface, in the cytoplasm and in the nucleus by a number of factors. Many of these factors are induced by BMP and inhibit the BMP pathway, thereby establishing negative feedback loops. Members of the BMP-Smad pathway can also interact with components of other signaling pathways. Moreover, MAP kinases can also transduce BMP signals.

competing with R-SMADs for binding of phosphorylated type I receptors (Hayashi et al., 1997; Imamura et al., 1997). Subsequently, they were found to recruit E3-ubiquitin ligases, known as SMAD ubiquitination regulatory factors 1 (Smurf1) and Smurf2, to the activated type I receptor, resulting in receptor ubiquitination and degradation, and ultimately termination of signaling (Shi and Massague, 2003). It has also been shown that *Smad6* and *Smad7* differ in the inhibitory effects they exert on the signaling initiated by the TGF-β super family such that *Smad6* preferentially inhibits by BMPs, whereas *Smad7* inhibits signaling by all TGF-β family members including TGF-β, activin and BMP. Furthermore, I-Smads may also have a nuclear role since *Smad6* has been shown to recruit the co-repressor, C-terminal binding protein (CtBP), to repress BMP-induced transcription (Lin et al., 2003). Moreover, the expression and duration of I-Smads are generally regulated at the transcriptional level and by post-translational modification. Nonetheless, the activity, subcellular distribution and stability of all three Smad subclasses are highly regulated and numerous Smad-interacting proteins have been identified (Moustakas et al., 2001).

Other studies have identified p38 mitogen-activated protein (MAP) kinase as a downstream effector of the TGF-β signaling pathway. MAP kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli and regulate various cellular activities including gene transcription, mitosis, differentiation and apoptosis (Pearson et al., 2001). Studies have also shown that BMP signals can be transduced by TGF-β activated kinase 1 (TAK1), a MAP kinase kinase kinase (MAPKKK), and TAK1 binding protein 1 (TAB1) that activates p38 MAPK (Yamaguchi et al., 1995). Additionally, signaling through the p38 MAPK intracellular pathway has previously been shown to regulate cartilage formation (Oh et al., 2000; Yoon et al., 2000). Specifically, during cartilage development, p38 signaling has been shown to be essential for chondroblast differentiation in that, inhibition of p38 signaling blocks

chondrogenesis (Oh et al., 2000). Accordingly, the addition of pharmacological p38 MAPK inhibitors to mouse primary mesenchymal limb cultures inhibits both SOX9 activity and cartilage formation (Hoffman et al., 2003).

BMPs are known to play essential roles in bone and cartilage development, heart development, neural patterning and a number of other events involved in embryogenesis. It has been proposed that BMP factors exert such diverse effects through the aforementioned interactions with SMAD proteins and other transcription factors to activate or repress target genes (Derynck and Zhang, 2003). GATA transcription factors have been shown to play critical roles in development, including cell-fate specification, regulation of differentiation and control of cell proliferation (Burch, 2005; Molkentin, 2000). Furthermore, mutation or modulation of *Gata* expression can result in human disease. GATA factors belong to an evolutionarily conserved family of zinc finger-containing proteins that recognize the consensus DNA sequence, (A/T) GATA (A/G) (Molkentin, 2000; Patient and McGhee, 2002). There are six mammalian GATA factors which are among the earliest transcription factors that have been implicated to be downstream of BMP signaling (Rossi et al., 2001; Schultheiss et al., 1997).

Studies have demonstrated that GATA transcription factors are downstream targets of BMP signaling. For example, BMP signaling results in the upregulation of *Gata4* expression in the pre-cardiac mesoderm of the chick (Schultheiss et al., 1997). Similarly, BMP4 signaling from the mesoderm upregulates *Gata4* expression in the prehepatic endoderm during mouse liver development (Rossi et al., 2001). Furthermore, studies suggest that GATA transcription factors act to integrate extracellular signals with nuclear signaling, responding either by interaction with Smads or with general transcriptional machinery. Although it has been shown that BMPs regulate *Gata* expression during embryogenesis in a number of tissues, the role of GATA transcription factors in chondrogenesis and the potential regulation of *Gata* gene expression by BMPs in the limb have not been investigated. Although downstream effectors of the TGF-

β/BMP signaling pathway have been well characterized, the targets and mechanisms that underlie their actions remain elusive. Herein, we describe a novel role for *Gata* transcription factors in early limb chondrogenesis.

1.9 Culture system

To effectively model the chondrogenic program, we have used primary cultures derived from the mesenchyme of E9.5-E11.5 murine limb buds. These cultures are plated as high-density micromass cultures which undergo condensation and differentiation to form distinct cartilage nodules which closely recapitulates those events occurring *in vivo* (Ahrens et al., 1977). Moreover, to efficiently evaluate the function of gene products within the chondrogenic program, a SOX9-responsive reporter was constructed that contains four re-iterated SOX9 binding sites (4 X 48 bp) upstream of a minimal type II collagen promoter (-89/+6) coupled to a luciferase gene (as previously described in (Lefebvre et al., 1997; Weston et al., 2002)). We have found that this reporter gene provides a reliable and accurate read-out on the status of chondroblast differentiation. This construct is transiently transfected into primary murine limb bud mesenchymal cultures and the activity of co-transfected expression cDNAs, factors or compounds is analyzed. Accordingly, we have found that factors which promote cartilage formation lead to increased reporter gene activity, whereas inhibitory factors decrease reporter gene activity.

Additionally, we have obtained a luciferase reporter of β-catenin-mediated transcriptional activation (obtained from R. Moon, UW). This reporter gene contains 8 TCF/LEF binding sites coupled to the firefly luciferase gene (pTA-Super-TOP-flash-luciferase) and allows us to measure activated cWNT signaling in primary mesenchymal cultures. Using the developing murine limb as a model system (E9.5-E11.5), two culture models have been used: (1) proximal mesenchymal cultures (PM), which give rise to numerous cartilage nodules within 4 days, and (2) distal mesenchymal cultures (DM), which contain a more homogenous chondroprogenitor

population and, thus, produce a much greater number of cartilage nodules. Consistent with this, distal-tip cultures exhibit a higher level of *Sox9* expression and increased basal SOX9 activity compared to stump cultures.

1.10 Rationale, hypothesis & objectives: Identification of GATA factors as a target of BMP signaling in early limb mesenchyme

Rationale

Primary limb mesenchymal (PLM) cultures provide a reliable and robust prochondrogenic response to BMPs, making this system an excellent model for deciphering the molecular basis of action of BMPs (Barna and Niswander, 2007; Hoffman et al., 2006). In recent studies we have identified the retinoic acid synthesis enzyme, *Aldh1a2* as a principal target of the BMP signaling pathway (Hoffman et al., 2006). More specifically, BMP4 functions by attenuating the expression of *Aldh1a2* at late stages of the chondrogenic program, thereby stimulating *Sox9* expression and activity, and ultimately elaborating the chondroblastic phenotype. Other studies have demonstrated an importance for BMP signaling during the earliest stages of the chondrogenic program when mesenchymal cells begin to coalesce and form condensations (Bandyopadhyay et al., 2006; Capdevila and Izpisua Belmonte, 2001; Pizette and Niswander, 1999; Yoon et al., 2005); however, the precise molecular mechanisms by which BMPs regulate such cellular behaviors early in the chondrogenic program remains unclear. We have recently found that there is a differential responsiveness of primary mesenchymal cultures to BMP4 which is dependent on the stage of development. More specifically, BMP4 inhibits chondrogenesis in primary mesenchymal limb cultures derived from the limb buds at the earliest stages of the chondrogenic program (E9.5 forelimbs, E10.5 hindlimbs and distal forelimbs) but promotes chondrogenesis in those cultures derived at later stages (E10.5 proximal forelimbs, E11.5 whole-limbs). An unbiased genome-wide strategy using microarrays was used to investigate the molecular basis of this responsiveness in various subpopulations of primary limb

mesenchymal cells. These analyses led to the identification of a number of downstream targets of BMP signaling early in chondrogenesis, including *Gata* transcription factors, which demonstrated the most profound changes in expression.

Hypothesis

BMP signaling negatively impacts chondrogenesis in the early limb mesenchyme through modulation of *Gata* gene expression.

Objectives

1. Cellular and molecular characterization of the activity of BMP signaling in early limb development.
2. Identification of BMP induced and repressed genes early in the chondrogenic program and determination of their functional relevancy in BMP signaling and chondrogenesis.

1.11 Rationale, hypothesis & objectives: Canonical WNT signals exhibit disparate effects on chondrogenesis which are associated with the stage of development

Rationale

Several factors belonging to the WNT signaling family have been shown to regulate the appearance of chondrogenic phenotypes in the developing limb. Previous studies have demonstrated that the canonical WNTs, WNT3a and WNT7a, inhibit chondrogenesis (Day et al., 2005; Hill et al., 2005; Rudnicki and Brown, 1997). A limitation of these studies may be that they focused on later stages of skeletal development and did not address the potential roles of WNT signaling in early skeletal development, particularly in the specification of mesenchymal cells to the chondrocytic lineage. Hence, if canonical WNT signals are important at the earliest stages of the chondrogenic program, then it is expected that cartilage formation will be significantly enhanced by canonical WNTs in cartilage-deficient early primary mesenchymal limb cultures. Furthermore, the AER secretes a wide range of signaling molecules, including WNTs, which are required for the coordination of growth and patterning of the limb, primarily in

more distal regions of the limb bud (reviewed in (Capdevila and Izpisua Belmonte, 2001)). In this regard, we seek to determine which ectodermal signals are important in specifying a chondrogenic fate in the early limb mesoderm. In fact, previous studies have suggested that the limb ectoderm exerts both stimulatory and inhibitory effects on cartilage differentiation depending on the stage of the underlying mesoderm (Hamburger and Hamilton, 1992). Specifically, the early limb mesoderm requires the presence of the ectoderm for its survival and for the formation of cartilage. At later stages, this dependence is lost and instead, the formation of cartilage is inhibited by the presence of the ectoderm (Solursh and Reiter, 1988). Hence, cartilage formation has long been known to require exposure to an ectoderm-derived factor and based on our studies WNT3a may play a role in this function.

We have found that there is a differential responsiveness of primary limb mesenchymal cultures to WNT3a which is dependent on the stage of development. Specifically, we have found that primary cultures derived from E11.5 proximal limb bud cultures with WNT3a (50 ng/ml) inhibits chondrogenesis as has been shown in previous studies. However, primary limb mesenchymal cultures established from early primary mesenchymal cultures (< E10.5 forelimbs or < E10.5 hindlimbs) form very little cartilage and this can be significantly enhanced by the addition of WNT3a (50 ng/ml). To decipher the role of WNT signaling in early limb development, we have established primary mesenchymal limb cultures from various regions of the murine E9.5 forelimb (FL) and E10.5 FL and hindlimb (HL). Interestingly, these cultures exhibited a wide range of responses to WNTs, from inhibition to stimulation of chondrogenesis. An unbiased genome-wide strategy using microarrays was employed to investigate the molecular basis of responsiveness to these subpopulations of primary limb mesenchymal cells. These analyses led to the identification of a number of downstream targets of WNT signaling early in chondrogenesis.

Hypothesis

Cartilage induction has long been known to require the exposure to an ectoderm-derived factor and we propose that the canonical WNT, WNT3a, performs this function.

Objectives

1. Cellular and molecular biological characterization of the activities of canonical and non-canonical WNT signaling pathways in chondrogenesis.
2. Identification of canonical and non-canonical WNT induced and repressed genes in the chondrogenic program and determination of their functional relevancy in WNT signaling and chondrogenesis.

1.12 Rationale, hypothesis & objectives: Comparative functional analysis of the mouse and *Fugu Sox9* promoters

Rationale

Campomelic dysplasia cases have underlined the extended and complex regulation of temporal and tissue-specific *Sox9* expression. To a large extent, recent progress has been made towards understanding the molecular biology of chondrogenesis and the critical role of *Sox9* in this program. Given the complexity and size of the *Sox9* promoter, it has been difficult to define essential regulatory elements.

Using a combination of molecular approaches and the developing mouse limb as a model system, we have analyzed the proximal and distal regions of the *Sox9* promoter in both mouse and *F. rubripes* and the results attained from these experiments have added to our current understanding of *Sox9* expression and cartilage development.

Hypothesis

Cartilage-specific expression of *Sox9* relies on elements within the proximal and distal regions of the *Sox9* promoter.

Objectives

1. To characterize the proximal and distal regions of the *Sox9* promoter.

2. To identify specific factors that modulate *Sox9* expression during chondrogenesis.

1.13 References

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CHAPTER II:
Identification of GATA factors as targets of BMP signaling in early limb mesenchyme

A version of this chapter will be submitted for publication. Karamboulas, K. and Underhill, T.M. Identification of GATA Factors as Targets of BMP Signaling in Early Limb Mesenchyme.

2.1 Introduction

Cartilage plays a fundamental role in the development of the appendicular skeleton, as bones within the limb are formed from a cartilage precursor and the cartilage, itself, forms from condensed mesenchyme. These condensations represent the earliest stages of limb patterning and ultimately give rise to mature bones of the limb (Hall and Miyake, 1992; Hall and Miyake, 1995; Hall and Miyake, 2000). Following condensation, mesodermal cells within this condensation differentiate into chondrocytes during outgrowth, such that proximal mesenchymal cells differentiate prior to more distal mesenchymal cells. This spatiotemporal regulation of mesenchyme differentiation into chondrocytes is a crucial step in endochondral bone formation in that it preserves the pattern of the bone primordia established earlier in limb development and provides a suitable matrix for subsequent ossification.

During limb outgrowth, signals that promote as well as inhibit chondrogenesis are important determinants of skeleton formation. Several members of the Transforming Growth Factor-β (TGF-β) superfamily figure prominently in the chondrogenic program, including the Bone Morphogenetic Proteins (BMPs). Indeed, BMP activity was first identified in demineralized bone extracts (Urist, 1965) and has subsequently been shown to induce cartilage formation both *in vivo* (Wozney et al., 1988) and *in vitro* (Chen et al., 1991; Chen et al., 1992; Wozney et al., 1988). Further evidence that BMPs play an important role in regulating chondrogenesis comes from recent studies describing the conditional deletion of either *Bmp*s or their receptors. Several *Bmp*s are expressed in the developing limb and deletion of *Bmp2* and *4* within the limb mesenchyme leads to a loss of precartilaginous condensations (Bandyopadhyay et al., 2006). Similarly, deletion of BMP receptors, *Bmpr1a* and *1b*, leads to an absence of all limb condensations and in those that do form the cells fail to differentiate into chondrocytes (Yoon et al., 2005). In addition, misexpression of the BMP antagonist NOGGIN (Zimmerman et al., 1996) leads to an inhibition of chondrogenesis by embryonic limb mesenchyme (Pizette and

Niswander, 2000; Weston et al., 2000), whereas *Noggin*-null animals present with increased cartilage (Brunet et al., 1998). This inhibition of cartilage formation with increased NOGGIN, appears to be due to a reduced formation of prechondrogenic condensations, suggesting that BMPs play a critical role in initiating mesenchyme cell condensation and subsequent cartilage differentiation (Pizette and Niswander, 2000) (Weston et al., 2000). Consistent with this idea, recent studies have demonstrated that BMP signaling is required for the "compaction" of chondroprogenitors whereby mesenchymal cell aggregates coalesce and form tight interactions that lead to the establishment of cartilage nodules (Barna and Niswander, 2007).

Critical to the chondrogenic program is the transcription factor SOX9. The *Sox9* gene belongs to a large family of transcription factors, which are characterized by a high-mobility group DNA binding domain similar to that of the testis-determining gene *Sry* (Lefebvre et al., 1997; Ng et al., 1997) (Wright et al., 1995). In chimeric animals, *Sox9*-null cells are excluded from developing cartilages (Bi et al., 1999). Subsequently, analysis of conditional null mutants of *Sox9* has demonstrated an absolute requirement for *Sox9* in the formation of precartilaginous condensations and in chondrocyte differentiation. Forced expression of *Sox9* in some cell types, leads to the expression of chondrocytic genes (Bell et al., 1997; Healy et al., 1999). Other *Sox* genes have been found to be expressed in the developing skeleton, and in particular L-*Sox5* and *Sox6* together are necessary for cartilage formation, but appear to function downstream of *Sox9* during chondroblast differentiation (Lefebvre et al., 2001; Lefebvre et al., 1998; Smits et al., 2001).

Primary limb mesenchymal (PLM) cultures provide a reliable and robust pro-chondrogenic response to BMPs, making this system an excellent model for deciphering the molecular basis of BMP action (Barna and Niswander, 2007; Hoffman et al., 2006). In recent studies we have identified the retinoic acid synthesis enzyme, *Aldh1a2* as a principal target of the BMP signaling pathway (Hoffman et al., 2006). More specifically, BMP4 functions by

attenuating the expression of *Aldh1a2* in the limb bud, thereby enhancing chondroblastic differentiation. In this manner, regions within the limb with chondrogenic potential are directed to alternative cell fates by activation of the retinoid signaling pathway. Other studies have demonstrated an importance for BMP signaling during the earliest stages of the chondrogenic program when mesenchymal cells begin to coalesce and form condensations (Bandyopadhyay et al., 2006; Pizette and Niswander, 2000; Yoon et al., 2005); however, the precise molecular mechanisms by which BMPs regulate such cellular behaviors in early limb development remains unclear. To gain insights into the actions of BMPs in early limb development at the onset of their expression, we have established primary cultures from various regions of the murine E9.5 forelimb (FL) and E10.5 FL and hindlimb (HL). Surprisingly, these cultures exhibited a wide range of responses to BMPs from inhibition to stimulation of chondrogenesis. An unbiased genome-wide strategy using microarrays was used to investigate the molecular basis of this responsiveness in these subpopulations of PLM cells. These analyses led to the identification of a number of downstream targets of BMP signaling early in chondrogenesis, including *Gata* transcription factors. Herein, we define a novel and unanticipated role for *Gata* transcription factors in the early appendicular chondrogenic program.

2.2 Methods and materials

Reagents

BMP4 and NOGGIN were purchased from R and D systems and resuspended according to the manufacturer's instructions. BMP4 was added to media at a concentration of 20 ng/ml, whereas NOGGIN was used at a concentration of 100 ng/ml (R and D Systems Inc.). SB 202190 (p38 inhibitor) was purchased from Sigma-Aldrich, resuspended according to the manufacturer's instructions and added to media a concentration of 10 μM.

Plasmid constructs

To assess chondrogenic activity, a SOX-responsive reporter was used. This reporter contains four reiterated SOX5, 6 and 9 binding sites (4X48 bp) upstream of a minimal type II collagen promoter (-89/+6) coupled to a luciferase gene [pGL3-4x48] (Weston et al., 2002). Full-length cDNA clones for mouse *Gata3* and *5* were obtained from the Ultimate ORF clone collection (InVitrogen) and recombineered into a Gateway compatible destination vector containing a CMV promoter (pcDNA3.1/nV5-DEST).

Establishment and transfection of primary mesenchymal cultures

PLM cultures from CD-1 embryonic age (E) E11.5 mouse limb buds were established as previously described (Hoffman et al., 2006). E10.5 PLM cultures were established from the PM and DM of the FL, and the DM of the HL from regions shown in figure 2.1C. After proteolytic digestion, cells were filtered through a Cell Strainer (40 μM; Falcon) to obtain a single cell suspension and resuspended at a density of 2 X 10^7/ml. For microarray analyses, 5 X 10 μl aliquots of this suspension were plated into a Nunc 35 mm tissue culture dish and allowed to adhere for 1 hr. Following this period, 2 mls of culture medium consisting of 60% F12, 40% DMEM and 10% FBS (Qualified-Invitrogen) was added to each well with or without 20 ng/ml BMP4 (R & D Systems) — this time was considered time 0 (T=0). Culture media was replaced on alternate days and cultures were maintained for a period of from 1-4 days. Alcian blue staining was carried out as described in (Weston et al., 2002; Weston et al., 2000).

For transfection, a new more efficient protocol was developed as the previous FuGene6-based method exhibited poor transfection efficiency in E10.5 limb-derived cells. The new protocol utilizes Effectene (Qiagen), supplemented with trehalose—this addition increases transfection efficiency > 3 fold (Garcha and Underhill, in preparation). Briefly, 0.25 μg of plasmid reporter, 0.025 μg pRL-SV40 (Promega) and 0.75 μg of expression vector (Stratagene,

Invitrogen) were combined for a total of ~ 1 μg of DNA. One microlitre of Enhancer solution is added to 15 μl EC buffer (Qiagen supplemented with 0.4 M trehalose) and combined with DNA. Following a 10' incubation, 5 μl of Effectene was added. Seven and a half microliters of this DNA/Effectene transfection mixture was transferred to sterile 1.5 ml microfuge tubes, followed by 40 μl of cell suspension. Cells and the transfection mixture were gently triturated, and 10 μl was used to seed a single well of a 24-well culture dish. The cells were allowed to attach for one hour, followed by the addition of 1 ml of media to each well. Extracts for luciferase analysis were collected on Day 1 or 2 as indicated. For experiments involving PLM cells alone or cells transfected with reporter genes only, factors or compounds were added at the time of media addition (T=0), unless otherwise indicated. For experiments involving co-transfections of cells with expression plasmids, factors and compounds were added up to 24 hours following transfection. Analysis of reporter gene activity was measured using the Dual Luciferase Assay System according to the manufacturer's instructions (Promega). Briefly, cells were washed once with PBS and lysed in 100 μl of Passive Lysis Buffer for 20 minutes and frozen at -80^{o}C overnight. Forty microliters of each lysate was then loaded into a 96-well plate and luciferase activity was determined using a luminometer (L-Max; Molecular Devices). Firefly luciferase was normalized against *Renilla* luciferase activity to control for differences in transfection efficiency.

Transcriptional profiling with microarrays: Experimental design and analysis

RNA was harvested from primary cultures using RNAeasy (Qiagen) according to the manufacturer's instructions as described previously (Hoffman et al., 2006). Following isolation, RNA was diluted to 125 ng/μl, the expression of *Sox9* and *Col2b* were measured using real-time PCR and RNA quality was examined on a Bioanalyzer 2100 (Agilent).

For each time point 2 biological replicates were analyzed. The Affymetrix mouse transcriptome arrays (MOE430 2.0) were used to generate transcriptional profiles from RNA collected at 24 and 48 hours treated with BMP4 (20 ng/ml) derived from E10.5 FL-DM, FL-PM and HL cultures. To eliminate the need for amplification prior to microarray analyses, > 1 μg of RNA was collected from cultures derived from ~280 embryos. One μg of RNA was labeled and hybridized to the chips using the manufacturer's recommended protocol (Affymetrix Inc.). Data sets were subsequently uploaded into GeneSpring for bioinformatic analysis and analyzed for the expression of individual genes (~ 39,000 on the chip set, with redundancy closer to 36,000 genes). All data sets were initially filtered to remove genes called absent by GeneSpring. Hierarchical clustering was carried out in GeneSpring with the GeneTree tool using Pearson correlation similarity measure.

Real-time PCR

To follow the expression of various gene transcripts, quantitative real-time PCR was carried out using the 7500 Fast Real-Time PCR System (Applied Biosystems). Some primers and TaqMan-minor groove binding (MGB)-probes were designed using PrimerDesigner 2.0 (Applied Biosystems). The primer/probe sets used for detection of *Sox9* and *Col2b* were used as described in Weston et al. (Weston et al., 2002). Primer and probe concentrations were optimized according to the manufacturer's instructions. Other primer and probe sets were purchased from the TaqMan gene expression collection (Applied Biosystems). Total RNA was isolated from primary cultures as described above. Quantification was carried out using ~10 ng of total RNA and the expression of all genes relative to endogenous *rRNA* was determined using TaqMan Ribosomal Control Reagents (Applied Biosystems) and the relative quantitation method.

Statistical analysis

All luciferase assays were performed in quadruplicate and repeated using three distinct preparations of PLM cells. Real-time PCR analysis was carried out in duplicate and repeated a minimum of 2 times with independent RNA samples. Luciferase reporter data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey post-test for multiple comparisons using GraphPad Prism, Version 4.0 (GraphPad Software Inc., San Diego, CA). Significance is represented as follows: *, $P < 0.05$; **, $P < 0.01$; #, $P < 0.001$. One representative experiment is shown for all luciferase and real-time PCR results.

2.3 Results

The BMPs play essential roles at multiple stages of the chondrogenic program. Embryos deleted for either *Bmp*s or their receptors present with several skeletal anomalies that arise through deficits in establishment of precartilaginous condensations. *Bmp*s (*2*, *4* and *7*) appear in the limb around embryonic age E10 (Bandyopadhyay et al., 2006) and to investigate their function in these early populations of limb mesenchymal cells we have employed the high-density micromass cell culture model. Examination of BMP4 responsivness in cultures from either the proximal or distal regions of the E11.5 limb reveals that BMPs stimulate cartilage nodule formation (Fig. 2.1A). Consistent with this, BMP4 also increases in a dose-dependent manner activity of a SOX-responsive reporter gene to 3 fold with 20 ng/ml treatment (Fig. 2.1A). These findings are similar with earlier reports and in keeping with BMP-mediated induction of *Sox5*, *6* and *9* expression (Hoffman et al., 2006). These three factors function together to regulate expression of *Col2a1*, and the SOX reporter is based on the common SOX5, 6 and 9 binding site within *Col2a1* (Lefebvre et al., 1997; Lefebvre et al., 1998).

BMPs exhibit stage-dependent chondrogenic activities

In the E10.5 limb, *Sox9* is expressed to varying extents, with the greatest expression in more proximal regions and the least in the distal mesenchyme (Fig. 2.1B). In contrast, *Sox6* a gene associated with chondroblast differentiation is for the most part evenly distributed throughout the fore and hindlimb at E10.5 (Fig. 2.1B). In comparison to E11.5 limb buds *Sox6* expression is significantly reduced (data not shown). The more uniform and weak expression of *Sox6* at E10.5 is reflective of the limited chondroblastic differentiation at this stage. The differential expression of these cartilage markers is reflective of the chondrogenic stage of cells within these regions, with more immature chondrogenic cells being found in the distal hindlimb mesenchyme in comparison to the other regions.

To assess BMP function in these different populations, cultures were established from 3 different regions, HL distal mesenchyme (HL-DM), FL distal mesenchyme (FL-DM) and proximal FL mesenchyme (FL-PM) (Fig. 2.1C). Under these conditions, microdissection of the limbs of ~ 12 embryos produces sufficient cells for 4, 4 and 10 micromass cultures from HL-DM, FL-DM and FL-PM, respectively. In contrast, to that observed in E11.5 cultures, addition of BMP4 (20 ng/ml) reveals that E10.5-derived cultures exhibit differential responsiveness to BMP4 (Fig. 2.1C). In HL cultures, BMP4 addition markedly inhibits cartilage nodule formation (Fig. 2.1C) and this is accompanied by a ~ 2-fold and ~ 4-fold reduction in *Sox9* and *Col2al* expression (Fig. 2.1D) and a 6-fold decrease in SOX reporter gene activity (Fig. 2.1E), whereas NOGGIN stimulates reporter gene activity (Fig. 2.1E). BMP4 also inhibited cartilage formation in FL-DM cultures, but to a lesser extent than observed in HL-DM. Consistent with this, *Sox9* and *Col2a1* showed little change (Fig. 2.1D), whereas SOX reporter activity was reduced to a similar extent as in HL-DM cultures (Fig. 2.1E). In contrast, in FL-PM cultures, BMP4 marginally increased cartilage nodule formation (Fig. 2.1C) and *Col2a1* expression (Fig. 2.1D), and had no significant affect on either *Sox9* expression (Fig. 2.1D) or activity (Fig. 2.1E). In

comparison to later stage cultures, the E10.5-derived cultures in general exhibit either an anti-chondrogenic or muted pro-chondrogenic response to BMP4 (Fig. 2.1C). Together, these results suggest that the limb mesenchyme exhibits stage-dependent responses to BMP signaling, that can be interpreted as representing a continuum of BMP responsiveness from negative to positive in immature to more mature chondrogenic precursors.

The observation that BMP responsiveness changed in accordance with the temporal appearance of these populations in the limb, suggested that time alone may influence BMP action. Cultures were incubated for 1 day following establishment prior to BMP addition. Under these conditions in all cultures, BMP4 stimulated cartilage nodule formation and SOX activity (Fig. 2.2). In HL-DM cultures, SOX reporter activity went from a ~ 5 fold decrease with BMP4 addition on Day 0, to a ~ 9-fold increase after addition 24 h later (Fig. 2.2B). Comparable trends, albeit with smaller increases in reporter activity were also observed in the FL populations. Similarly, the expression of *Sox6*, *Sox9* and *Col2* in Day 0 versus Day 1 treated cultures was increased (Fig. 2.2C). Thus, the responsiveness of limb mesenchymal cells to BMPs is dictated by their stage of development.

To investigate the molecular basis of stage-dependent responses of limb mesenchymal cells to BMP4, genome-wide expression profiling with microarrays was employed. Cells were isolated from the three different regions and cultured in the presence or absence of BMP4 for 24 and 48 h, and transcriptional profiles were generated through hybridization of processed RNA to Affymetrix MOE430 2.0 chips. Consistent with the qPCR described earlier, cartilage-associated genes (*Agc1*, *Sox6*, *Col2a1*) were decreased in HL-DM samples following addition of BMP4, and exhibited little or no change in FL-DM samples, whereas they were increased in FL-PM samples (Fig. 2.3). To identify genes exhibiting differential responsiveness in the populations, fold cut-off analyses were used to select genes that exhibited a 2-fold change in expression following BMP addition in HL and FL-PM samples. A gene list of 288 genes was generated and

Pearson correlation analysis was used to cluster genes based on their expression patterns (Fig. 2.4). Not surprisingly, numerous genes involved in chondrogenesis exhibit differential expression and several of the known BMP-responsive genes, such as *Noggin* were also up-regulated under all conditions (Fig. 2.4, box 1). In contrast to *Noggin*, the expression of *Bmp2* and *7* were down-regulated across all BMP4-treated samples (Fig. 2.4, box 3). Further, several transcription factors associated with limb development or skeletogenesis were also differentially expressed including, *Pax1* and *9*, *Runx2*, *Zic3*, *Twist1*, *Alx3*, *Meox2*, *Foxc1* and *2*, and *Dlx2* (Table 2.1). In general, the microarray data closely agreed with the observed biological activities of BMP4 in the different limb cell populations.

_Gata_s are target genes of the BMP signaling pathway

Further bioinformatic inspection of the transcriptional profiles, revealed that multiple *Gata* genes (*Gata2*, *3*, *5*, and *6*) were also induced following BMP4 addition, with HL samples exhibiting greater changes in expression then FL samples. In contrast, analysis of microarrays from E11.5-derived cultures revealed no significant changes in the expression of *Gata1*, *2*, *3* and *6* in response to BMP4 addition, whereas *Gata4* and *5* were called absent and showed no change (data not shown). *Gata*s have been shown to be regulated by BMPs in other developmental systems (Andree et al., 1998; Maeno et al., 1996; Schultheiss et al., 1997) and there is a link between GATA factors and *Sox9* expression in the developing testes (Bouma et al., 2007; Manuylov et al., 2007) (Tevosian et al., 2002). A recent study has shown that *Gata6* is expressed in the limb, within precartilaginous condensations, however other *Gata*s with the exception of *Gata*2 were not detected (Alexandrovich et al., 2007). To assess potential involvement of *Gata* genes in limb development, it would be first important to establish that *Gata*s are in fact expressed in the limb. For this purpose, quantitative PCR was used to quantitate *Gata* expression in sub-regions of the E10.5 fore and hindlimb. *Gata5* was found to be expressed in a distal-proximal gradient in the HL and FL, with a 9-fold increase in expression

in the proximal versus distal limb bud (Fig. 2.5A). *Gata6* exhibits a similar pattern of expression in the HL, but changes little in the FL, whereas *Gata3* exhibits little change within the limb. Overall, *Gata5* and *6* are more abundantly expressed in the E10.5 HL versus the FL. All 3 *Gata*s could be detected in the E9.5 limb, whereas in the E11.5 limb, *Gata3* was abundantly expressed and *Gata5* and *6* exhibited reduced expression in comparison to the E10.5 HL, with *Gata5* only being detected in the most proximal section. Thus, *Gata5* is expressed transiently in the early limb.

Expression of *Gata3*, *5* and *6* was also evaluated in primary cultures following BMP4 and/or NOGGIN (100 ng/ml) treatment (Fig. 2.5B). Following a 24 h treatment with BMP4, *Gata3*, *5* and *6* were induced, ~ 10, 36 and 26-fold, respectively in HL-DM cultures. *Gata3* and *6* expression were also induced by BMP4 in FL cultures, albeit to a lower level then observed in the HL-DM cultures. NOGGIN alone had a minimal effect on *Gata* expression, but in combination with BMP4, negated BMP4-mediated induction of *Gata* gene expression. Together, these findings parallel the expression profiles observed with microarray analysis and further reinforce the observation that *Gata*s are robustly induced in response to BMP4 treatment. Further, consistent with *in vivo Gata* expression patterns, the HL-derived cultures abundantly expressed *Gata*s, especially *Gata5*, and these cells also exhibited the greatest induction of *Gata*s following BMP4 addition.

BMPs function through modulation of SMAD activity and/or the MAPK signaling pathways including the p38 MAPKs (Nohe et al., 2003; Shi and Massague, 2003). Previous studies have demonstrated that inhibition of p38 MAPK inhibits chondroblast differentiation and cartilage formation (Oh et al., 2000; Weston et al., 2002). To assess the role of p38 MAPK signaling in BMP-mediated induction of *Gata*s, cultures were treated with the p38 MAPK inhibitor SB202190 (10 μM). Similar to other reports, SB202190 addition attenuates SOX reporter gene activity by > 20 fold and inhibits the ability of BMP4 to stimulate SOX activity in

all cultures (data not shown). Interestingly, BMP-mediated induction of the *Gata*s is only marginally impacted if at all by SB202190 (especially in comparison to the extent of the effect of SB202190 on the SOX reporter activity), suggesting that this pathway is unlikely involved in BMP regulation of *Gata* expression. Further, under these conditions, if the p38 MAPK pathway was playing a role in *Gata* expression, then addition of SB 202190 to HL-DM cultures may have at least partially inhibited the anti-chondrogenic activities of BMPs, and this was not the case.

Treatment of HL-DM cultures with BMP4 inhibits chondrogenesis, whereas the FL populations exhibit little or slightly positive chondrogenic response to BMP addition. These observations parallel the general trends observed with BMP induction of *Gata*s, with the HL cultures exhibiting the greatest increase in *Gata* expression upon BMP4 addition. As shown earlier, adding BMP4 24 h following culture initiation can reverse its anti-chondrogenic activities. If *Gata*s are in part involved in mediating the chondrogenic activities of BMPs in early limb mesenchyme, then it might be expected that BMP4-mediated *Gata* induction would be muted if BMP4 was added 24 h following culture establishment. This was indeed the case. HL-DM cultures exhibit the largest increase (12-60 fold) in *Gata* expression following BMP4 addition at time 0, and if added at 24 h post-culture initiation, then *Gata* expression is only induced 2-3 fold (Fig. 2.6). Similar trends are observed in both FL cultures. Together, these findings demonstrate that the extent of BMP4-mediated *Gata* induction coincides with its anti-chondrogenic activities. Thus, it might be postulated that high *Gata* expression attenuates expression of the chondroblastic phenotype.

Manipulation of *Gata5* inhibits chondroblast differentiation

To assess the relationship of *Gata* expression to chondrogenesis, we over-expressed *Gata5* in the presence and absence of BMP4 in E10.5-derived cultures and followed SOX activity (Fig. 2.7). SOX activity in previous studies has been found to tightly correlate with cartilage formation (Hoffman et al., 2006; Muramatsu et al., 2007; Weston et al., 2002; Weston

et al., 2003). As shown above, treatment of PLM cultures with BMP4 on Day 0 reduced SOX reporter activity (lysed on Day 1) to varying extents in the different cell populations, with the HL-DM cultures exhibiting the greatest decline in activity. Expression of *Gata5* (and *Gata6*, data not shown) also significantly reduced reporter gene activity by 50-60%, and in combination with BMP4, reporter activity was further marginally decreased in comparison to BMP4 alone (Fig. 2.7). In contrast, addition of BMP4 on day 1 with lysis on day 2, was either neutral or stimulated reporter gene activity. Under these conditions, expression of *Gata5* reduced reporter gene activity in all cell populations to ~ 20% of controls and this was partially rescued by BMP4. Together, these results demonstrate that increased expression of *Gata5* can phenocopy BMP activity in the early limb mesenchyme and that continued expression of *Gata5* inhibits chondroblast differentiation.

2.4 Discussion

Multiple *Bmp*s are expressed in the early limb and recent genetic evidence suggests that *Bmp*s play a minor role if any in limb patterning, but are critical to the establishment of the chondrogenic elements. Similarly, deletion of the *Bmpr* type I receptors leads to reduced *Sox9* expression and deficiencies in condensation formation. Herein, we have identified diverse roles for BMP signaling in the early limb, with BMPs negatively impacting chondrogenesis in limb mesenchyme from the early limb bud. At slightly later stages, BMPs stimulate the formation of condensations and subsequent cartilage nodules. These disparate activities appear to be linked tightly to *Gata* expression, as in situations where chondrogenesis is inhibited by BMP4 *Gata*s are strongly induced and this is accompanied by decreased expression and activity of *Sox6* and *9*. In cultures derived from the E11.5 limb and treated with BMP4, *Gata* expression exhibits little change, further underscoring that the BMP induction of *Gata* expression is a characteristic of the early limb mesenchyme.

BMP signaling, *Gata*s and chondrogenesis

BMPs play an essential role in activating the chondrogenic program within somitic mesoderm. Studies over the last several years have established a model where BMP signaling reinforces a positive regulatory loop between *Nkx3-2* and *Sox9*, which promotes and sustains *Sox9* expression culminating in chondroblast differentiation (Murtaugh et al., 1999; Murtaugh et al., 2001; Zeng et al., 2002). In this model, *Nkx3-2* functions as a transcriptional repressor to repress the expression of a gene X, which in turn negatively regulates *Sox9* expression. Continued expression of NKX3-2 inhibits gene X expression thereby promoting derepression of *Sox9* and its expression. In this manner, *Nkx3-2* functions to promote chondrogenic competency of the somitic mesoderm enabling BMPs to promote expression of the chondrogenic program. *Nkx3-2* expression can also drive the chondrogenic program in cultured limb mesenchyme (our unpublished results). However, in the limb *Nkx3-2* is expressed after *Sox9* and *Nkx3-2* null mutants present with axial malformations, but strikingly normal limbs suggesting an alternative program is operating in appendicular skeletogenesis (Tribioli et al., 1997; Tribioli and Lufkin, 1999).

Gata5 is transiently expressed during limb development, and of the three *Gata*s analyzed, *Gata5* exhibits the greatest magnitude change in response to BMP4. Further, the extent of induction is greatest in the more immature prechondrogenic cells from the distal mesenchyme of the hindlimb. Consistent with the transient nature of *Gata5* expression *in vivo*, BMP4 induction of *Gata5* is also transient. Expression of *Gata5* reduces the activity of a *Col2a1*-based SOX reporter gene, indicative of impaired chondroblast differentiation. Similarly, *Gata*s are induced to varying extents by BMP4 in the various E10.5 populations and the extent of these inductions is proportional to the "anti-chondrogenic" activity of BMP4. Together, these various observations suggest that *Gata5* and possibly the other *Gata*s, at least partly mediate the "anti-chondrogenic" actions of BMPs in the early limb mesenchyme. Interestingly, it has been

recently reported that *Gata6* when over-expressed in 7 day-old differentiating ATDC5 cultures had no effect on the expression of *Col2a1* or other cartilage-associated genes (Alexandrovich et al., 2007). However, as shown herein the *Gata*s appear to act within a specific window of skeletogenesis to regulate the chondrogenic program.

In other developmental systems, *Gata*s have been shown to play a role in cell fate specification. In the developing heart, *Gata5* functions in induction of the myocardial program, whereas other *Gata*s are more critical for subsequent differentiation (Peterkin et al., 2007). In this system, the *Gata*s are expressed in a hierarchical network analogous to that observed in the early limb, with *Gata5* being expressed early followed by *Gata6* and *3*. Robust BMP induction of *Gata5* in a stage-dependent manner in the early limb is also certainly consistent with this model. However, expression of *Gata5* or BMP4 addition inhibits the appearance of chondroblasts. Recent studies, have also shown that *Gata*s function in a dose-dependent manner to regulate *Sox9* expression in gonadogenesis and in heart development (Bouma et al., 2007; Xin et al., 2006). As *Gata5* is transiently expressed in the early limb bud during establishment of prechondrogenic populations, its continued or increased expression may interfere with subsequent chondroblast differentiation. Likewise a similar scenario may be operating with BMP4 addition, where *Gata5* is induced > 60-fold within 24h. Collectively these findings suggest that attenuation of *Gata5* is necessary for expression of the chondroblastic phenotype. In this manner, *Gata*s may function in the early limb to prevent the premature differentiation of chondroprogenitors, thereby allowing expansion of these populations. However, these results do not preclude a role for *Gata*s in chondrogenic specification in the limb akin to that of *Nkx3.2* in the axial skeleton. In fact, the early and transient expression of *Gata5* in the limb and its acute sensitivity to BMPs is congruent with this function.

Null mutants have been generated for *Gata3*, *5* and *6*, and these mutants are either embryonic lethal before appendicular skeletogenesis or don't present with any apparent skeletal

defects. This is not entirely surprising given redundancy in *Gata* function and the fact that we have identified at least 4 *Gata*s (*Gata2*, *3*, *5* and *6*) in the early limb. Further, in the heart where *Gata*s are co-expressed and critical for cardiac morphogenesis, it has been challenging to evaluate the role of any one *Gata* factor in this process (Brewer and Pizzey, 2006).

BMPs play fundamental roles in the establishment of the precartilaginous condensations in the developing skeleton. Delineation of the molecular mechanisms underlying BMP action processes will be vital for understanding the processes regulating establishment of the cartilaginous anlagen and identification of the *Gata* factors as likely contributors to this process is a significant step forward. The next challenge will be in defining how *Gata*s regulate *Sox9* and possibly *Sox5*/*6* expression and consequently, the emergence of chondrogenic cells.

2.5 Acknowledgements

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2.6 Figures

Figure 2.1. BMP4 exhibits stage-dependent activities in cultures derived from early limb mesenchyme-region specific differences. A. Schematic representation of the regions used from E11.5 murine limbs to generate whole-limb (WL) and distal-tip (DM) cultures. BMP4 (20 ng/ml) stimulates cartilage formation in both WL and DM cultures as determined by alcian blue staining. Consistent with the increase in alcian blue staining, the activity of a SOX responsive reporter (pGL3(4X48)) also increases in a dose-dependent fashion with increasing concentrations of BMP4 (0 – 20 ng/ml) in WL and DM cultures. B. Quantitative RT-PCR of limb sections from E10.5 limb buds shows that the chondrogenic genes *Sox6* and *Sox9* are expressed to varying extents throughout the E10.5 limb, with the highest levels of expression in more proximal regions as compared to the distal mesenchyme. C. Graphical depiction of the sections used from E10.5 murine limbs to generate hindlimb distal mesencyhme (HL-DM), forelimb distal mesenchyme FL-DM) and forelimb proximal mesenchyme (FL-PM) cultures. BMP4 (20 ng/ml) exhibits substantially different activities in these 3 cultures. In HL-DM cultures, BMP4 exhibits anti-chondrogenic activity, alcian blue staining of cartilage nodules is substantially reduced, as is (D) *Sox9* and *Col2* expression and (E) SOX reporter activity. In contrast, BMP4 addition to FL-PM cultures leads to a modest increase in all of these measures, and FL-DM present with an intermediate response between HL-DM and FL-PM. (D) The addition of the BMP antagonist, NOGGIN (100 ng/ml) has a relatively minor effect, but it does stimulate SOX activity in the HL-DM cultures. Bar in A, C,1 mm.

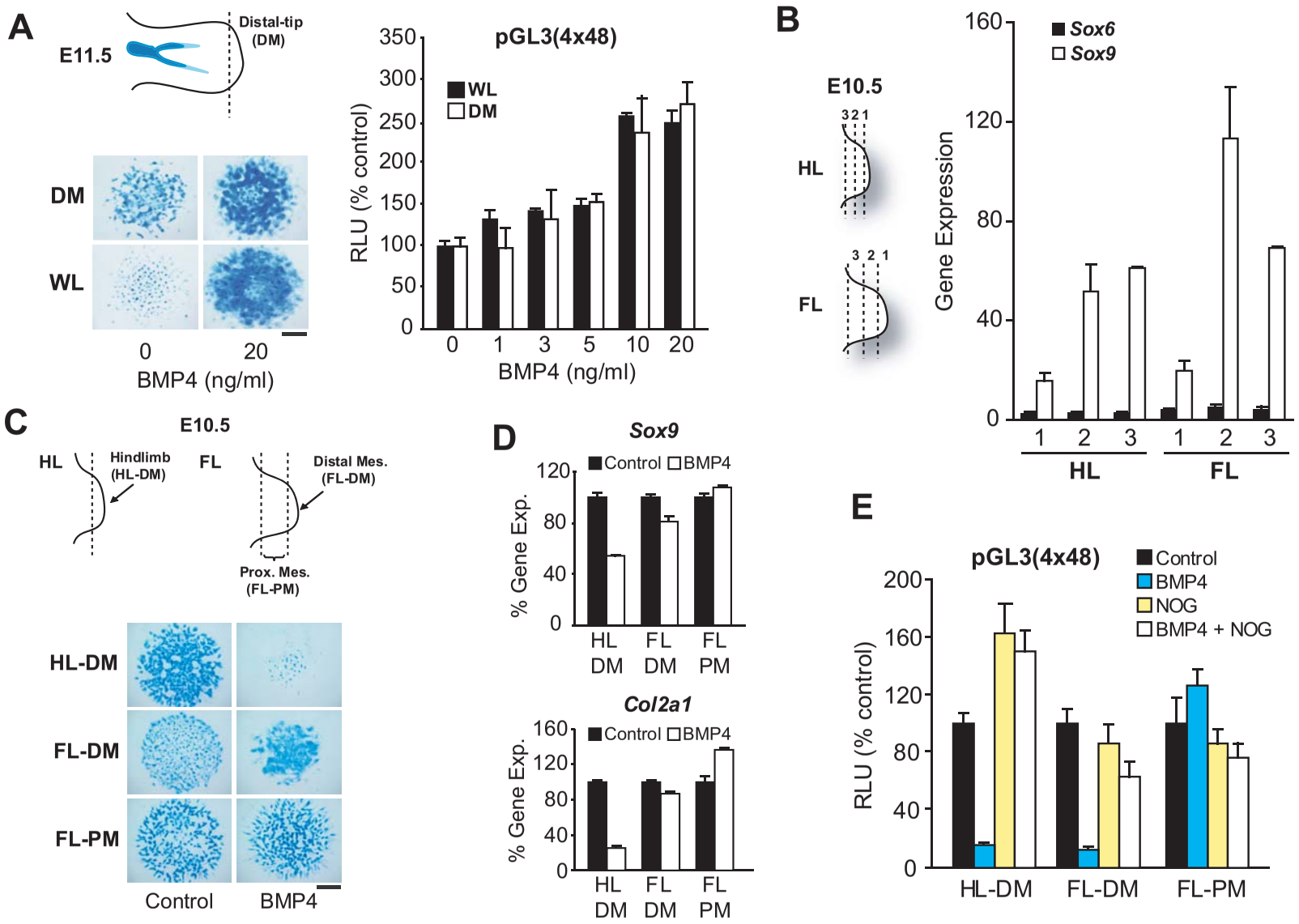
A
E11.5
Distal-tip
(DM)
DM
WL
0
20
BMP4 (ng/ml)
pGL3(4x48)
WL
DM
RLU (% control)
350
300
250
200
150
100
50
0
0 1 3 5 10 20
BMP4 (ng/ml)
B
E10.5
HL
FL
3 2 1
Sox6
Sox9
Gene Expression
160
120
80
40
0
1 2 3 1 2 3
HL
FL
C
E10.5
HL
Hindlimb
(HL-DM)
FL
Distal Mes.
(FL-DM)
Prox. Mes.
(FL-PM)
HL-DM
FL-DM
FL-PM
Control
BMP4
D
Sox9
Control BMP4
% Gene Exp.
120
80
40
0
HL DM
FL DM
FL PM
Col2a1
Control BMP4
% Gene Exp.
160
120
80
40
0
HL DM
FL DM
FL PM
E
pGL3(4x48)
Control
BMP4
NOG
BMP4 + NOG
RLU (% control)
200
160
120
80
40
0
HL-DM
FL-DM
FL-PM

Figure 2.2. BMP4 responsiveness of E10.5 cultures exhibits stage-dependent activities in cultures derived from early limb mesenchyme-time dependence. A. BMP4 exhibits disparate activities in E10.5 cultures, both stimulating and inhibiting cartilage formation. BMP4 addition 1 day following culture initiation leads to a more uniform response, with all cultures exhibiting increased amounts of alcian blue stained nodules. B, Similar to that observed with alcian blue staining, BMP4 increases SOX reporter activity in all cultures irrespective of origin when added 1 day following establishment. With the exception of the HL-DM cultures, NOGGIN addition leads to decreased reporter activity, with the FL-PM cultures being the most negatively impacted. C, Quantitative RT-PCR analysis of *Sox6*, *Sox9* and *Col2*, confirms what is observed with alcian blue staining and reporter gene analyses. The inhibitory activities of BMPs are mitigated following addition after 1 day in culture. Bar, 1 mm.

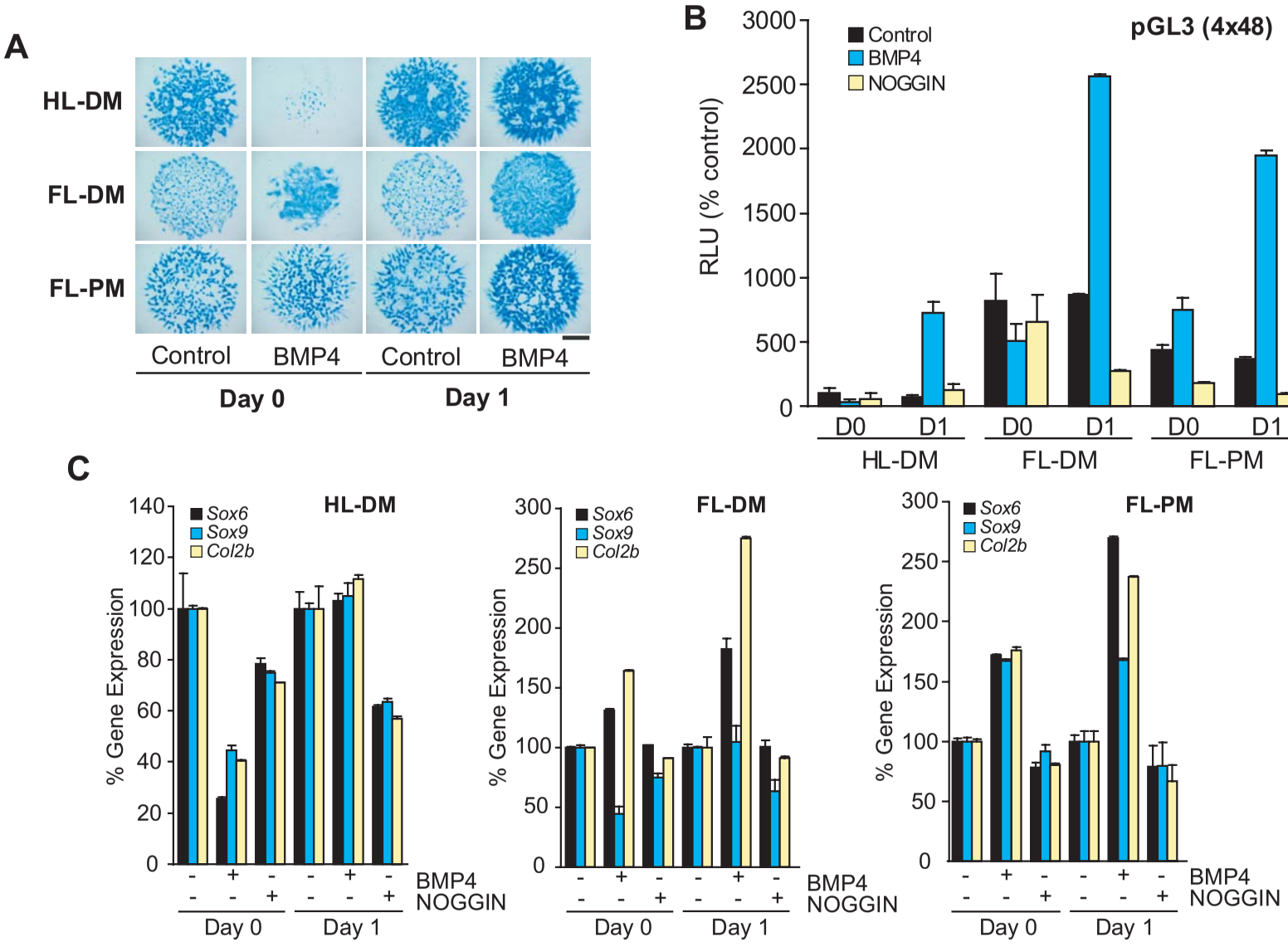
A
HL-DM
FL-DM
FL-PM
Control
BMP4
Control
BMP4
Day 0
Day 1
B
pGL3 (4x48)
Control
BMP4
NOGGIN
RLU (% control)
3000
2500
2000
1500
1000
500
0
D0
D1
D0
D1
D0
D1
HL-DM
FL-DM
FL-PM
C
HL-DM
FL-DM
FL-PM
Sox6
Sox9
Col2b
% Gene Expression
BMP4
NOGGIN
Day 0
Day 1

Figure 2.3. Microarray analysis of BMP4 action reveals that the three cell populations exhibit markedly different chondrogenic responses. *Agc1*, *Col2a1* and *Sox6* expression were analyzed in microarray data sets generated from RNA collected at 24 and 48h following BMP4 addition in HL-DM, FL-DM and FL-PM cultures. These 3 cell populations exhibit different responses to BMP4, ranging from inhibition (HL-DM) to stimulation (FL-PM) of *Agc1*, *Col2a1* and *Sox6*.

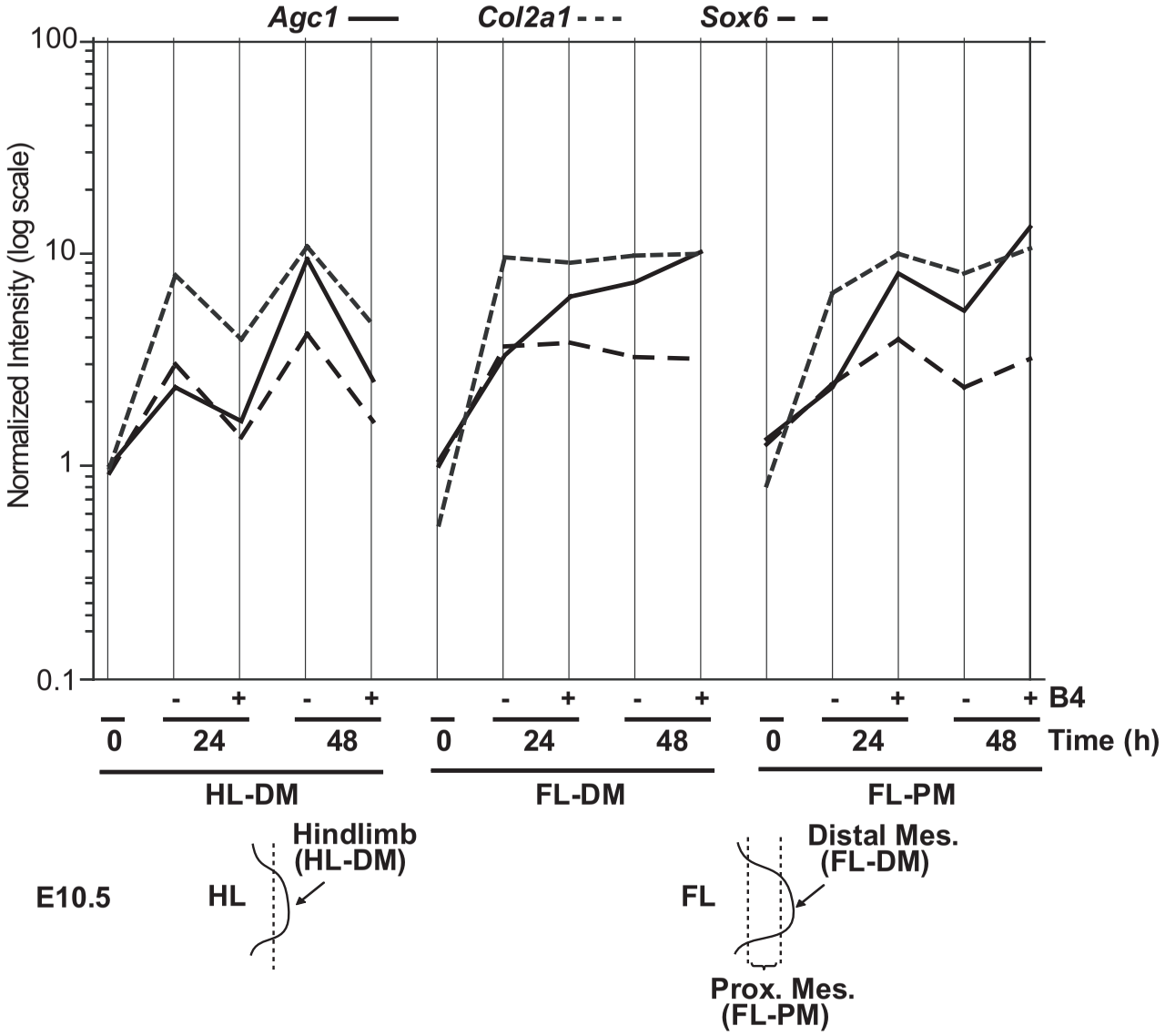
Agc1
Col2a1
Sox6
100
10
1
0.1
Normalized Intensity (log scale)
- + - + - + - + - + - + B4
0 24 48 0 24 48 0 24 48 Time (h)
HL-DM
FL-DM
FL-PM
E10.5
HL
Hindlimb
(HL-DM)
FL
Distal Mes.
(FL-DM)
Prox. Mes.
(FL-PM)

Figure 2.4. Elucidation of the genetic programs underlying BMP action in early primary mesenchymal limb cultures. Affymetrix mouse transcriptome arrays (MOE430 2.0 chips) were used to generate transcriptional profiles from RNA collected at 24 and 48 hours following treatment with BMP4 (20 ng/ml), derived from E10.5 HL, FL-DM and FL-PM cultures. Left panel, hierarchical clustering of genes that exhibited a > 2 fold expression change in HL-DM and FL-PM samples following 24 hr BMP treatment. A list of 288 genes was generated after the cutoff was applied and genes common to the 2 samples selected, and these were clustered using Pearson correlation similarity measure. Right panel, the boxed regions in the left panel were expanded to highlight different patterns of expression. Box 1 represents genes that co-clustered with *Noggin* and are generally upregulated in response to BMP4 in all cultures. Box 2 contains a cluster of genes which are up-regulated in the HL-DM samples, but exhibit little or decreased expression in the FL samples. Three *Gata*s *2*, *5*, and *6* are found within this cluster. Box 3 contains genes that were down-regulated by BMP addition, and these include *Bmp2* and *Bmp7*. Genes of interest are highlighted in red.

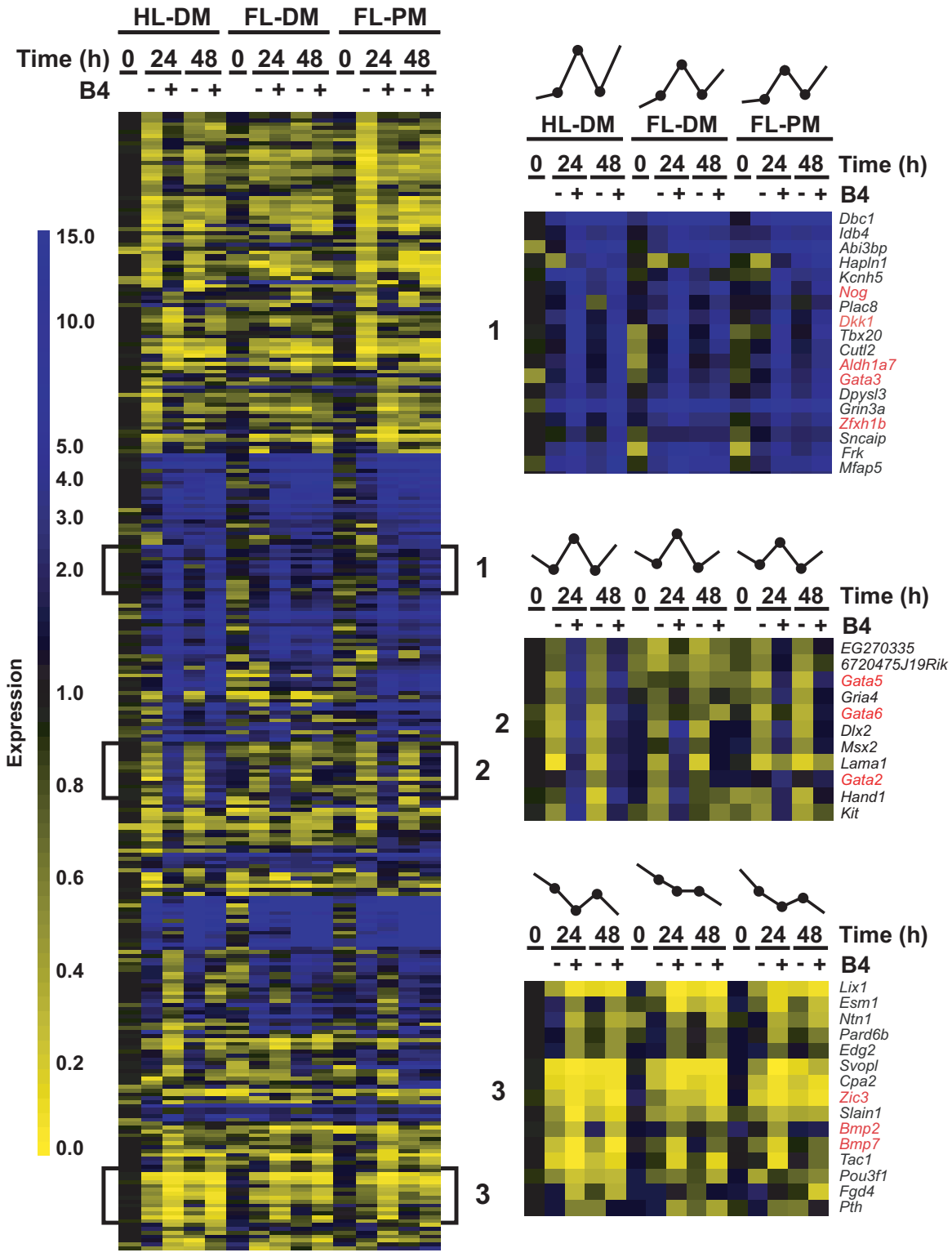
HL-DM
FL-DM
FL-PM
Time (h)
0 24 48 0 24 48 0 24 48
B4
- + - + - + - + - + - +
Expression
15.0
10.0
5.0
4.0
3.0
2.0
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
Dbc1
Idb4
Abi3bp
Hapln1
Kcnh5
Nog
Plac8
Dkk1
Tbx20
Cutl2
Aldh1a7
Gata3
Dpysl3
Grin3a
Zfxh1b
Sncaip
Frk
Mfap5
EG270335
6720475J19Rik
Gata5
Gria4
Gata6
Dlx2
Msx2
Lama1
Gata2
Hand1
Kit
Lix1
Esm1
Ntn1
Pard6b
Edg2
Svopl
Cpa2
Zic3
Slain1
Bmp2
Bmp7
Tac1
Pou3f1
Fgd4
Pth

Figure 2.5. Multiple *Gata* genes are expressed in the early limb and their expression is up-regulated by BMP4. A. Quantitative RT-PCR of limb sections from E10.5 forelimbs and hindlimbs shows that *Gata3*, *Gata5*, and *Gata6* are broadly expressed within the limb particularly and *Gata5* exhibits a gradient of expression from low in the distal region to high in the proximal portion of the HL and FL. nd, 40 cycles of qPCR and *Gata5* transcript not detected. B-D. *Gata3, 5* and *6* are all induced to varying extents in the various cultures, with the HL-DM cultures exhibiting the greatest expression of all analyzed *Gata*s. In the HL-DM cultures, *Gata5* expression is induced > 35 fold within 24 h. NOGGIN reverses the effect of BMPs, but has very little effect on *Gata* expression alone. C. BMPs influence gene expression through modulation of the p38 MAPK signaling pathway. Addition of a p38 MAPK α/β inhibitor, SB202190 (SB) inhibits chondroblast differentiation in HL-DM cultures (left panel), but has no effect on BMP4-mediated induction of *Gata3*, *5* or *6* (right panel). For B and C, real-time quantitative PCR was used to follow gene expression in RNA samples collected from cultures treated with BMP4 (20 ng/ml) for 24 h with or without SB202190 (10 μM).

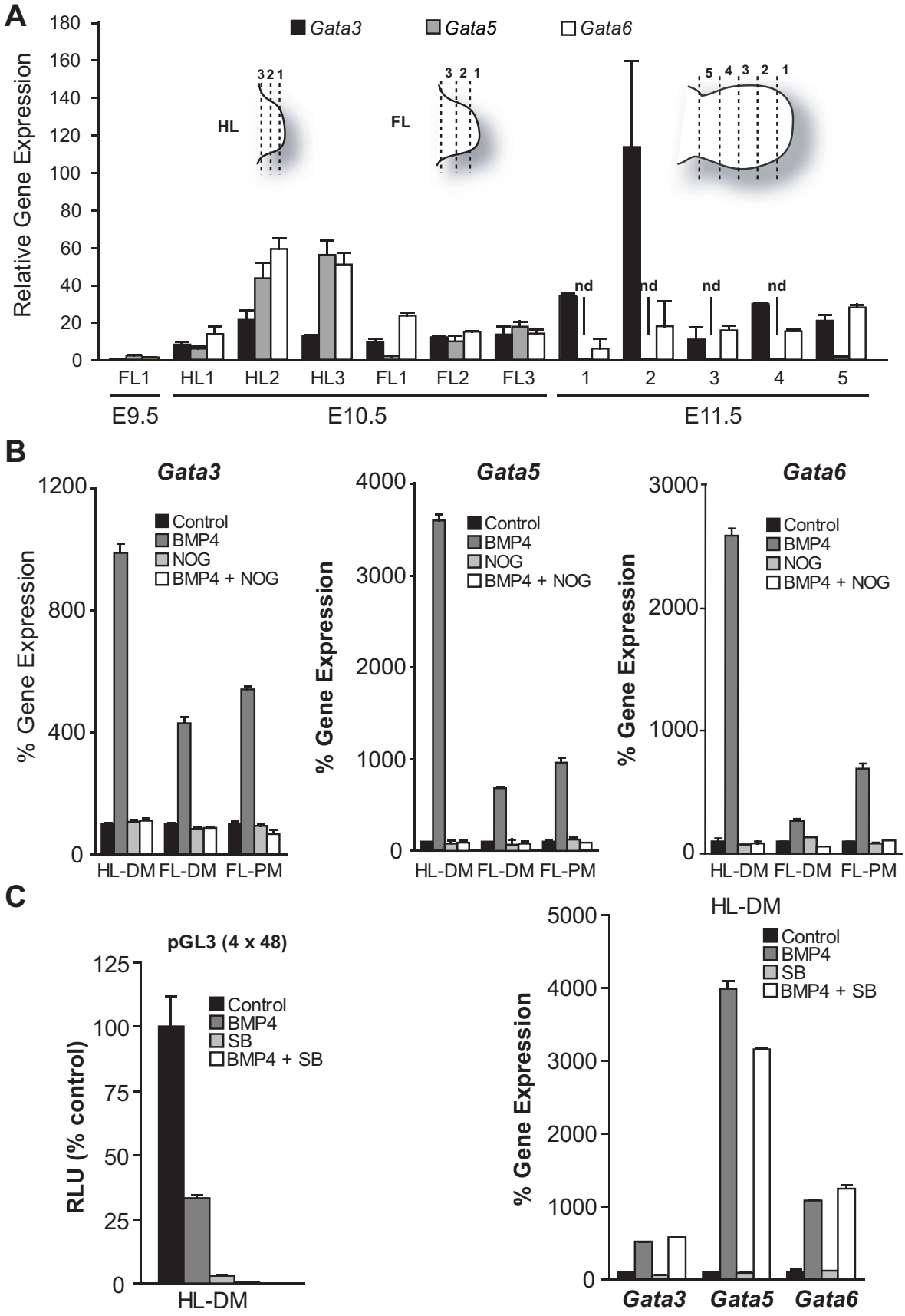
A
Gata3
Gata5
Gata6
Relative Gene Expression
HL
FL
nd
FL1
HL1
HL2
HL3
FL1
FL2
FL3
1
2
3
4
5
E9.5
E10.5
E11.5
B
Gata3
Gata5
Gata6
% Gene Expression
Control
BMP4
NOG
BMP4 + NOG
HL-DM
FL-DM
FL-PM
C
pGL3 (4 x 48)
RLU (% control)
SB
BMP4 + SB
HL-DM
Gata3
Gata5
Gata6

Figure 2.6. BMP4-mediated *Gata* induction is stage dependent. A-C. Upon treatment of primary cultures with BMP4 (20 ng/ml) at time 0, quantitative real-time PCR reveals that *Gata3*, *5* and *6* exhibit large changes in expression in HL-DM, FL-DM and FL-PM cultures, with HL-DM cultures displaying the largest induction in expression (12-60 fold). However, if BMP4 (20 ng/ml) is added 24 h following culture initiation, *Gata3*, *5* and *6* are only increased 2-3 fold.

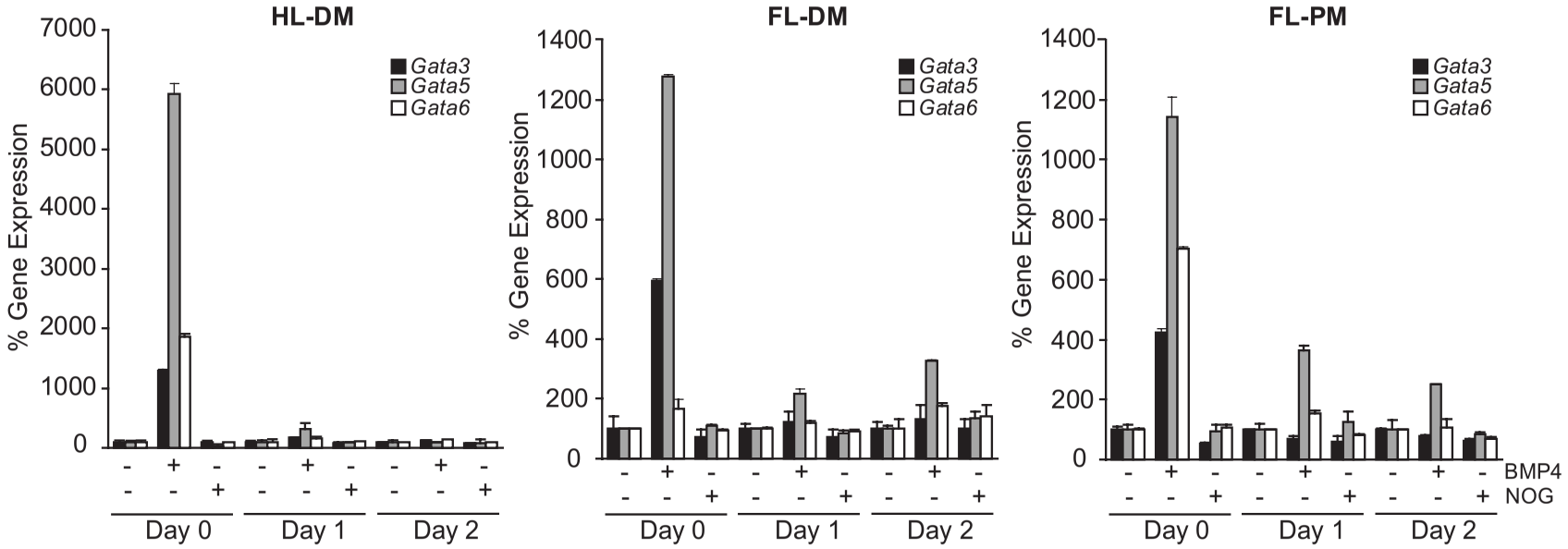
HL-DM
FL-DM
FL-PM
% Gene Expression
Gata3
Gata5
Gata6
BMP4
NOG
Day 0
Day 1
Day 2

Figure 2.7. *Gata5* regulates SOX activity in PLM cultures and mimics BMP action. Left panel, E10.5 cultures were co-transfected with a SOX-responsive reporter gene and an expression plasmid encoding *Gata5*, treated with BMP4 on Day 0 and luciferase activity was measured on Day 1. Right panel, E10.5 cultures were transfected on day 0, treated with BMP4 on day 1 and assayed on day 2. In all cases, expression of *Gata5* reduces reporter gene activity. Significance is in comparison to control cultures and is represented as follows: **, $P < 0.01$; #, $P < 0.001$.

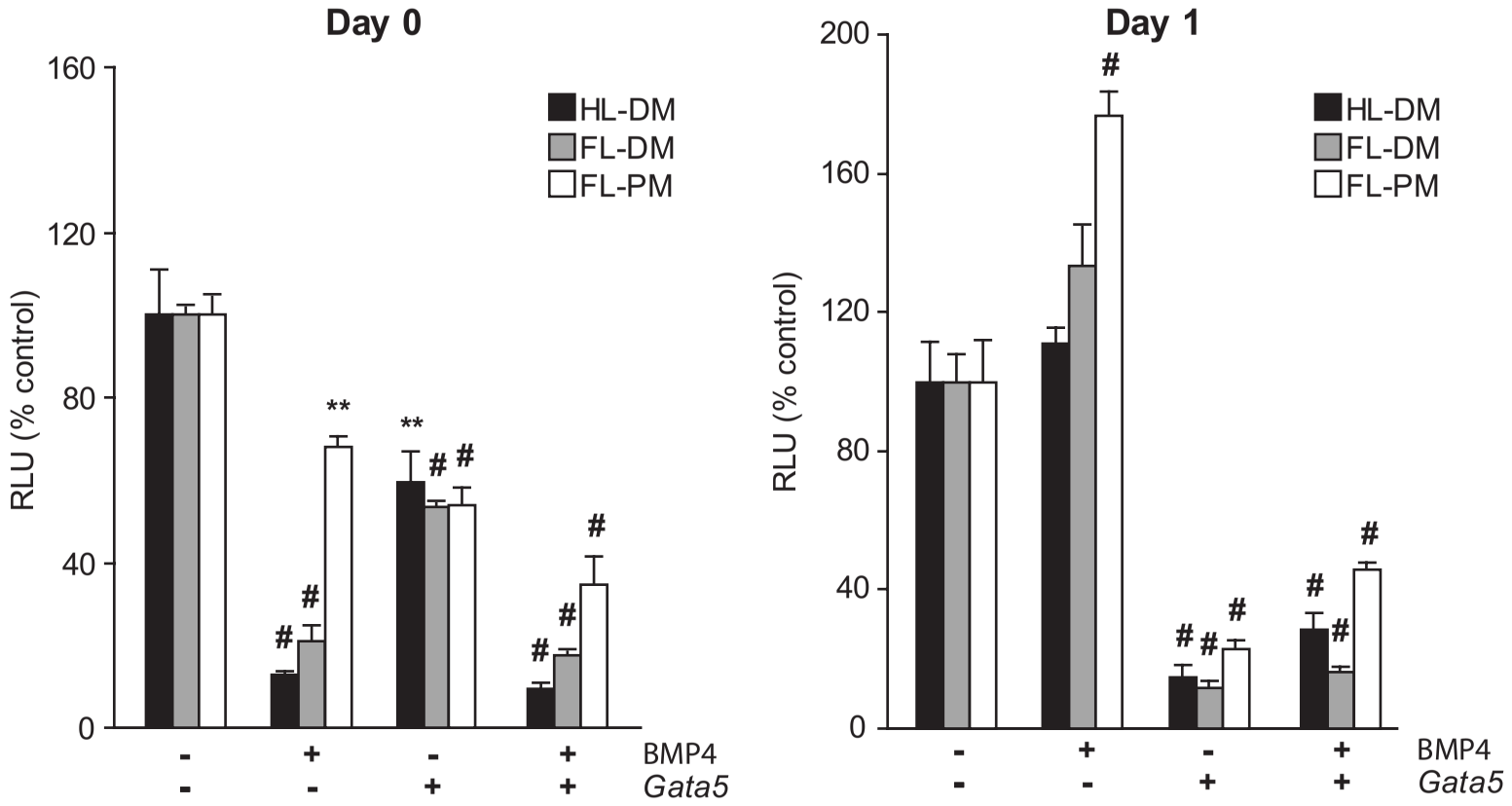
Day 0
Day 1
RLU (% control)
HL-DM
FL-DM
FL-PM
BMP4
Gata5

Table 2.1: RT-qPCR analysis of selected genes in E10.5 PLM cultures

	HL-DM		FL-DM		FL-PM	
	BMP 4 (20 ng/ml)		BMP 4 (20 ng/ml)		BMP 4 (20 ng/ml)	
	Fold Change		Fold Change		Fold Change	
	12 h	24 h	12 h	24 h	12 h	24 h
Sox6		0.2		1.0		2.2
Col1a1	3.8	3.5	2.4	2.5	1.8	1.6
Sox9	1.0	0.9	0.9	1.5	1.1	1.9
Col2b	1.0	0.7	0.8	1.9	0.9	2.1
Nog	6.8	6.9	5.0	9.7	7.6	8.8
Runx2	1.8	1.3	1.3	0.8	0.5	0.5
Gata3	4.4	3.8	4.3	4.5	6.4	3.6
Gata5	28.0	74.9	13.5	46.0	13.2	13.4
Gata6	6.9	13.1	2.7	4.5	12.5	10.1
Zic3	0.6	0.6	0.5	0.4	0.6	0.2
Mdfi	1.5	1.1	1.2	1.5	1.5	1.6
Twist1	0.4	0.3	0.3	0.3	0.4	0.3
Alx3	2.7	3.3	1.0	1.3	0.5	0.5
Dkk1	5.1	6.0	6.8	13.2	16.1	9.4
Dkk2	1.5		0.6		0.7	
Pax1	2.5		1.5		1.2	
Pax9	0.3		0.1		0.4	
Meox2	0.3		0.0		0.1	
Fzd9	1.6		0.9		1.2	
Foxc1	1.1		0.2		0.4	
Foxc2	1.8		0.8		0.9	
Kit	5.7		5.4		8.2	
Dlx2	5.9		6.9		10.8	
Aldh1a7	3.8		6.4		6.1	

Fold change is determined by normalization to untreated control culture.

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CHAPTER III:
Canonical WNT signaling exhibits stage-dependent activities in skeletogenesis

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3.1 Introduction

Limb outgrowth occurs in a proximal-distal direction, whereby the femur and humerus form first, followed by the formation of more distal elements such as the digits (reviewed in (Tabin and Wolpert, 2007)). This proximal-distal outgrowth and subsequent patterning of the limb bud depends on ectodermal signals, particularly those emanating from the apical ectodermal ridge (AER) (Saunders, 1998). Previous studies have suggested that the limb ectoderm exerts both stimulatory and inhibitory effects on cartilage formation, depending on the stage of the underlying mesoderm (Hamburger and Hamilton, 1992). Specifically, the early mesoderm requires the presence of the ectoderm for the formation of cartilage. For example, explanted limb buds from early mouse embryos lacking the ectoderm fail to form cartilage. At later stages, this dependence is lost and instead, the formation of cartilage is inhibited by the presence of the ectoderm (Solursh and Reiter, 1988).

The AER secretes a number of signaling molecules including a group of secreted signaling molecules that have been implicated as key regulators of skeletogenesis in the developing limb which are encoded by members of the wingless (WNT) gene family (Dealy et al., 1993; Galceran et al., 1999; Hartmann and Tabin, 2000; Kawakami et al., 2000; Kawakami et al., 1999; Kengaku et al., 1997; Yamaguchi et al., 1999). WNTs can be divided into two general classes based on their activation of β-catenin. The first class signals through the canonical WNT pathway in which binding of WNT ligand to its receptor leads to the stabilization of an effector protein (β-catenin), allowing it to accumulate in the nucleus where it interacts with LEF/TCF transcription factors to activate target genes (Akiyama, 2000; Eastman and Grosschedl, 1999). In the absence of canonical WNT, β-catenin is phosphorylated by glycogen synthase kinase-3 (GSK3) and degraded through the proteasome. The second class of WNTs, termed non-canonical WNTs, operate through the modulation of other pathways, some of which involve attenuation of canonical WNT signaling (Kohn and Moon, 2005; Weidinger and Moon, 2003). In the

developing limb, both WNT signaling pathways are essential, as null mutants of both canonical WNT and non-canonical WNT genes, *Wnt3* and *Wnt5a*, respectively lead to a spectrum of severe limb malformations (Barrow et al., 2003; Yamaguchi et al., 1999) and mutations in *Wnt3* have been associated with tetra-amelia in humans (Niemann et al., 2004). Further, canonical WNTs are expressed in the limb ectoderm at the onset of limb development in both the chick and mouse, and misexpression of a canonical WNT in the body wall is sufficient to induce ectopic limb development (Barrow et al., 2003; Hartmann and Tabin, 2000; Kawakami et al., 2001). In more recent studies, several groups have shown that activation of the canonical WNT signaling pathway represses chondrogenesis in limb mesenchymal cells *in vitro* and *in vivo* (Day et al., 2005; Hill et al., 2005).

The formation of the endochondral skeleton is dependent upon SOX9, a transcription factor belonging to the SRY-related HMG-box gene family (Akiyama et al., 2002; Bi et al., 1999). Analyses of conditional null mutants of *Sox9* have demonstrated a requirement for SOX9 in the formation of precartilaginous condensations and in chondrocyte differentiation (Akiyama et al., 2002). Further, in some cell types heterologous expression of *Sox9* is sufficient to drive expression of chondrocytic genes (Bell et al., 1997; Healy et al., 1999). Interestingly, SOX9 has also been shown to interfere with canonical WNT signaling, by promoting the degradation of nuclear β-catenin (Akiyama et al., 2004). Thus, it has been suggested that SOX9 can suppress canonical WNT signals thereby promoting chondrogenesis.

Several factors belonging to the WNT signaling family have been shown to regulate the appearance of the chondrogenic phenotypes in the developing limb. Herein we show that primary limb mesenchymal (PLM) cultures exhibit differential responsiveness to a canonical WNT, WNT3a, that is dependent on the stage of development. Specifically, we have found that PLM cultures established from < E10.5 forelimbs or < E10.5 hindlimbs form very little cartilage and this can be significantly enhanced by the addition of WNT3a. To decipher the role of WNT

signaling in early limb development, we have established PLM cultures from various regions of the murine E9.5 forelimb (FL) and E10.5 FL and hindlimb (HL). Interestingly, these cultures exhibited a wide range of responses to WNTs, from inhibition to stimulation of chondrogenesis, reminiscent to that observed in the classic limb ectoderm-mesenchyme co-culture experiments. Consistent with previous reports, we have also found that continued activation of β-catenin pathway inhibits chondroblast differentiation, however, we have also discovered that a transient and/or weak canonical WNT signal can promote the initial stages of chondrogenesis, those involving the elaboration of chondroprogenitors.

3.2 Materials and methods

Reagents

WNT3a was purchased from R & D systems and resuspended according to the manufacturer's instructions and used at a concentration of 5-100 ng/ml. SB-216763 (GSK-3 inhibitor) was obtained from Sigma-Aldrich, dissolved in dimethyl sulfoxide (DMSO) following the manufacturer's instructions and was added to media at a concentration of 1-5 μm. All other chemicals were tissue culture grade from Sigma-Aldrich.

Plasmid constructs

To assess chondrogenic activity, the SOX9-responsive reporter was used. This reporter contains four reiterated SOX9 binding sites (4x48 bp) upstream of a minimal type II collagen promoter (-89/+6) coupled to a luciferase gene [pGL3-4x48] (Weston et al., 2002). To measure canonical WNT signaling, a reporter plasmid containing 16 TCF/LEF binding sites coupled to the firefly luciferase gene [pTA-Super-TOP-Flash Luciferase] was used (obtained from R. Moon, UW). Complementary DNA clones for *Runx2* and *Runx2-ΔEF* were kindly provided by Jaro Sodek and Arthur Sampaio, respectively.

Transgenic reporter mice

Reporter mice expressing a transgene whose expression is dependent upon activated TCF/LEF and β-catenin were kindly provided by Dr. F. Rossi (University of British Columbia). These mice develop normally and contain a *LacZ* reporter gene coupled to response elements of the TCF/LEF family of transcription factors (Mohamed et al., 2004). Embryonic age (E) 9.5 – E11.5 mice were dissected and stained for β-galactosidase activity as previously described (Weston et al., 2002; Weston et al., 2000). The morning of the plug was considered E0.5. For sections, embryos were fixed extensively in 3.7% formaldehyde in PBS. Limb buds were subsequently removed, embedded in 3% agarose and sectioned into 50-60 μm sections (3-4 sections/limb) with a vibratome.

Establishment, analysis and transfection of primary mesenchymal cultures

PLM cultures from CD-1 E10.5 – E11.5 mouse limb buds were established as previously described (Hoffman et al., 2006). E10.5 PLM cultures were established from PM and DM of the FL, and the DM of the HL from regions shown in Figure 3.1B. After proteolytic digestion, cells were filtered through a Cell Strainer (40 μM; Falcon) to obtain a single cell suspension and resuspended at a density of 2 X 10^7 cells/ml. For microarray analyses five 10 μl aliquots of this suspension were plated into a Nunc 35 mm tissue culture dish and allowed to adhere for 1 hr. Following this period, 2 mls of culture medium consisting of 60% F12, 40% Dulbecco's modified Eagle's medium (DMEM) and 10% FBS (Qualified-Invitrogen) was added to each well with or without 50 ng/ml WNT3a (R & D Systems) - this time was considered time 0 (T=0). Culture media was replaced on alternate days and cultures were maintained for a period of 1-4 days. Alcian blue staining was carried out as described in (Weston et al., 2002; Weston et al., 2000). Peanut agglutinin (PNA) staining was used to visualize mesenchymal cellular condensations. Micromass cultures were grown on glass coverslips and treated at time 0 with

WNT3a at 50 ng/ml. Cultures were fixed on day 1 in 4% paraformaldehyde (PFA) at 4°C for 30 min. Coverslips were then rinsed with PBS and subsequently incubated overnight at 4°C with 10 μg/ml rhodamine-labeled PNA diluted in PBS (Vector Laboratories). Cultures were washed again with PBS and PNA distribution was visualized using fluorescence microscopy.

To evaluate cell proliferation, BrdU incorporation was followed in cultures treated for 24 h with WNT3a (50 ng/ml). Cells were incubated with WNT3a for 23.5 h, and BrdU was added for 0.5 h prior to fixation. BrdU was detected according to the manufacturer's instructions (Roche Applied Science) and the number of BrdU-positive cells were enumerated from a minimum of 4 fields per slide.

For transfection, we have developed a new protocol that utilizes Effectene (Qiagen), supplemented with trehalose – this addition increases transfection efficiency > 3 fold in primary mesenchymal cells. This modification was necessary to enable efficient transfection of DM cells and E10.5-derived cells. Briefly, 0.25 μg of plasmid reporter, 0.025 μg pRL-SV40 (Promega) and 0.750 μg of expression vector (Stratagene, Invitrogen) were combined for a total of ~1 μg of DNA. One microlitre of Enhancer solution is added to 15 μl EC buffer (Qiagen, supplemented with 0.4 M trehalose) and combined with DNA. Following 10 min incubation, 5 μl of Effectene was added. Seven and a half microliters of this DNA/Effectene mixture was transferred to sterile 1.5 ml microfuge tubes, followed by 40μl of cell suspension. Cells and the transfection mixture were gently triturated, and 10 μl was used to seed a single well of a 24-well culture dish. The cells were allowed to attach for one hour, followed by the addition of 1 ml of media to each well. Extracts for luciferase analysis were collected on Day 1 or Day 2 as indicated. For experiments involving PLM cells alone or cells transfected with reporter genes only, factors or compounds were added at the time of media addition (T=0), unless otherwise indicated. For experiments involving co-transfection of cells with expression plasmids, factors and compounds were added

up to 24 hours following transfection. Analysis of reporter gene activity was preformed using the Dual Luciferase Assay System according to the manufacturer's instructions (Promega). Briefly, cells were washed once with PBS, lysed in 100 μl of Passive Lysis Buffer for 20 minutes and frozen at – 80^{o}C overnight. Forty microliters of each lysate was then loaded into a 96-well plate and luciferase activity was determined using a luminometer (L-Max; Molecular Devices). Firefly luciferase was normalized against *Renilla* luciferase activity to control for differences in transfection efficiency.

Transcriptional profiling with microarrays: Experimental design and analysis

RNA was harvested from primary cultures using RNAeasy (Qiagen) according to the manufacturer's instructions as described previously (Hoffman et al., 2006). Following isolation, RNA was diluted to 125 ng/ml, the expression of *Sox9* and *Col2* were measured using real-time PCR and RNA quality was examined on a Bioanalyzer 2100 (Agilent).

For microarray experiments, two biological replicates were analyzed per time point. The Affymetrix mouse transcriptome arrays (MOE430 2.0) were used to generate transcriptional profiles from RNA collected at 24 and 48 hours treated with WNT3a (50 ng/ml) derived from E10.5 FL-DM, FL-PM and HL cultures. To eliminate the need for amplification prior to microarray analyses, > 1 μg of RNA was collected from cultures derived from ~ 300 embryos. One μg of RNA was labeled and hybridized to the chips using the manufacturer's recommended protocol. Data sets were subsequently uploaded into GeneSpring for bioinformatic analysis and analyzed for the expression of individual genes (~ 39,000 on the chip set, with redundancy closer to 36,000 genes). All data sets were initially filtered to remove genes called absent by GeneSpring. Hierarchical clustering was carried out in GeneSpring with the GeneTree tool using Pearson correlation similarity measure.

Real-time PCR

To quantify the expression of various gene transcripts, quantitative real-time PCR was carried out using the 7500 Fast Real-Time PCR System (Applied Biosystems). Some primers and TaqMan-minor groove binding (MGB)-probes were designed using PrimerDesigner 2.0 (Applied Biosystems). The primer/probe sets used for detection of *Sox9* and *Col2* were used as described in Weston et al. (Weston et al., 2002). Primer and probe concentrations were optimized according to the manufacturer's instructions. Other primer and probe sets were purchased from the TaqMan gene expression collection (Applied Biosystems). Total RNA was isolated from PLM cultures as described above. Quantification was carried out using ~10 ng of total RNA and the expression of all genes relative to endogenous *rRNA* was determined using TaqMan Ribosomal Control Reagents (Applied Biosystems).

Statistical analysis

All luciferase assays with the exception of the TOP/SUPER-TOP (repeated twice) were performed in quadruplicate and repeated using three distinct preparations of PLM cells. Real-time PCR analysis was carried out in duplicate and repeated a minimum of 2 times with independent RNA samples. One representative experiment is shown for all luciferase and real-time PCR results.

3.3 Results

Several studies have demonstrated a critical role for WNT signaling in the developing limb, with an absence of canonical or non-canonical signaling negatively impacting skeletogenesis. Interestingly, canonical WNTs also appear to share many of the properties of the "diffusible ectodermal signal" in inducing cartilage formation in early limb mesenchyme. Canonical WNTs are expressed at the onset of limb development in the ectoderm and they certainly inhibit chondrogenesis in later stage limb mesenchyme. Thus, we sought to address the

role of canonical WNT signaling in early limb mesenchyme (E9.5-E10.5). Activation of the canonical WNT signaling pathway in early limb mesenchyme was analyzed in reporter mice harboring a LacZ transgene driven by a TCF/LEF response element (Mohamed et al., 2004). TCF/LEF-*LacZ* reporter mice show areas of activated canonical WNT signaling distributed throughout the entire E9.5 forelimb (Fig. 3.1A). At later stages (E10.5, E11.5) *LacZ* expression becomes confined to distal regions of both the forelimb and hindlimb (Fig. 3.1B, 3.1C). Sectioning of the limbs revealed extensive transgene expression in the mesenchyme of E9.5 limbs, in a distal (high) to proximal gradient (Fig. 3.1A). Similar patterns of expression were observed in the E10.5 and E11.5 limbs, with the expression of the transgene being progressively confined to the distal mesenchyme underlying the ectoderm with increasing age (Fig. 3.1B, 3.1C). Consistent with these observations, basal Super-TOP-flash (a canonical WNT responsive reporter) activity is increased 6-fold in those cultures derived from the distal region (DM) of E11.5 limb buds in comparison to cultures derived from more proximal regions (PM) (Fig. 3.1D). In aggregate these results show that the canonical WNT signaling pathway is activated in the mesenchyme underlying the ectoderm, a source of canonical WNTs.

To investigate the role of canonical WNT signaling in the limb mesenchyme, E10.5 and E11.5 limb buds were micro-dissected into regions that overlapped with the distal limb populations that were transgene-positive or more proximal regions that exhibited limited or undetectable transgene expression. In this manner, PLM cultures were established from three distinct regions of the E10.5 forelimb and hindlimb: HL distal mesenchyme (HL), FL distal mesenchyme (FL-DM) and proximal FL mesenchyme (FL-PM) (Fig. 3.1B) and the E11.5 limb bud was dissociated into either distal (DM) or whole-limb (WL) populations. WL populations of the E11.5 limb were used to enable comparison to existing published reports (Reinhold et al., 2006). In E9.5 forelimb cultures, WNT3a addition (50 ng/ml) promotes cartilage nodule

formation and this is accompanied by a ~ 2-fold and ~ 3-fold increase in *Sox9* and *Col2a1* expression, respectively (Fig. 3.1A).

In E10.5 HL and FL-PM cultures, treatment with WNT3a (50 ng/ml) results in a decrease in cartilage nodule formation, whereas FL-DM cultures display little to no change in cartilage nodule formation upon treatment with WNT3a (50 ng/ml) (Fig. 3.1B). Primary cultures derived from E11.5 whole limbs (WL) exhibit decreased cartilage nodule formation upon treatment with WNT3a (50 ng/ml) consistent with earlier reports. In contrast, E11.5 DM cultures show a marked increase in cartilage nodule formation (Fig. 3.1C). Consistent with the results described above, treatment of E11.5 DM cultures with WNT3a (50 ng/ml) promotes chondrogenesis that correlates with an increase in the activity of a *Col2a1*-based reporter gene containing SOX5/6/9 binding elements (pGL3-4x48) (Fig. 3.1E) (Lefebvre et al., 1998). This reporter has been found to provide a reliable readout on SOX5/6/9 activity and the status of chondroblast differentiation (Hoffman et al., 2006; Weston et al., 2002). However, there is a progressive decrease in SOX-reporter gene activity in cultures derived from more proximal regions of the developing limb, treated with WNT3a (50 ng/ml).

Quantitative RT-PCR of limb sections from E10.5 limb buds shows that *Sox9* is expressed to varying extents throughout the E10.5 limb, with the highest level of expression in more proximal regions of the limb and the least in the distal mesenchyme, with the distal E10.5 HL showing the least expression of *Sox9* (Fig. 3.1E). This expression pattern of *Sox9* is consistent with the proximal-distal direction of chondroblast differentiation and the ~ 0.5 day delay in hindlimb versus forelimb development. Quantitative RT-PCR also shows that regulators of cWNT signaling (*Axin2*, *Nkd2*, *Dkk1*) are also widely expressed throughout both the fore and hind limb (Fig. 3.1E).

To further investigate the role of WNT3a in the limb mesenchyme, E11.5 primary DM and WL cultures were established and cell proliferation was followed by BrdU labeling of

untreated and WNT3a-treated cultures 24h after WNT3a addition. In comparison to untreated controls, the WNT3a-treated DM cultures exhibited an ~ 2.5-fold increase in the number of BrdU-labeled cells; whereas, there was a small (~ 0.4-fold) increase in the number of BrdU-labeled cells in WL cultures (Fig. 3.2A). The appearance of pre-cartilaginous condensations is one of the earliest morphological events in chondrogenesis (Thorogood and Hinchliffe, 1975). Rhodamine-labeled PNA was used to examine the effect of WNT3a of pre-cartilaginous condensation formation. WNT3a (50 ng/ml) inhibits condensation of chondrogenic cells in primary mesenchymal cultures derived from WL cultures of E11.5 mouse limb buds (Fig. 3.2B). However, cultures derived from the DM of fore and hind limbs show a marked increase in the appearance of precartilaginous condensations upon treatment with WNT3a (50 ng/ml) (Fig. 3.2B). Furthermore, treatment of WL E11.5 cultures with various concentrations of WNT3a (0-100 ng/ml) inhibited chondrogenesis in a dose-dependent manner as determined by decreased SOX-reporter gene activity and decreased alcian blue staining (Fig. 3.2C). In contrast, treatment of DM cultures with increasing amounts of WNT3a (0-100 ng/ml) enhances chondrogenesis in a dose-dependent manner as observed by increased SOX-reporter gene activity and increased cartilage nodule formation (Fig. 3.2C). Moreover, WNT3a (0-100 ng/ml) increases Super-TOP-flash reporter gene activity in a dose-dependent fashion, in both WL and DM cultures, with greater increases in reporter gene activity observed in WL cultures (Fig. 3.2D).

Lithium chloride (LiCl) is an inhibitor of GSK3 and has been used to activate canonical WNT signaling. Treatment of E11.5 DM cultures with LiCl (0-16 mM) increases SOX-reporter gene activity, whereas treatment of WL cultures with LiCl (0-16 mM) decreases SOX-reporter gene activity in a dose-dependent manner (Fig. 3.3A). Further analysis, demonstrates that LiCl mimics the effects of WNT3a in both DM and WL cultures (Fig. 3.3B). Treatment of E11.5 DM and WL cultures with increasing amounts of LiCl (0-16 mM) also activates Super-TOP-flash reporter gene activity in a dose-dependent fashion (Fig. 3.3C); however, greater reporter gene

activity is observed in WL and DM cultures with WNT3a addition in comparison to LiCl (Fig. 3.3D). It has been demonstrated that LiCl has multiple non-competitive targets in addition to GSK-3β (Klein and Melton, 1996; Stambolic et al., 1996); thus, the effects of a synthetic GSK-3β specific inhibitor, SB216763 (SB), were examined in PLM cultures. Similar to that observed in the E11.5 DM cultures with LiCl, addition of SB (0-2 μm) to E10.5 HL-DM and FL-DM primary cultures promotes chondrogenesis, as observed by increased SOX-reporter gene activity (Fig. 3.3E). FL-PM cultures show little to no change in SOX9-reporter gene activity following the addition of SB (1-2 μM) (Fig. 3.3E). Furthermore, with 1 μM SB treatment Super-TOP-reporter gene activity is appreciably increased in HL-DM cultures, with little or no increase in either FL-DM or FL-PM cultures (Fig. 3.3F). Chemical activators of the canonical WNT signaling pathway, LiCl and SB both stimulate SOX reporter gene activity, suggesting that activation of β-catenin is a component of the pro-chondrogenic activity of WNT3a.

Axin2 is a negative regulator of the canonical WNT signaling pathway and is typically upregulated following activation of the cWNT signaling pathway. Consistent with this, *Axin2* was upregulated in all WNT3a-treated cultures as determined by microarray analysis and qPCR (data not shown). Overexpression of *Axin2* has been shown to effectively inhibit canonical WNT signaling, and this approach was used to further assess the function of cWNT signaling in chondrogenesis. As shown above, addition of WNT3a to either WL or DM cultures leads to inhibition and activation, respectively of the SOX reporter gene activity (Fig. 3.4). Interestingly, overexpression of *Axin2* increases reporter gene activity in both cultures, as well as E10.5 limb-derived cultures. Treatment of *Axin2*-transfected WL, FL-PM and to a lesser extent HL-DM cultures with WNT3a partially rescues the negative effect of WNT3a on SOX reporter gene activity. Unexpectedly, in the DM and FL-DM cultures, reporter gene activity was further increased (Fig. 3.4). Furthermore, heterologous expression of *Axin2* attenuates WNT3a

induction of Super-TOP reporter (Figure 3.4). These results further suggest that chondrogenesis is stimulated by either a transient and/or weak canonical WNT signal.

To investigate the molecular basis of stage-dependent responses of limb mesenchymal cells to WNT3a, genome-wide expression profiling with microarrays was employed. Cells were isolated from the aforementioned three regions of E10.5 limbs and cultured in the presence or absence of WNT3a (50 ng/ml) for 24 and 48 h, and transcriptional profiles were generated through hybridization of processed RNA to Affymetrix MOE430 2.0 chips. To identify genes exhibiting differential responsiveness in the 3 populations of cells, fold cut-off analyses were used to select genes that exhibited a 2-fold change in expression following WNT3a addition in HL and FL-PM samples. A list of genes was generated and Pearson correlation analysis was used to cluster genes based on their expression patterns (Fig. 3.5). There are a number of genes which change more than ~ 10-fold upon treatment of primary mesenchymal cultures with WNT3a (50 ng/ml). Consistent with qPCR analysis described earlier, cartilage-associated genes (Fig. 3.5, Box1 and 2) were down-regulated to varying extents in all samples following the addition of WNT3a (50 ng/ml). These trends were consistent with the expected expression of these genes at the different stages of the chondrogenic program. Further, several transcription factors associated with limb development or skeletogenesis were also differentially expressed including, *Pax1* and *9*, *Twist1*, *Zic3*, *Alx3* and *Runx2* (Table 3.1). Many of these genes (*Pax1* and *9*, *Twist1*, and *Alx3*) exhibit different degrees of change in the three cultures in response to WNT3a treatment. Canonical WNT target genes, *Axin2* and *Nkd2* (Fig. 3.5, Box 3) were induced by WNT3a under all conditions, and these genes tightly co-clustered with other WNT3a-induced genes including the limb mesenchyme expressed transcription factors *Msx2* and *Zfp503*.

Further bioinformatic inspection of the microarray data sets, revealed that expression of the runt-related transcription factor-2 (*Runx2*) expression was notably decreased (~ 3.5 - 6 fold) in all 3 cell populations upon treatment of these cultures with WNT3a (50 ng/ml) (Fig. 3.6A).

Runx2 is a transcription factor that has been shown to play an important role in osteogenesis and chondrocyte maturation. *Runx2* is expressed in osteo-chondro bipotential progenitors, osteoprogenitors and differentiating osteoblasts. Once bipotential progenitors commit to the chondrocytic lineage, *Runx2* expression declines. *Runx2*-deficient (*Runx2*$^{-/-}$) mice completely lack bone formation and show disturbed chondrocyte maturation (Inada et al., 1999). Furthermore, over-expression of *Runx2* or the dominant-negative form of *Runx2* accelerates or delays chondrocyte maturation, respectively (Ueta et al., 2001). Expression of *Runx2* was evaluated in primary cultures following WNT3a treatment (Fig. 3.6B). Following a 24 h treatment with WNT3a (50 ng/ml), consistent with the microarray findings, *Runx2* expression was down-regulated ~ 5-fold, 6-fold and 3-fold, in HL, FL-DM and FL-PM cultures respectively (Fig. 3.5B). Further, quantitative PCR was used to quantitate *Runx2* expression in sub-regions of the E10.5 forelimb and hindlimb. *Runx2* was expressed at low levels throughout the HL and in a distal-proximal gradient in the FL, with a 8-fold increase in expression in the proximal versus distal limb bud (Fig. 3.6C). In contrast, *Sox9* exhibited greater expression in the proximal regions of both fore and hind limbs.

To evaluate the role of *Runx2* expression in early chondrogenesis, we over-expressed *Runx2* and measured SOX-reporter gene activity. Co-transfection of 1μg of *Runx2* expression plasmid decreased reporter gene activity ~ 40%, 30% and 19% in HL-DM, FL-DM and FL-PM cultures respectively (Fig. 3.6D). Co-transfection of an expression plasmid containing a mutant form of *Runx2* moderately increased SOX9-reporter gene activity in HL-DM and FL-DM cultures (Fig. 3.6D). Increased expression of *Runx2* inhibits SOX9 activity and this is consistent with its proposed function *in vivo*, WNT3a significantly decreases *Runx2* expression and thereby could be acting indirectly through attenuation of *Runx2* to promote chondrogenesis.

3.4 Discussion

Classical studies carried out in the chick embryo over two decades ago established an important role for the ectoderm in promoting cartilage formation in the early underlying mesenchyme. Interestingly, at slightly later stages the ectoderm inhibited cartilage formation (Gumpel-Pinot, 1980; Solursh and Reiter, 1988). Shortly thereafter, additional studies revealed that a "diffusible factor" appeared to be released from the ectoderm that stimulated chondrogenesis in a stage-dependent manner (Solursh and Reiter, 1988). However, the nature of this factor has remained elusive. Herein we show that canonical WNTs exhibit the properties consistent with this "factor" as described in earlier studies. A canonical WNT, WNT3a promotes chondrogenesis in more immature chondrogenic cells, while inhibiting chondroblast differentiation in more advanced chondrogenic cells. Further, several *Wnt*s have been reported to be expressed in the early limb ectoderm and activation of the canonical pathway has been detected in the subjacent mesenchyme. Collectively, these findings highlight a novel function for WNT signaling in promoting the initial stages of chondrogenesis.

Limb initiation in the chick involves the sequential activation of *Wnt* expression in the underlying mesoderm followed by expression of *Wnt3a* in the overlying ectoderm. *Wnt3a* subsequently induces the expression of *Fgf8* in the ectoderm, which is important for maintenance of *Fgf10* expression in the subjacent mesenchyme (Kawakami et al., 2001). Inhibition of canonical WNT signaling in the ectoderm of either chick or mouse leads to the disruption of the AER and severe limb truncations (Barrow et al., 2003; Kengaku et al., 1998). Numerous studies carried out *in vitro* and *in vivo* have convincingly demonstrated that activation of the canonical WNT signaling pathway in the limb interferes with chondroblast differentiation and in some instances it appears that "chondrogenic cells" are redirected to an osteoblast fate (Hill et al., 2005). All of these studies involved sustained activation of the WNT pathway and were typically performed in > E10.5 cell populations. Consistent with these reports, we also find that

activation of canonical WNT signaling interferes with chondroblast differentiation, especially in later stage cultures. In contrast, cells from the early limb (E9.5) that exhibit limited chondrogenic potential are robustly induced to form cartilage nodules upon WNT3a addition and this is accompanied by increased expression of *Sox9* and *Col2a1*. So how can these rather disparate observations be reconciled? Firstly, it should be noted that the ability of WNT3a to stimulate chondrogenesis is stage and/or population dependent. Secondly, the pro-chondrogenic activity of WNTs or activators of β-catenin-mediated signaling was dose-dependent, with higher doses inhibiting chondrogenesis. Thirdly, a weak or transient canonical WNT signal appears to be important.

Chondroblast differentiation occurs in a proximal-distal gradient with fewer differentiated cells appearing in the more distal regions (Tabin and Wolpert, 2007). The least differentiated cells with the highest proliferation index are the mesenchymal cells located subjacent to the apical ectodermal ridge, a source of WNTs. It is also this population of cells that we have found exhibit a pro-chondrogenic response to WNT3a, whereas slightly more proximal populations exhibit markedly decreased cartilage formation in response to WNT3a. Moreover, WNT3a stimulates the formation of prechondrogenic condensation in distal limb populations. Consistent with earlier reports (Gumpel-Pinot, 1980; Solursh and Reiter, 1988), early limb mesenchyme (E9.5) exhibits a pro-chondrogenic response to this ectoderm-released factor. The distal mesenchyme in older limbs also shares these properties and this likely relates to its earlier position within the chondrogenic program. Together, these studies established that canonical WNT signals exhibit distinct activities associated with the chondrogenic stage, however, this only partly explained the pro-chondrogenic activities of canonical WNTs.

High concentrations of WNTs or LiCl that are associated with increased activation of β-catenin responsive reporters, decreased SOX9-reporter gene activity and reduced cartilage formation. Furthermore, co-transfection of a WNT3a-expressing plasmid also consistently led to

decreased chondrogenesis, irrespective of the cell population (data not shown). Collectively, these studies indicated that high or sustained activation of the canonical WNT signaling pathway inhibited chondroblast differentiation. This suggested that either a weak or transient canonical WNT signal may be important in regulating chondrogenesis. The results from the *Axin2* over-expression experiment were critical in defining this role. In this experiment, the cells were co-transfected with the *Axin2* construct along with the reporter, plated and WNT3a added shortly thereafter. Thus, at culture initiation the cells are exposed to WNT3a, whereas *Axin2* would be expressed at low levels if at all. Over time *Axin2* expression increases and attenuates canonical WNT signaling, resulting in reduced Super-TOP reporter gene activity. In this manner, β-catenin would be activated transiently, subsequently declining to near control levels. This explanation agrees well with the observation that WNT3a and *Axin2* over-expression both stimulate SOX9 reporter gene activity in distal mesenchyme and that together they induce an even greater increase in reporter activity in comparison to the individual treatments. Further, *Axin2* is expressed in the distal limb bud (Jho et al., 2002). In the context of limb development it is likely that WNT5a which is expressed in a proximal-distal gradient and antagonizes canonical WNT signaling plays this role in promoting chondroblast differentiation (Topol et al., 2003). It should be noted that in WNT5a null animals, appreciable β-catenin signaling extends into the underlying mesenchyme (Topol et al., 2003). Together, these studies suggest that canonical WNTs not only play an essential role in establishing and maintaining the AER, but also exhibit context-dependent pro-chondrogenic activities.

To gain further insights into WNT function in the limb, genome-wide expression profiling was employed to identify WNT modulated genes. *Pax1* was previously shown to be down-regulated following attenuation of β-catenin signaling (Hill et al., 2006), consistent with this, WNT3a increases the expression of *Pax1* in addition to *Pax9*. *Pax1* and *9* are expressed in the limb precartilaginous condensations, and are subsequently down-regulated during

chondroblast differentiation (LeClair et al., 1999). *Pax1* and *9* are differentially induced/repressed in the various E10.5 mesenchymal populations, in response to WNT3a addition, with the DM cultures exhibiting the greatest increase in *Pax9* expression, in some instances > 100 fold in 24 hrs. *Pax9* knockouts present with limb defects, characterized by a supernumary pre-axial digit (Peters et al., 1998), whereas *Pax1* null mutants exhibit scapular abnormalities (Wilm et al., 1998). The ability of WNT3a to stimulate the expression of these pre-chondrogenic markers is consistent with their proposed action in promoting precartilaginous condensation. Similarly, WNTs have also been shown to regulate *Twist1* expression, and this gene has been previously shown to be a WNT target (Reinhold et al., 2006). Further, over-expression of *Twist1* inhibited chondroblast differentiation in ATDC5 cells, but it is unclear whether it has an earlier role in skeletogenesis. In the osteogenic program, *Twist1* is expressed in osteoprogenitors and is subsequently down-regulated during differentiation, as continued *Twist1* expression interferes with osteoblast differentiation (Bialek et al., 2004). Whether Twist1 operates in a similar capacity in the chondrogenic program is unclear. *Twist1* knockouts present with severe limb defects as a consequence of failed limb outgrowth (Loebel et al., 2002; O'Rourke et al., 2002).

Runx2 plays an essential role in skeletal development, and null mutants present with an absence of osteoblasts and impaired chondrocyte hypertrophy (Ducy et al., 1997). *Runx2* is expressed in early chondroprogenitors or bi-potential osteochondral progenitors and is subsequently down-regulated during chondroblast differentiation (Yoshida and Komori, 2005). Continued expression of *Runx2* impairs cartilage formation and contributes to the expression of an osteogenic fate (Yoshida and Komori, 2005). Canonical WNTs have been shown to promote osteogenesis, however in all of the limb mesenchymal cultures, WNT3a significantly down-regulates *Runx2*. Over-expression of *Runx2* decreases SOX9 reporter gene activity, indicating that WNTs may function in part through influencing the expression of negative regulators of

chondrogenesis. Consistent with the chondrogenic modulatory activity of WNTs, numerous additional genes implicated in skeletal development are impacted by WNT signaling, including, *Alx3, Meox2, Foxc1, Foxc2* and *Dlx2*. In this manner, WNT signaling operates through multiple target genes in a context-dependent fashion to regulate the emergence of the prechondrogenic phenotype.

As discussed earlier, several studies have convincingly demonstrated that activation of the canonical WNT signaling pathway inhibits chondroblast differentiation, however, that doesn't preclude a role for this pathway at earlier stages in the chondrogenic program. Consistent with this premise, several recent reports, have shown that canonical WNT signals can positively regulate chondrogenesis. For instance, in fracture repair models, inhibition of canonical WNT signaling inhibited chondrogenesis in the callous (Chen et al., 2007) and it has been suggested that canonical WNT signals are important in enabling differentiation of mesenchymal cells to the chondrocytic lineage. Further, canonical WNT and LiCl both stimulate chondrogenesis in pericyte cultures (Kirton et al., 2007). In addition, TGFβ1 stimulation of cartilage differentiation of human marrow stromal cells was associated with activation of the canonical WNT signaling pathway and potentiated by the addition of LiCl (Zhou et al., 2004). A common theme emerges from these various studies; canonical WNT signals have a role in directing the differentiation of multi-potential stem or progenitor cell populations to the chondrocytic lineage. A similar role for canonical WNT signals in early limb development has been defined herein, however, the precise mechanisms underlying this activity need further resolution.

3.5 Acknowledgements

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Regional Genomics Centre, in particular David Carter for carrying out the microarray experiments. In addition, we would like to thank Dr. Cal Roskelley for comments on the manuscript. KK was supported by a doctoral fellowship from the Canadian Arthritis Network. This research was funded by grants to TMU from the Canadian Institutes of Health and TMU holds an Investigator award from The Arthritis Society.

3.6 Figures

Figure 3.1. Identification and characterization of sites of canonical WNT signaling in the limb mesenchyme: evidence for differential responsiveness to canonical WNT signals. A-C (Top panels). TCF/LEF-*LacZ* reporter mice show areas of activated cWNT signaling distributed through much of the E9.5 forelimb. At later stages (E10.5-E11.5) *LacZ* expression becomes restricted to the distal region of the developing limb. Limb buds were removed, embedded in 3% agarose and sectioned into 50-60 μm sections (3-4 sections/limb) with a vibratome. Sectioning of the limbs show that *LacZ* expression is present in the subjacent mesenchyme. Red arrows define *LacZ* positive regions in the limb bud. A, treatment of PLM cultures derived from E9.5 forelimbs with WNT3a (50 ng/ml) stimulates chondrogenesis as determined by alcian blue staining. Consistent with the increase in alcian blue staining, *Sox9* and *Col2b* expression are also elevated in WNT3a-treated cultures; determined by quantitative real-time PCR. B, schematic representation of the regions used from E10.5 murine limbs to generate hindlimb distal mesenchyme (HL-DM), forelimb distal mesenchyme (FL-DM) and forelimb proximal mesenchyme (FL-PM) cultures. In HL-DM and FL-PM cultures, WNT3a (50 ng/ml) exhibits anti-chondrogenic activity whereby alcian blue staining of cartilage nodules is substantially reduced. In contrast, WNT3a addition to FL-DM cultures minimally affects cartilage nodule formation. C, graphical depiction of the regions used from E11.5 murine limbs to generate whole-limb (WL) and distal mesenchymal (DM) cultures. Treatment of PLM DM cultures with WNT3a (50 ng/ml) stimulates chondrogenesis as indicated by the increase in alcian blue staining. Treatment of PLM cultures derived from E11.5 whole limb buds with WNT3a (50 ng/ml) inhibits chondrogenesis. D, basal cWNT signaling is higher in E11.5 DM cultures as determined by transfection of PLM DM and PM cultures with a reporter plasmid containing 16 TCF/LEF binding sites (pTA-Super-TOP). E, treatment of E11.5 limb-derived cultures show treatment of E11.5 PLM DM cultures (#1) with WNT3a (50 ng/ml) increases SOX reporter gene

activity (pGL3-4x48), whereas there is a progressive decrease in chondrogenic activity in cultures derived from more proximal regions of the developing limb. F, Quantitative-PCR of limb sections from E10.5 limb buds show that *Sox9* is expressed to varying extents throughout the E10.5 limb, with the highest level of expression in more proximal regions compared to more distal mesenchyme. Negative regulators of cWNT signaling are also broadly expressed throughout both the forelimb and hindlimb as determined by qPCR.

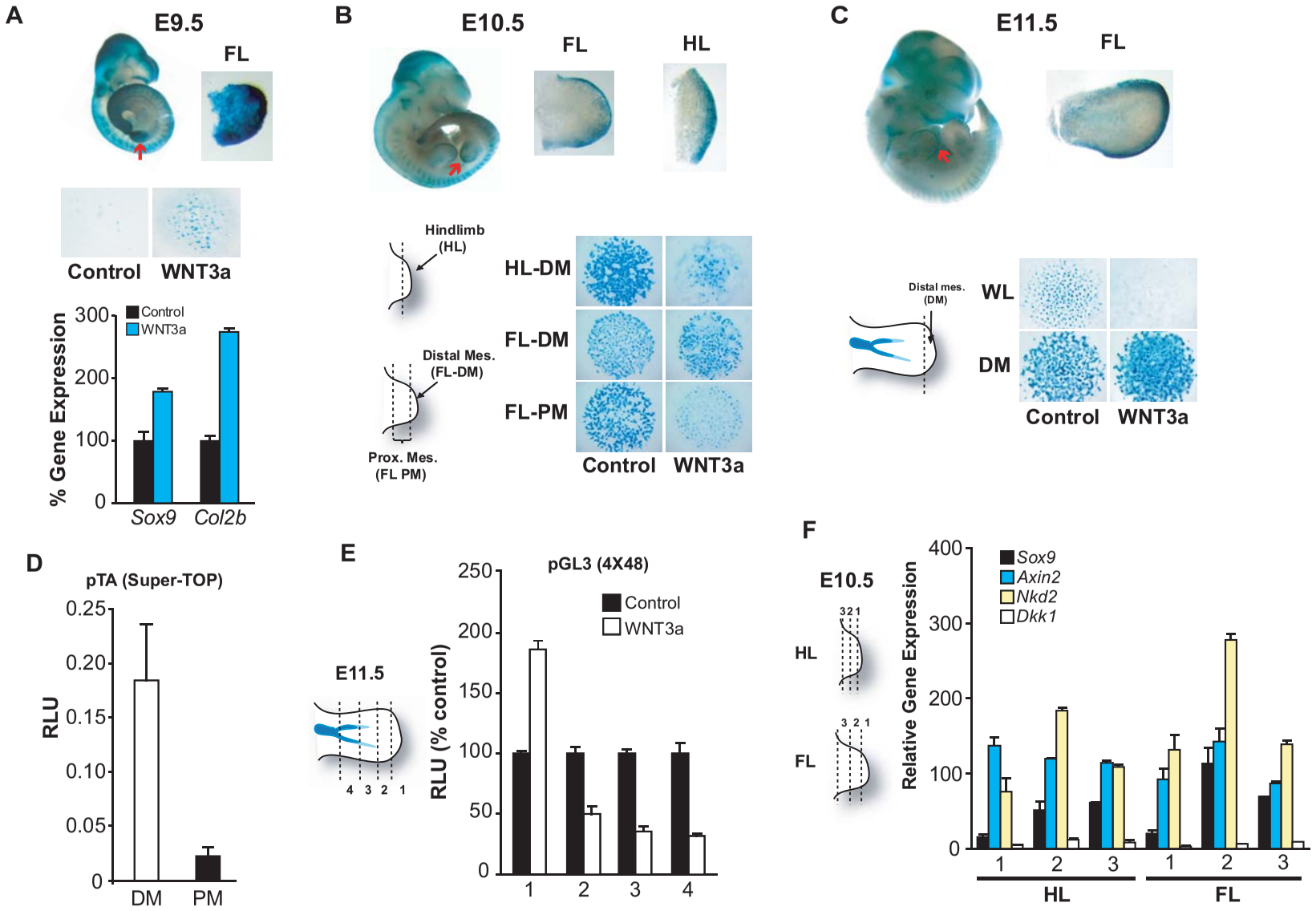
A
E9.5
FL
Control
WNT3a
% Gene Expression
300
200
100
0
Control
WNT3a
Sox9
Col2b
B
E10.5
FL
HL
Hindlimb
(HL)
Distal Mes.
(FL-DM)
Prox. Mes.
(FL PM)
HL-DM
FL-DM
FL-PM
Control
WNT3a
C
E11.5
FL
Distal mes.
(DM)
WL
DM
Control
WNT3a
D
pTA (Super-TOP)
RLU
0.25
0.20
0.15
0.10
0.05
0
DM
PM
E
E11.5
4 3 2 1
pGL3 (4X48)
RLU (% control)
250
200
150
100
50
0
Control
WNT3a
1
2
3
4
F
E10.5
HL
FL
3 2 1
Relative Gene Expression
400
300
200
100
0
Sox9
Axin2
Nkd2
Dkk1
1
2
3
HL
FL

Figure 3.2. Proximal and distal mesenchymal populations exhibit differential responsiveness to WNT3a. A, cell proliferation of E11.5 DM cells is increased more than in WL cells after 24h treatment with WNT3a (50 ng/ml). Cell proliferation was measured with a BrdU incorporation assay. B, rhodamine-labeled peanut agglutinin (PNA) staining demonstrates that WNT3a (50 ng/ml) inhibits condensation of chondrogenic cells in E11.5 WL cultures. DM-derived cultures display an increase in condensation of chondrogenic cells upon treatment with WNT3a (50 ng/ml). C, treatment of WL cultures with various concentrations of WNT3a (0-100 ng/ml) inhibits chondrogenesis in a dose-dependent manner as determined by decreased SOX reporter gene activity and decreased alcian blue staining. DM cultures supplemented with increasing amounts of WNT3a (0-100 ng/ml) exhibit a dose-dependent increase in cartilage formation; both SOX reporter gene activity and alcian blue staining are increased. D, WNT3a (0-100 ng/ml) addition to either WL or DM cultures activates TOP-flash reporter gene activity in a dose-dependent manner.

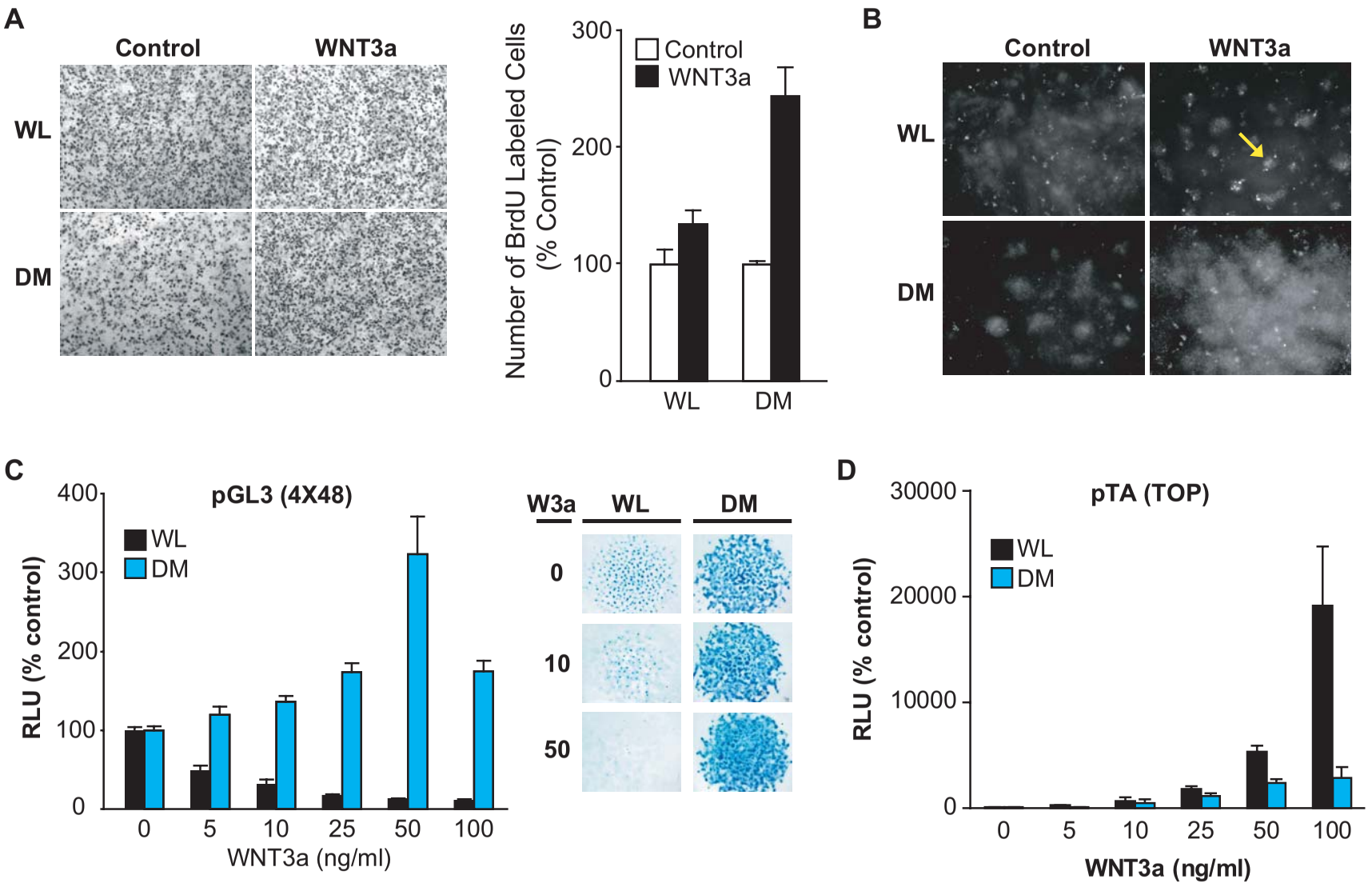
A
Control
WNT3a
WL
DM
Number of BrdU Labeled Cells
(% Control)
300
200
100
0
Control
WNT3a
WL
DM
B
Control
WNT3a
WL
DM
C
pGL3 (4X48)
RLU (% control)
400
300
200
100
0
WL
DM
0
5
10
25
50
100
WNT3a (ng/ml)
W3a
WL
DM
0
10
50
D
pTA (TOP)
RLU (% control)
30000
20000
10000
0
WL
DM
0
5
10
25
50
100
WNT3a (ng/ml)

Figure 3.3. Inhibitors of GSK-3 mimic the effects of WNT3a in various limb mesenchyme sub-populations. A, treatment of E11.5 DM and WL cultures with lithium chloride (0-16 mM) increases and decreases, respectively, SOX reporter gene activity in a dose-dependent manner. B, LiCl (6 mM) and WNT3a (50 ng/ml) exhibit similar activities in E11.5 WL and DM cultures as determined by SOX reporter gene activity. C, treatment of PLM cultures with LiCl (0-16 mM) activates Super-TOP-flash reporter gene activity in a dose-dependent manner in both E11.5 WL and DM cultures. D, LiCl (6 mM) and WNT3a increase Super-TOP reporter gene activity in E11.5 PLM cultures. E, addition of a GSK3 inhibitor, SB216763 (SB) to E10.5 HL-DM and FL-DM PLM cultures increases SOX reporter gene activity. FL-PM cultures show little change in SOX reporter gene activity in the presence of SB. F, Super-TOP reporter gene activity is weakly induced by SB treatment in all 3 types of cultures.

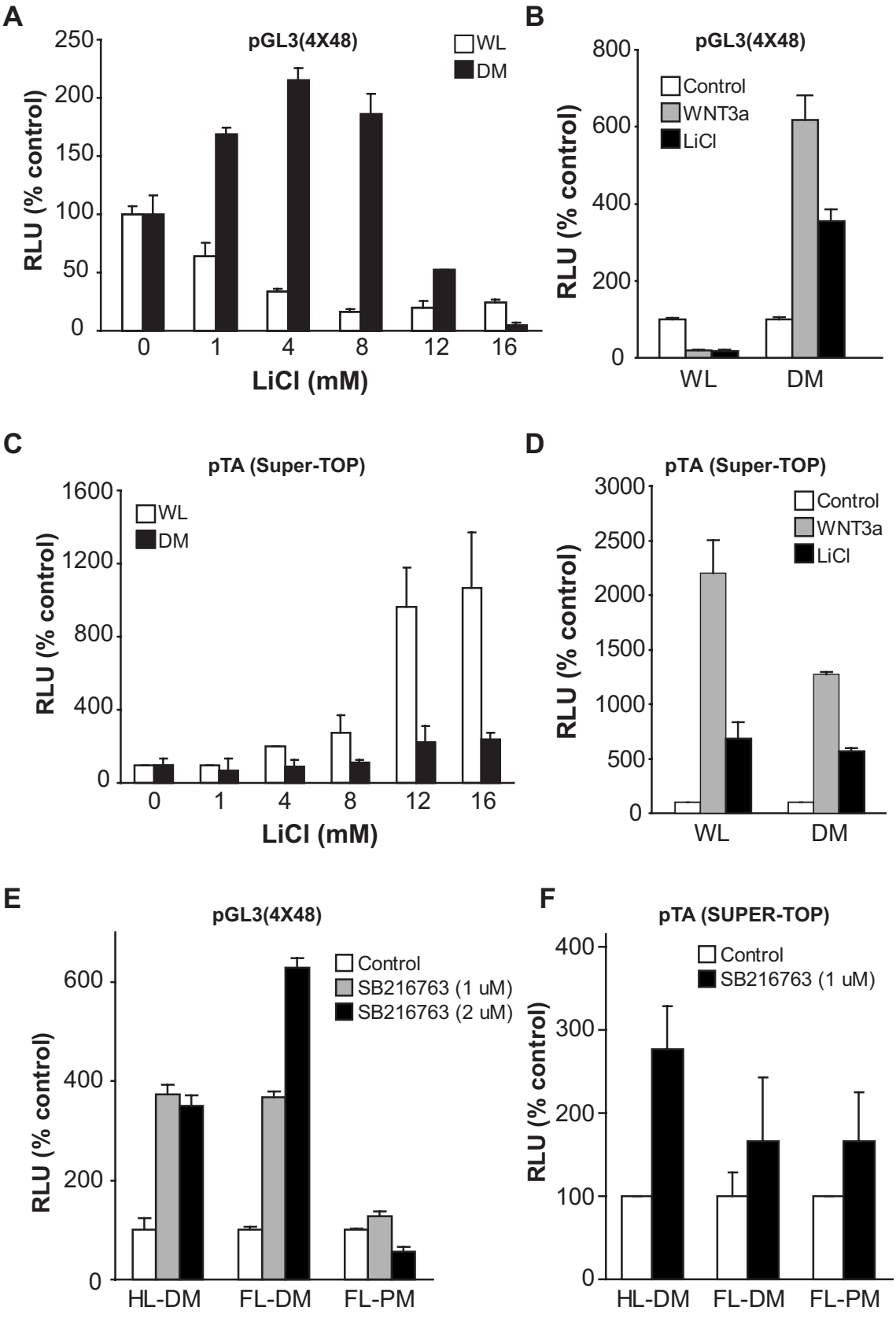
A
pGL3(4X48)
WL
DM
RLU (% control)
250
200
150
100
50
0
0
1
4
8
12
16
LiCl (mM)
B
pGL3(4X48)
Control
WNT3a
LiCl
RLU (% control)
800
600
400
200
0
WL
DM
C
pTA (Super-TOP)
WL
DM
RLU (% control)
1600
1200
800
400
0
0
1
4
8
12
16
LiCl (mM)
D
pTA (Super-TOP)
Control
WNT3a
LiCl
RLU (% control)
3000
2500
2000
1500
1000
500
0
WL
DM
E
pGL3(4X48)
Control
SB216763 (1 uM)
SB216763 (2 uM)
RLU (% control)
600
400
200
0
HL-DM
FL-DM
FL-PM
F
pTA (SUPER-TOP)
Control
SB216763 (1 uM)
RLU (% control)
400
300
200
100
0
HL-DM
FL-DM
FL-PM

Figure 3.4. Inhibition of canonical WNT signaling by expression of *Axin2* promotes SOX reporter activity. A, heterologous expression of *Axin2* in E10.5 limb-derived cultures increases SOX reporter activity and reverses decreased reporter gene activity observed with WNT3a addition in FL-PM and to a lesser extent HL-DM cultures, whereas *Axin2* induced reporter activity is potentiated by WNT3a addition in FL-DM cultures. B, WNT3a (50 ng/ml) stimulates and reduces SOX reporter activity in E11.5 DM and PM cultures, respectively and SOX reporter activity in WNT3a-treated cultures is increased by over-expression of *Axin2*. Right panel, heterologous expression of *Axin2* attenuates WNT3a induction of Super-TOP reporter.

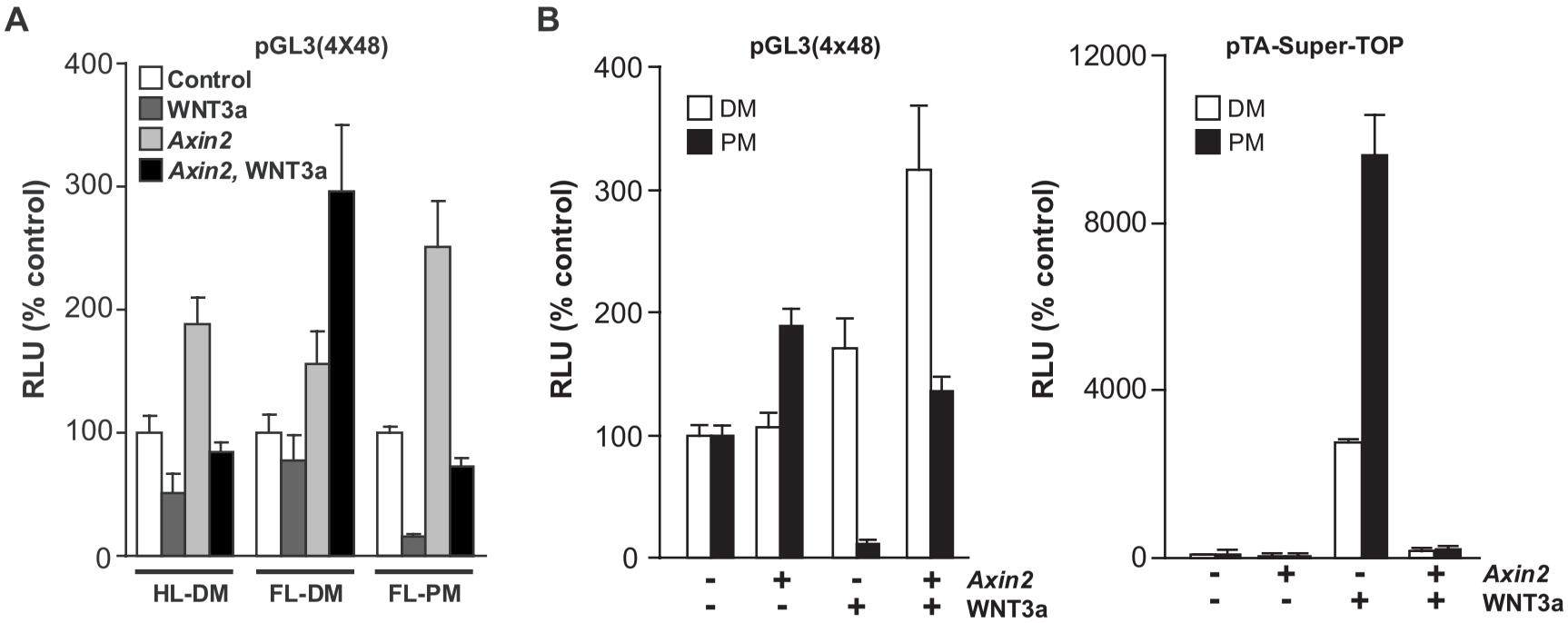
A
pGL3(4X48)
Control
WNT3a
Axin2
Axin2, WNT3a
RLU (% control)
400
300
200
100
0
HL-DM
FL-DM
FL-PM
B
pGL3(4x48)
DM
PM
RLU (% control)
400
300
200
100
0
- + - +
- - + +
Axin2
WNT3a
pTA-Super-TOP
DM
PM
RLU (% control)
12000
8000
4000
0
- + - +
- - + +
Axin2
WNT3a

Figure 3.5. Elucidation of the genetic programs underlying WNT action in primary mesenchymal limb cultures. Affymetrix mouse transcriptome arrays (MOE430 2.0 chips) were used to generate transcriptional profiles from RNA collected at 24 and 48 hours of untreated (-) or WNT3a (50 ng/ml)-treated E10.5 HL-DM, FL-DM and FL-PM cultures. Left panel, hierarchical clustering of genes that exhibited a $\geq$ 2 fold expression change in HL-DM and FL-PM samples following 24 hr WNT3a (50 ng/ml) treatment. A list of genes was generated after the cutoff was applied and genes common to the 2 samples were selected, and these were clustered using Pearson correlation similarity measure in GeneSpring. Right panel, the boxed regions in the left panel were expanded to highlight different patterns of expression. Box 1 represents genes that co-cluster with *Sox6* and *Sox9* and are down-regulated to varying extents in response to WNT3a. Box 2 contains genes that co-cluster with *Col9a2* and are down-regulated in response to WNT3a treatment. The genes highlighted in red represent cartilage-associated genes. Box 3 includes genes that co-cluster with *Axin2* (in blue) and are generally up-regulated upon treatment with WNT3a.

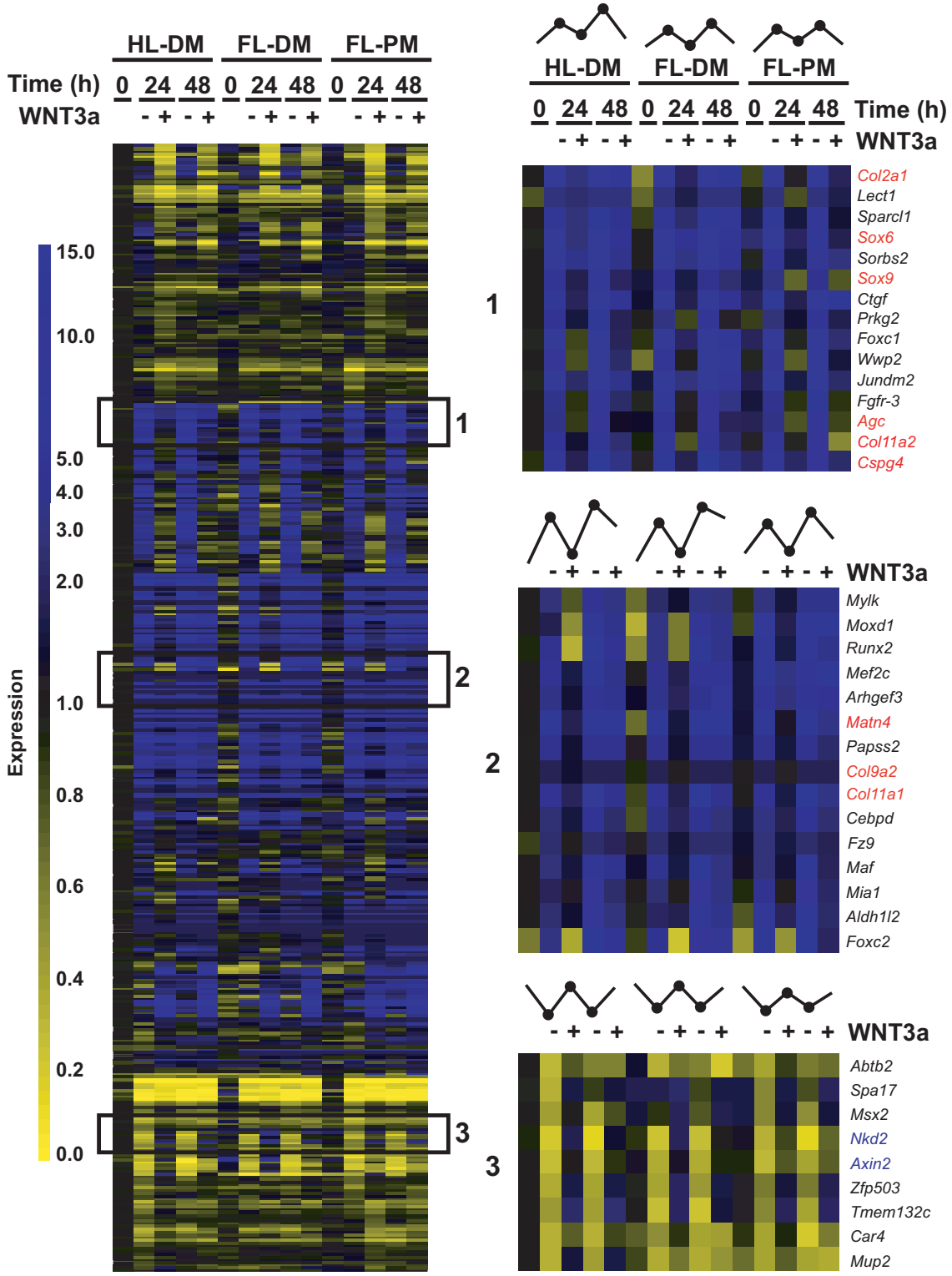
HL-DM
FL-DM
FL-PM
Time (h)
0 24 48 0 24 48 0 24 48
WNT3a
- + - + - + - + - + - +
Expression
15.0
10.0
5.0
4.0
3.0
2.0
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
Col2a1
Lect1
Sparcl1
Sox6
Sorbs2
Sox9
Ctgf
Prkg2
Foxc1
Wwp2
Jundm2
Fgfr-3
Agc
Col11a2
Cspg4
Mylk
Moxd1
Runx2
Mef2c
Arhgef3
Matn4
Papss2
Col9a2
Col11a1
Cebpd
Fz9
Maf
Mia1
Aldh1l2
Foxc2
Abtb2
Spa17
Msx2
Nkd2
Axin2
Zfp503
Tmem132c
Car4
Mup2

Figure 3.6. *Runx2* is expressed in the early limb, is down-regulated by WNT3a and modulates the chondrogenic program. A, bioinformatic analysis of microarray data-sets reveals that *Runx2* is down-regulated by WNT3a treatment at both 24 and 48h in various E10.5 limb-derived cultures. B, treatment of HL-DM, FL-DM and FL-PM with WNT3a (50 ng/ml) results in decreased expression of *Runx2* as determined by qPCR. C, expression of *Runx2* and *Sox9* in various regions of the E10.5 fore and hind limb. Regions are indicated on the left and obtained through micro-dissection. Transcript abundance was measured using qPCR. D, co-transfection of an expression plasmid for *Runx2* reduces SOX reporter activity in all E10.5 PLM cultures, whereas expression of a truncated *Runx2* (deleted for the transcriptional repression domain) moderately increases SOX reporter activity.

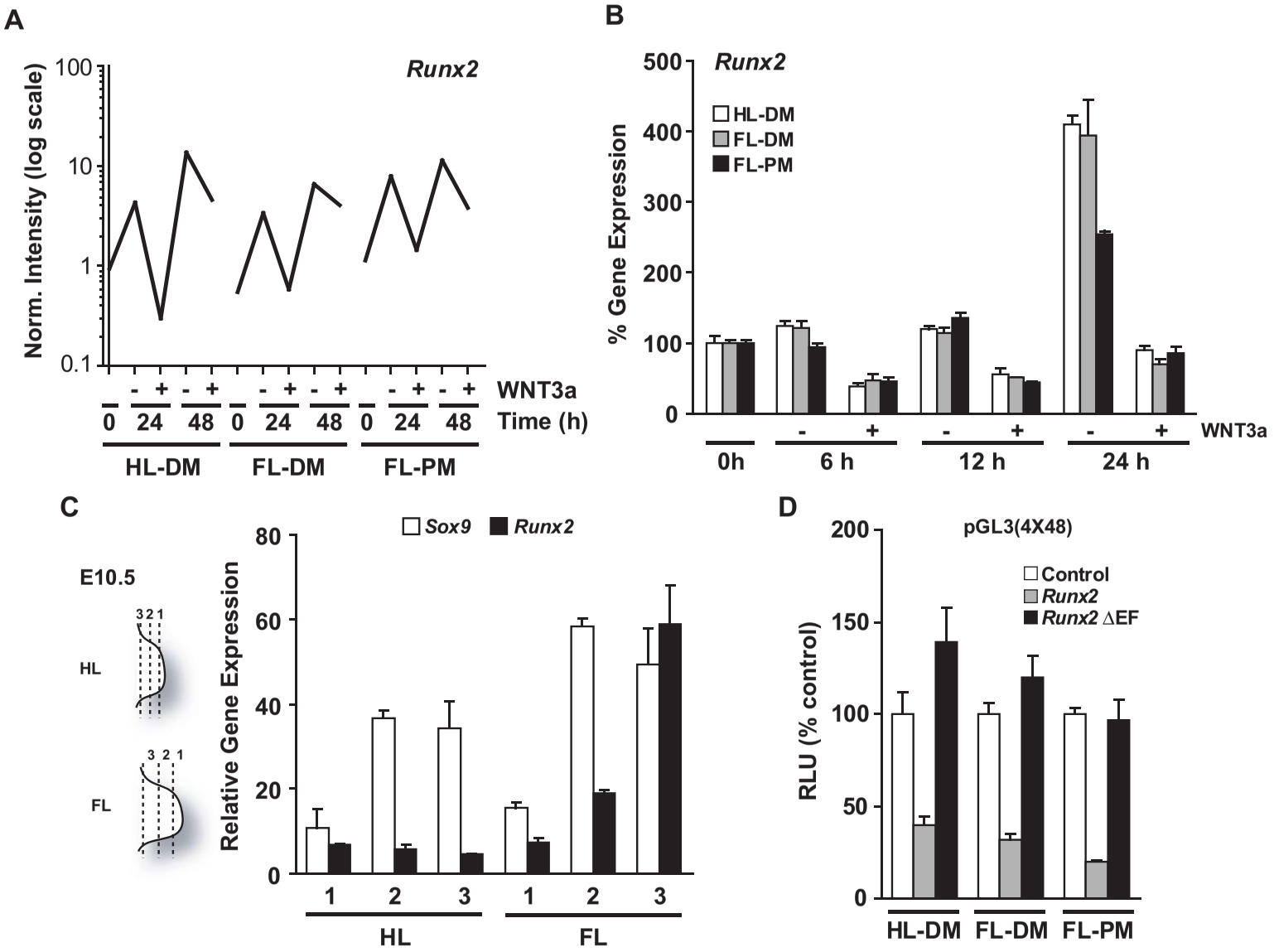
A
Runx2
Norm. Intensity (log scale)
100
10
1
0.1
- + - + - + - + - + - +
WNT3a
0 24 48 0 24 48 0 24 48
Time (h)
HL-DM
FL-DM
FL-PM
B
Runx2
HL-DM
FL-DM
FL-PM
% Gene Expression
500
400
300
200
100
0
- + - + - +
WNT3a
0h
6 h
12 h
24 h
C
Sox9
Runx2
E10.5
3 2 1
HL
3 2 1
FL
Relative Gene Expression
80
60
40
20
0
1 2 3 1 2 3
HL
FL
D
pGL3(4X48)
Control
Runx2
Runx2 ΔEF
RLU (% control)
200
150
100
50
0
HL-DM
FL-DM
FL-PM

Table 3.1: RT-qPCR analysis of selected genes in E10.5 PLM cultures

	HL		FL-DM		FL-PM	
	WNT3a (50 ng/ml)		WNT3a (50 ng/ml)		WNT3a (50 ng/ml)	
	Fold Change		Fold Change		Fold Change	
	12 h	24 h	12 h	24 h	12 h	24 h
Bmpr1a		1.2		1.9		0.8
Bmpr1b		0.6		0.3		0.5
Pax1	1.7	7.8	2.6	62.4	2.9	10.5
Pax9	1.9	13.3	2.7	123.6	0.4	6.6
Gata3	1.0	0.9	0.9	0.5	0.7	0.7
Gata5	0.1	0.5	0.2	0.3	0.4	0.8
Gata6	0.7	0.5	0.7	0.8	0.5	0.7
Zic3	1.9	2.5	1.1	2.1	1.6	1.8
Mdfi	0.7	1.0	0.6	0.9	0.7	1.0
Runx2	0.5	0.2	0.5	0.2	0.3	0.3
Twist1	2.3	4.1	2.5	2.0	2.7	0.9
Alx3	2.7	2.3	1.6	2.4	1.8	4.2
Dkk1	1.6	1.0	1.1	0.8	1.6	1.6
Dkk2	2.2		1.6		2.4	
Meox2	1.3		4.0		2.1	
Kit	0.5		0.5		0.4	
Fzd9	1.1		0.9		0.8	
Foxc1	0.5		0.2		0.2	
Foxc2	1.5		1.0		0.5	
Dlx2	0.4		0.4		0.4	
Aldh1a7	0.4		0.2		0.4	

* Fold change is determined by normalization to untreated control culture.

3.7 References

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CHAPTER IV:
Comparative functional analysis of the mouse and *Fugu Sox9* promoters

A version of this chapter will be submitted for publication. Karamboulas, K., Hodge, L. and Underhill, T.M. Comparative Functional Analysis of the Mouse and *Fugu Sox9* Promoters.

4.1 Introduction

Much of the vertebrate skeleton develops through endochondral ossification, whereby a cartilaginous template is initially formed for subsequent mineralization and replacement by bone (Olsen et al., 2000). Cartilage formation, or chondrogenesis, involves a series of events, including the condensation of mesenchymal chondroprogenitor cells. Following the formation of precartilaginous condensations, cells differentiate into chondroblasts which begin elaborating a cartilaginous extracellular matrix (ECM) rich in collagens II (*Col2a1*), IX (*Col9a1*, *Col9a2* and *Col9a3*) XI (*Col11a1*, *Col11a2*), *aggrecan* (*Agc*) and other proteoglycans (Muir, 1995). *Col2a1* is the most abundant extracellular protein made by chondrocytes which starts to be expressed immediately following mesenchymal condensation formation and thus represents an early marker of chondrocyte differentiation (Cheah et al., 1991; Ng et al., 1993; Vuorio and de Crombrugghe, 1990). Transgenic mice harboring a mutated type II collagen gene display abnormal skeleton formation (Vandenberg et al., 1991). In attempts to identify cis-regulating elements of *Col2a1*, a 48 base pair enhancer element within the first intron of *Col2a1* was identified and shown to be sufficient to drive cartilage-specific expression of a *lacZ* reporter gene in transgenic mice (Zhou et al., 1998). Studies have been carried out to identify potential transcription factors that may control and activate *Col2*. Furthermore, reiteration of this 48 bp enhancer element, strongly increased promoter activity in transiently transfected rat chondrosarcoma (RCS) cells and mouse primary chondrocytes but not in C3H10T1/2 fibroblasts (Lefebvre et al., 1996a). Additionally, this element was used to identify the transcription factor, SRY-box containing gene 9 (*Sox9*) which binds a consensus sequence within this 48 bp element (Bell et al., 1997; Lefebvre et al., 1997; Ng et al., 1997a).

Sox9 belongs to a large family of transcription factors which are characterized by a high-mobility group (HMG) DNA-binding domain, similar to that of the master testis-determining gene, *Sry* (sex-determining region of Y chromosome) (Denny et al., 1992; Gubbay et al., 1990;

Sinclair et al., 1990). Comparable with other SOX (SRY-like HMG box) and HMG containing proteins (Connor et al., 1994; Love et al., 1995; Werner et al., 1995), *Sox9* binds to DNA within the minor groove of the double helix (Lefebvre et al., 1997). During mouse embryogenesis, *Sox9* is expressed in all cartilage primordia and cartilages, and in other tissues such as the central nervous system, heart, pancreas and urogenital systems (Kent et al., 1996; Love et al., 1995; Morais da Silva et al., 1996; Ng et al., 1997b; Zhao et al., 1997). Furthermore, *Sox9* is expressed during skeletal development in mesenchymal cells, pre-chondrocytes and differentiated chondrocytes, but not in hypertrophic chondrocytes (Zhao et al., 1997). *In situ* hybridization during mouse embryogenesis further reveals that the expression pattern of *Sox9* slightly precedes but greatly resembles that of *Col2a1* (Zhao et al., 1997). Subsequently two additional SOX proteins, SOX5 and SOX6 along with SOX9 have been shown to cooperatively regulate *Col2a1* expression in developing cartilages (Lefebvre et al., 1998).

Studies initially revealed the importance of *Sox9* in chondrogenesis with the identification of heterozygous mutations in, or translocations around *Sox9* in human patients with campomelic dysplasia (CD), a severe from of chondrodysplasia that is often associated with XY sex reversal (Foster et al., 1994; Wagner et al., 1994). Several of the reported CD cases result from translocations and inversions with breakpoints outside of the open reading frame (ORF) of *Sox9*. Some of these breakpoints have been mapped to locations at least 50 kb and further from the transcriptional start site of *Sox9* (Foster et al., 1994; Kwok et al., 1995; Wagner et al., 1994). Another study revealed the presence of breakpoints in CD patients to regions spanning 140-950 kb proximal to the start site, by using bacterial-artificial-chromosome (BAC) and P1-artificial-choromosome clones that contained 1.2 Mb upstream sequence of *Sox9* (Pfeifer et al., 1999). Nonetheless, each breakpoint is believed to result in disruption in spatial and temporal expression of *Sox9*. These observations demonstrate a fundamental role of *Sox9* in chondrogenesis, and also highlight the complex nature of its regulation.

Given the complexity and size of the *Sox9* promoter, it has been difficult to define essential regulatory elements. Identification of these *cis*-acting elements would provide information about the tissue- and temporal-specific regulation of *Sox9*. To identify potential regulatory elements upstream of *Sox9*, a comparative genomic analysis was performed between the human, mouse and *Fugu rubripes* (*Fugu)* genomes. This approach has been validated in several studies and is useful in delineating regulatory elements because over 430 million years of evolution between *Fugu* and mammals, essential sequences required for common functions should be highly conserved (Aparicio et al., 1995; Miles et al., 1998). In previous studies, comparative analysis of large intergenic sequences upstream of *Sox9* in human, mouse and *Fugu* revealed the presence of five short highly conserved elements, (E1-E5) within the *Sox9* promoter (Bagheri-Fam et al., 2006; Bagheri-Fam et al., 2001).

Complete transcriptional control of *Sox9* is likely to involve a combination of distal and proximal, enhancer and silencer elements which bind both cell-specific and ubiquitous transcription factors. To a large extent, recent progress has been made towards understanding the molecular biology of chondrogenesis and the critical role of *Sox9* in this program. Delineation of regulatory elements in the *Sox9* promoter and determining molecular factors that bind to these elements is critical when defining the regulation of *Sox9*. Using a combination of molecular approaches and the developing mouse limb as a model system, we have analyzed the proximal and distal regions of the *Sox9* promoter in both mouse and *Fugu*, and results attained from these experiments have added to our current understanding of *Sox9* expression and cartilage development.

4.2 Materials and methods

Cloning of *Sox9* promoter fragments

DNA fragments encompassing the proximal promoter region of the *Mus musculus (M. musculus) Sox9* gene were generated by PCR amplification of DNA from a BAC clone (GenBank accession number: AC040983). Primers were designed to amplify a 2 kb fragment (-1852 bp to +107 bp of mouse *Sox9*), 3 kb fragment (-2953/+107 bp), and 5 kb fragment (-2952/+1750 bp). *Sox9* genomic DNA was PCR amplified using High Fidelity Elongase Enzyme Mix (Invitrogen – Life Technologies). Other mouse and human BAC clones (GenBank accession numbers AC040983, AC053470 and AC005144) were used as templates to amplify upstream *Sox9* conserved elements E1-E5 (Bagheri-Fam et al., 2001).

Fugu Sox9 proximal promoter fragments were PCR amplified from BAC clone DNA (GenBank accession number: AF329945; obtained from MRC GeneService). Primers were designed to amplify a 4 kb fragment using the Expand Long Template PCR system (Roche) (Table 4.3).

Cloning of *Sox9* promoter constructs and plasmid construction

All *M. musculus* Sox9 promoter 5' deletion constructs were sub-cloned into a modified pGL3-basic vector termed pGL3-NXBH (Promega). The *Not*I site in the pGL3-basic vector was removed by digestion, blunt-end filled and religated. An adaptor (sense: 5'-AGCTGCGGCC GCTATCTAGATAAGATCTTA- 3', antisense: 5'- AGCTTAAGATCTTATCTAGATAGCG GCCGC- 3') was inserted into the *Hind*III site of the multiple cloning site (MCS) such that the order of restriction sites was 5'- MCS-*Not*I-*Xba*I-*Bgl*II-*Hind*III- 3'. The 5kb, 3kb, and 2kb *Sox9* PCR fragments were initially sub-cloned into pGEMT-easy (Stratagene) (Table 4.1) and subsequently sub-cloned as Not1 fragments into pGL3-NXBH upstream of the *luciferase* gene and named pGL3-MmS-5, pGL3-MmS-3 and pGL3-MmS-2 respectively. To make the *Sac*I

(pGL3-MmS-*Sac*I) (-193/+107 bp) and *Nhe*I (pGL3-MmS-*Nhe*I) (-73/+107 bp) constructs, the pGL3-MmS-2 promoter construct was digested with *Sac*I and *Nhe*I, respectively, and religated.

PCR fragments (Table 4.2) containing E1-E5 were initially sub-cloned into pGEMT-easy and then sub-cloned upstream of the 2kb fragment in the pGL3-MmS-2 promoter construct. Primers for E1-E5, were designed to contain unique restriction sites on the 3' ends for further cloning purposes. Briefly, E5 was introduced into the *Xho*I site of a linearized pGL3-MmS-2 and E4 was ligated into the *Spe*I restriction site contained on the 3' end of E5. Subsequently, E3 was ligated into the *Pst*I site on the 3' end of E4. The new plasmid was labeled pGL3-MmS-2-E5/E4/E3. Secondly, E1 was sub-cloned into the *Mlu*I site in pGL3-NXBH and then E2 was sub-cloned into the *Eco*RI site on the 3' end of E1 to make pGL3-MmS-2-E1/E2. Variants of these constructs were made by removing different elements, E1-E5, by restriction digestion.

All *Fugu Sox9* promoter constructs were sub-cloned into a modified pGL3-basic vector termed pGL3-AXHN (Promega). The *Not1* and *Xba1* sites in the pGL3-basic vector were removed by digestion, blunt-end filled and religated. An adaptor (sense: 5'- GATCTTAGGCG CGCCTATCTAGATAAAGCTTTAGCGGCCGCTT-3', antisense: 5'-AATCCGCGCGGAT GATCTATTTCGAAATCGCCGGCGAATCGA-3') was inserted into the HindIII site of the MCS such that the order of restriction sites was 5'-MCS-*Asc*I-*Xba*I-*Hind*III-*Not*-3'. The proximal 4 kb fragment was initially sub-cloned into pGEMT-easy, digested with *Not*I and subsequently sub-cloned into pGL3-AXHN, upstream of the *luciferase* gene (pGL3-FrS-4). To make the 2kb construct, pGL3-FrS-4 was digested with Mlu1 and religated (pGL3-FrS-2). PCR fragments (Table 4.4) of distal fragments (~ 5 kb) of the promoter were initially sub-cloned into pGEMT-easy and then sub-cloned upstream of the 4 kb fragment of the pGL3-FrS-4 construct. Primers designed for these regions (A, B, C) included restriction sites on either end. Briefly, region A was sub-cloned into the *Asc*I restriction site; region B was sub-cloned into the *Xba*I/*Hind*III restriction sites and region C was sub-cloned into the *Hind*III site of the modified

vector, pGL3-AXHN upstream of the *luciferase* gene. Variations of these constructs were made by the addition or removal of different regions by endonuclease digestion.

To identify possible regulatory elements within the *Sox9* promoter, sequences were analyzed using rVISTA (Regulatory VISTA) which combines a transcription factor binding site database search with a comparative sequence analysis (Loots et al., 2002). Results identified several potential binding sites for transcription factors such as Kruppel-like factor-4 (KLF-4) and Muscle-Segment like Homeobox 1 (MSX-1). Full-length MGC cDNA clones for *Klf-4*, *Msx-1* and *Msx-2* were obtained from the MRC Geneservice (Babraham, Bioincubator; Babraham, Cambridge, UK).

Establishment and transient transfection of primary limb mesenchymal cultures

Limb mesenchymal cells were harvested from embryonic age (E) 11.25-E11.75 mouse embryos as previously described (Weston et al., 2000). For transient transfections, cells were resuspended at a density of ~ 2.0 x 10^7 cells/ml and mixed with a DNA/FuGene6 mixture in a 2:1 ratio. FuGene6-DNA mixtures were prepared according to manufacturer's instructions (Roche Biomolecular). Briefly, 0.5 μg of plasmid reporter, 0.25 μg of expression vector, 0.25 μg pKS II (Stratagene) and 0.05 μg of pRLSV40 (Promega) were combined for a total of ~1 μg DNA in 50 μl media and FuGene6. Twenty microlitres of the DNA/FuGene6 mixtures was transferred to sterile 1.5 ml eppendorf tubes, followed by 40 μl of cell suspension. Cells were gently triturated, and 10 μl was used to seed a single well of a 24-well culture dish. The cells were allowed to attach for one hour in a humidified CO_2 incubator, followed by the addition of 1 ml of media to each well. Culture media contained 40% Dulbecco's modified Eagle's medium (DMEM) and 60% F12 supplemented with 10% fetal bovine serum (Gibco-BRL) and antibiotics. Media was changed 24 hours post-transfection and if necessary, compounds or factors were added.

Forty-eight hours post-transfection, analysis of reporter gene activity was preformed using the Dual Luciferase Assay System according to the manufacturer's instructions (Promega). Briefly, cells were washed once with PBS and lysed in 100 μl of Passive Lysis Buffer for 20 minutes. Forty microlitres of each lysate was then loaded in a 96-well plate and luciferase activity was determined by a luminometer (L-Max; Molecular Devices). Firefly luciferase activity was normalized against *Renilla* luciferase activity to control for differences in transfection efficiency. Luciferase experiments were performed a minimum of three times and one representative experiment is shown for all luciferase results.

Culture conditions and transient transfections of cell lines

RCS and COS-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL) and antibiotics at 37°C in 5% CO_2. C3H10T1/2 cells were maintained in Basal Medium Eagle's (BME) media (Gibco-BRL) supplemented with 10% FBS and antibiotics. ATDC5 cells were cultured in DMEM/Ham's F-12 hybrid medium (Gibco-BRL) containing 5% FBS and liquid media supplement (ITS) (Sigma).

For transfection purposes, cells were plated in 12-well tissue cultures plates at 2-5 $\times$ 10^4 cells/well approximately 24 hours before transfection. Transient transfections were performed using FuGene6 transfection reagent according to manufacturer's instructions (Roche Biomolecular). Briefly, each well of cells was transfected with FuGene6 and a total of 0.5 μg DNA, including 0.3 μg of reporter, 0.2 μg expression vector, and 0.05 μg pRLSV40. Luciferase assays were preformed ~ 48 h following transfection as described above, with the exception of using 150 μl/well of Passive Lysis Buffer (Promega) to lyse cells.

Electrophoretic mobility shift assays (EMSAs)

Nuclear extracts were prepared according to the protocol of Andrews *et. al* (Kent et al., 1996). Briefly, cells were washed twice with cold TBS and scraped in TBS. Cells were

collected into eppendorf tubes and centrifuged to form pellets. Pellets containing 1-3 $\times 10^7$ cells were resuspended in 400μl of cold Buffer A (10 mM Hepes [pH 7.9], 10 mM KCl, 0.1 mM EDTA [pH 8.0], 1 mM DTT, AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride), and 0.5 mM of protease inhibitors: NaF, NaOrtho. Cells were incubated on ice for 15 minutes before 25 μl of 10% Triton-X was added. After mixing and centrifugation, pellets were resuspended in cold Buffer C (20 mM Hepes [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, AEBSF, and 0.5 mM of protease inhibitors: sodium fluoride (NaF) and sodium orthovanadate (NaOrtho)). After vortexing and centrifugation, the supernatant containing nuclear extracts was stored in aliquots at -80°C. Nuclear extracts were quantified using the BCA protein assay kit (Pierce) with minor modifications. Complementary oligonucleotides were annealed to form double-stranded probes. Probe A (between –193 and – 134 bp relative to the mouse *Sox9* transcription start site) was modified to contain *Not* I overhangs on each end: 5'-GGCCGCCCCCTCCCTTCTCCTCCCCT CCTGTCTCGTCACCCAACCCGGAGCCACAATCCTCCCCAGGC-3'. Probe B (–133 and – 73 bp of mouse *Sox9*) was modified to include *Xho* I sites on each end: 5'-TCGAGCCCCC CTTCCAAAATCCGGTCCAATCAGCGACTTGCCAACACTGATGACTCAAGAGC - 3'. Mutant probe B was prepared with oligonucleotides that replaced CCAAT with TTAAT. Double-stranded DNA probes were radiolabelled by end-filling with DNA polymerase I (Klenow fragment, Life Technologies, Inc.) in the presence of [α-^{32}P]dCTP. Protein-DNA binding reactions were performed in a total volume of 10 μl of gel-shift binding buffer (15 mM Tris [pH 7.5], 75 mM NaCl, 1.5 mM EDTA [pH 7.5], 1.5 mM DTT and 7.5% glycerol) with 10 μg of nuclear extracts in the presence of labeled nucleotide (≥200,000 cpm) for 20 min. at room temperature. In competition reactions, unlabeled oligonucleotides were used in a 100-fold molar excess compared to the probe and incubated for 10 min before adding probe. For supershifting experiments, 2 μg of NFY-B (G-2) antibody (Santa Cruz Biotechnology, Inc.) and nuclear

extracts were pre-incubated for 1 hour at room temperature. Samples were loaded onto 4% polyacrylamide gels with 1×TBE (50 mM Tris-borate, 20 mL of 0.5M EDTA [pH 8.0]) at 100 V for 3.5 hours. Gels were dried for 1 hour at 72°C and autoradiographed.

4.3 Results

It has been shown repeatedly that *Sox9* plays a critical role during chondrogenesis (Lefebvre et al., 1997; Ng et al., 1997a; Wunderle et al., 1998); however, little is known about the regulation of its expression. To gain a broader understanding of the early stages of chondrogenesis, it is necessary to delineate the molecular networks directly affecting *Sox9* expression and gene activity by analyzing its promoter region.

Localization of the *M. musculus Sox9* minimal promoter

Taking into consideration the large and extensive *Sox9* promoter region, it was first necessary to define the most proximal region, as it may contain several essential elements required for transcription. To this end, a mouse BAC clone containing a DNA fragment encompassing the *Sox9* locus was used as a template to amplify 5 kb (-2952/+1750 bp), 3 kb (-2952/+107 bp), and 2 kb (-1853/+107 bp) fragments, relative to the murine *Sox9* transcriptional start site as identified previously (Kanai and Koopman, 1999). These fragments were each sub-cloned upstream of the *luciferase* gene in the reporter vector pGL3-NXBH and various 5' deletions were made to the -2 kb promoter construct by restriction digests to generate nested deletions. Minimal luciferase activity was observed when the pGL3-MmS-5 (5 kb) or pGL3-MmS-3 (3 kb) constructs were transiently transfected into primary mesenchymal cultures. However, upon transfection of the pGL3-MmS-2 promoter construct, there was a marked increase in luciferase activity. This 2 kb *Sox9* promoter fragment was sub-cloned into pGL3-NXBH in both forward and reverse orientations and transient transfections were performed in primary mesenchymal cultures, C3H10T1/2 cells and COS-P7 cells. Only the forward, 5' to 3'

oriented, fragment exhibits significantly increased luciferase activity compared to the reverse constructs. Furthermore, the pGL3-MmS-2 promoter activity is significantly higher in primary mesenchymal cultures and in C3H10T1/2 cells in comparison to COS-P7 cells (Figure 4.1C). Furthermore, both the addition of 1 kb upstream and the deletion of the region between the *Sac*I (-193 bp) and *Nhe*I (-73 bp) sites abolished much of this activity (Figure 4.1B). These results indicate the presence of positive regulatory elements between –1853 and –73 bp from the transcriptional start site. Furthermore, a smaller construct, pGL3(–44/+107) still produced some weak luciferase activity; whereas pGL3(–44/+6), still containing the putative TATA-like box (Kanai and Koopman, 1999), abolished any remaining weak activity (Figure 4.1B). Nonetheless, the basal luciferase activities of the smaller constructs were significantly lower than that of the pGL3-MmS-*Sac*I. These findings suggest that the putative TATA box previously reported may not play a crucial role in regulating basal transcriptional activity (Kanai and Koopman, 1999). However, it may be possible that other elements further upstream of the transcriptional start site are required in combination with a TATA-box protein complex to initiate transcription.

Characterization of the 120 bp region of the *Sox9* promoter

Since a significant decrease in luciferase activity was observed with the deletion of the *Sac*I-*Nhe*I (-193 to –73 bp) region, we further characterized this region. Transient transfections were performed in primary mesenchymal cultures and C3H10T1/2 cells with the pGL3-MmS-*Sac*I and pGL3-MmS-*Nhe*I constructs. Luciferase activity was significantly extinguished in primary mesenchymal cultures (Figure 4.1B) and C3H10T1/2 cells (Figure 4.1D) when this 120 bp region of the promoter was deleted, indicating that this region plays an important role in *Sox9* transcription. To identify positive regulatory elements, the sequence of the 120 bp region was analyzed by TFSEARCH against the TRANSFAC database (Heinemeyer et al., 1998) and results identified several potential binding sites for transcription factors, such as a CCAAT binding site, two GATA sites and a SRY-like site. In addition, a sequence comparison and alignment of

human and mouse *Sox9* proximal promoter DNA, performed using the ClustalW multiple sequence alignment program revealed high homology between these two sequences (Thompson et al., 1994). Furthermore, an extended comparison between human, mouse and *Fugu* proximal promoter sequences revealed high sequence homology only between mouse and human sequences in a large region of the proximal region (Figure 4.2). Since the genome of *Fugu* is highly compact, a comparison may prove to be difficult in this region even though conserved elements may be present; however a CCAAT binding site was also identified in the *Fugu Sox9* proximal promoter (Figure 4.2).

Since there was no obvious site that may be responsible for the transactivation of the promoter, EMSAs were performed to determine if any nuclear proteins bound the 120 bp sequence using two 60 bp probes, A and B, that spanned the 120 bp region (Figure 4.3A). Incubation of nuclear extracts from several cell lines with probe B resulted in a specific DNA-protein complex (Figure 4.3B). To verify binding specificity of the complex, the probe B/protein complex was incubated with 100-molar excess cold probe or 100-molar excess of an unrelated consensus oligo for NFκB. Results demonstrated that excess unlabeled probe B could out-compete the complex, but not the NFκB oligo, indicating binding specificity of the protein complex for probe B (Figure 4.3B and data not shown).

In an attempt to identify potential proteins in the complex, the sequence of probe B was closely examined using TRANSFAC. Since there was a potential binding site for CCAAT/enhancer binding protein-β (C/EBP-β) in the 60 bp sequence, we attempted without success to out-compete the complex with an excess of a C/EBP-β consensus oligo (data not shown).

Mutational analysis of the 60 bp enhancer element

Another potential binding site between –133 and –73 bp on the *M. musculus Sox9* promoter was the CCAAT site. Thus, an oligonucleotide that replaced CCAAT with TTAAT

was made. To examine the consequence of mutating the CCAAT site, competitive EMSAs were performed. Interestingly, increasing amounts of cold probe B*mut* (B*) could not dissociate the labeled probe B/nuclear protein complex (Figure 4.3C). Moreover, labeled probe B* incubated with the same nuclear extracts could not form the specific complex (Figure 4.3C). These results implied that the CCAAT site in probe B was potentially responsible for the DNA/protein complex observed in the EMSAs.

It has been previously shown in many eukaryotic genes that the transcription factor, CCAAT-binding factor (CBF/NFY) binds specifically to CCAAT-sequences (Currie, 1997; Lin et al., 2003; Maity and de Crombrugghe, 1998; Mantovani et al., 1992). NFY is made up of three subunits, NFY-A, NFY-B, and NFY-C and all are necessary for DNA binding (Currie, 1997; Lin et al., 2003; Maity and de Crombrugghe, 1998). To test if NFY formed a complex on probe B, super-shift experiments were carried out by incubation of the nuclear extracts with an anti-NFY-B antibody. The addition of anti-NFY-B antibody was able to super shift the probe B/protein complex (Figure 4.3D). Oligonucleotides for this 60 bp region were also designed and sub-cloned upstream of the luciferase gene in the modified pGL3-NXBH vector. Transient transfections of the mutated CCAAT motif (pGL3-B*) in primary mesenchymal cultures results in decreased luciferase activity in comparison to luciferase activity observed for the vector alone (pGL3-NXBH) or the wild-type construct (pGL3-B). Furthermore, co-transfection of pGL3-B with *Nfy-a, -b, -c* in primary mesenchymal cultures results in a further increase in luciferase activity compared to control; whereas there is no change in luciferase activity upon co-transfection of pGL3-B* with *Nfy-a, -b, -c* (Figure 4.3E). Together, these results confirmed the identity of the complex as NFY/CCAAT and support an important role for activation of the *Sox9* promoter.

Highly conserved distal elements affect proximal promoter activity

To further examine the transcriptional regulation of *Sox9* by potential distal enhancers and repressors, we examined conserved distal elements along the *Sox9* promoter. In addition to the homology in the proximal promoter, five highly conserved elements were identified upstream of the *Sox9* transcriptional start site (Bagheri-Fam et al., 2006; Bagheri-Fam et al., 2001). The pGL3-MmS-2 reporter construct was used to identify enhancer and/or silencer elements by sub-cloning the conserved elements (E1-E5) upstream of this sequence (Figure 4.4). Using human and mouse BAC clones and PCR, conserved elements E1-E5 were amplified and sub-cloned upstream of the minimal 2 kb promoter. When transiently transfected into primary mesenchymal cells, luciferase activity was highest in the pGL3-MmS-2 reporter construct alone compared to constructs containing E1 alone (pGL3-MmS-E1), E5/E4/E3/E1 (pGL3-MmS-E5/E4/E3/E1), E5/E4 (pGL3-MmS-E5/E4), or E4 alone (pGL3-MmS-E4) (Figure 4.4B). Activity of the reporter construct containing elements E1 and E2, pGL3-MmS-2-E1/E2, was marginally higher than pGL3-MmS-2 alone (Figure 4.4B). These results indicate the presence of positive regulatory element(s) in E2 that increase basal luciferase activity of the mouse *Sox9* proximal promoter, and repressive element(s) in E4 (Figure 4.4B).

Characterization of the *Sox9* proximal promoter in *Fugu rubripes*

With 430 million years of evolution between mammals and *Fugu*, comparative analysis has been a useful tool in identifying genes and gene regulatory elements (Armes et al., 1997; Baxendale et al., 1995; Coutelle et al., 1998; Elgar et al., 1996; Mason et al., 1995; Sandford et al., 1997; Venkatesh and Brenner, 1995). When transiently transfected into primary mesenchymal cultures, highest basal luciferase activity was observed with the pGL3-FrS-4 promoter construct compared to the pGL3-FrS-2 promoter construct, whereas little activity was observed with a construct containing the 4kb promoter fragment in the reverse orientation (Figure 4.5A). Transient transfections were also performed in various cell lines to assess if the

transcriptional activity of pGL3-FrS-4 was cell-type specific. The pGL3-FrS-4 construct resulted in markedly decreased luciferase activity in C3H10T1/2 cells (Figure 4.5A), which express *Sox9* weakly and in COS-P7 cells (Figure 4.5A), which do not express *Sox9*, compared to primary cultures. These differences indicate the presence of specific *cis*-elements in the 4kb promoter region that are acting uniquely in primary and C3H10T1/2 cultures, similar to the pGL3-MmS-2 mouse *Sox9* proximal promoter.

Analysis of various upstream *Fugu rubripes* promoter fragments in different cell lines

The remainder of the putative *Fugu Sox9* promoter was divided into three fragments of approximately 5 kb in length (A, B, C) (Figure 4.5B). Each of these fragments was amplified and sub-cloned alone or in combination upstream of the 4 kb proximal promoter fragment in the pGL3-FrS-4 construct. Transient transfection of these constructs in primary mesenchymal cultures resulted in an increase in luciferase activity with regions A and B, suggesting the presence of putative enhancer elements within this region. On the other hand, region C resulted in slightly decreased luciferase activity, indicating the presence of potential repressor elements within this region (Figure 4.5C). When transfected into different cell lines (COS-P7 and C3H10T1/2), these promoter constructs exhibited different activities, suggesting that the differences in luciferase activity likely reflect cell-type specific differences in regulation (Figure 4.5C).

Transcriptional regulation of the element E4 of the *Sox9* promoter

We focused on the conserved element E4 since its presence greatly reduced activity of the mouse 2 kb *Sox9* proximal promoter. This element is located within the "A" fragment of the *Fugu Sox9* promoter (Figure 4.5B). The E4 sequence was analyzed using both rVISTA and TFSEARCH against the TRANSFAC database (Heinemeyer et al., 1998). Results indicated that this region contains putative KLF-4 and MSX-1 conserved binding sites (human, mouse and

Fugu; data not shown). Previously in the lab, transcriptional profiling with Affymetrix U74 v2 chips A and B was performed to identify differential gene expression in primary mesenchymal cultures in response to BMP4 (20 ng/ml) (Hoffman et al., 2006). This microarray data demonstrates a progressive increase in *Klf-4* and *Msx-1* throughout the chondrogenic program (Figure 4.6A). Co-transfection of both *Klf-4* and *Msx-1* with pGL3-MmS-2-E4 exhibit increased luciferase reporter activity; however, co-transfection of *Msx-2* does not alter pGL3-MmS-2-E4 luciferase activity (Figure 4.6B). Conversely, co-transfection of *Klf-4*, *Msx-1* or *Msx-2* with pGL3-FrS-4A (which contains the element E4) does not affect the activity of the *Fugu* 4 kb proximal promoter (Figure 4.6C).

This microarray data also suggests that the expression of both *Klf-4* and *Msx-1* is modulated by BMP4 (Figure 4.6D). Co-transfection of *Klf-4* with a SOX9-responsive reporter (pGL3-4x48) results in decreased SOX9-reporter gene activity, even when primary mesenchymal cultures are treated with BMP4 (Figure 4.6E). Similarly, co-transfection of *Msx-1* with the SOX9-reporter gene (pGL3-4x48) results in decreased reporter gene activity, even in the presence of BMP4 (Figure 4.6F); however, co-transfection of *Msx-2* has no effect on pGL3-Mm2-E4 reporter gene activity (Figure 4.6G).

4.4 Discussion

During chondrogenesis, *Sox9* is expressed in a sex- and tissue-specific manner that includes all cartilages (Kent et al., 1996; Love et al., 1995; Morais da Silva et al., 1996). *Sox9* has emerged as a central player in regulating the initial stages of the chondrogenic program. In previous studies, it has been shown in mouse chimeras using *Sox9*$^{-/-}$ embryonic stem cells that *Sox9*-null cells are excluded from the developing cartilages. A few *Sox9*$^{-/-}$ cells remained in the periphery of condensations, but these cells did not express chondrogenic markers such as *Col2a1* (Bi et al., 1999). Subsequent studies in which *Sox9* was conditionally removed from limb

mesenchymal cells or in mesenchymal cells following condensation has demonstrated a requirement for *Sox9* in both precartilaginous condensation formation and chondrocyte differentiation (Akiyama et al., 2002). Moreover, forced expression of *Sox9* in some cell types, leads to the expression of chondrocytic genes (Bell et al., 1997; Healy et al., 1999). *Sox9* is therefore considered to be both necessary and in some instances sufficient for cartilage formation, and thus has been termed a master regulator of the chondrogenic program.

Complexity of the promoter

We first analyzed the most proximal region of the mouse *Sox9* promoter by using a series of promoter deletion constructs. Luciferase data indicates that the first 2 kb of the mouse *Sox9* promoter drove the highest expression compared to other deletion constructs in primary mesenchymal cultures. When compared to the pGL3-MmS-2 deletion construct, the basal luciferase activity of the pGL3-MmS-3 construct was ~7-fold lower, suggesting the presence of repressor/silencer elements within this region. Moreover, deletion of the region between the *Sac*I and *Nhe*I sites (–193 to –73 bp) abolished nearly all luciferase activity in primary mesenchymal cultures and in various stable cell lines. For example, when transiently transfected into C3H10T1/2 cells, the pGL3-MmS-*Sac*I construct results in the highest luciferase activity compared to other deletion constructs. A previous study also showed that this 120 bp region was essential for maximal promoter activity in C3H10T1/2 and L3T3 cells and gonadal tissues (Kanai and Koopman, 1999). Specifically, this region drove higher levels of *Sox9* expression in testicular than in ovarian somatic cells and liver cells, which suggests the presence of sex- and tissue-specific *cis*-regulatory elements in the 120 bp sequence.

Using DNA I hypersensitivity assays, it also appears that an open chromatin structure upstream of the promoter is necessary for *Sox9* trans-activation but is not sufficient for the tissue-specific regulation of its transcription level (Kanai and Koopman, 1999). These studies also revealed the presence of a conserved putative TATA-like box positioned –23 bp from the

mouse *Sox9* transcriptional site that may be important in regulating *Sox9* expression. Most typical eukaryotic promoters contain a TATA box within the core region that is implicated as general transcriptional machinery. It has been shown that the recruitment of TATA-binding protein (TBP) to the TATA box is a key step in subsequently recruiting the RNA polymerase II holoenzyme, believed to be necessary for transcription of the target gene (Pugh, 2000). Nonetheless, it has also been confirmed that for gene-specific regulation, a multitude of binding sites for specific activators are required within the promoter region. To this end, our luciferase data conferred that the deletion of the 120 bp region abolished nearly all promoter activity independently of the cell type. These results indicate that this 120 bp region (–193 to –73 bp) appears to contain other important regulatory sequences, other than the TATA box, that are essential for transcriptional control of *Sox9.*

NFY expression

To investigate the importance of this 120 bp region in the transcription of *Sox9*, EMSAs were used to identify any DNA-protein complexes formed in this region. These experiments revealed that a complex was produced in the region -133 to -73 bp from the transcriptional start site. To isolate the exact sequence responsible for this complex formation, consensus oligonucleotides for various factors were used in competitive reactions without success. However, when the CCAAT motif (beginning at -108 bp) was mutated, the formation of this DNA-protein complex was entirely abolished.

CCAAT motifs have been found in many higher eukaryotic promoters between -50 and -110 bp from the transcriptional start site and this position is usually highly conserved (Bucher, 1990; Chodosh et al., 1988; Dorn et al., 1987; Hatamochi et al., 1988). Previous studies have shown that the mammalian CCAAT-binding factor (CBF; also called NF-Y) binds to the CCAAT motif in many eukaryotic promoters (Bucher, 1990; Chodosh et al., 1988; Dorn et al., 1987; Hatamochi et al., 1988; Maity and de Crombrugghe, 1998; Mantovani et al., 1992). NFY

is a heterotrimeric protein containing three subunits: NFY-A. NFY-B and NFY-C, whereby NFY-A and NFY-C first interact with each other to form a heterodimer before binding to the B subunit (Lefebvre et al., 1996b). Furthermore, NFY is thought to be a transcriptional activator, since mutations in any nucleotide within the CCAAT sequence abolishes NFY binding, resulting in decreased transcriptional activity (Coustry et al., 1995; Milos and Zaret, 1992; Roy and Lee, 1995; Wright et al., 1994)

To this end, we were able to supershift the DNA-protein complex previously described, when the NFY-B antibody was incubated with the nuclear extracts. Although this data suggests the presence of the protein as NFY, further studies should be preformed to determine the specificity of the NFY-B antibody. It has also been shown that it is not only the CCAAT motif which is required for the NFY/DNA complex but also the flanking sequence and these flanking sequences affect the affinity of NFY for DNA, which underlines the means by which NFY may regulate transcription of various eukaryotic genes (Bi et al., 1997). Nevertheless, we have identified the CCAAT motif in the proximal promoter of *Sox9* in mouse, which is required for the regulation of transcription and determined that it forms a DNA-protein complex with NFY. Furthermore, transient transfections in mesenchymal cell cultures indicate that the CCAAT box is required for transcriptional activation, since luciferase activity is abolished upon transfection with a construct containing a mutated CCAAT motif.

The functional importance of CCAAT boxes is variable amongst different classes of promoters. Relatively simple TATA-less promoters, containing one or two additional activator sites, absolutely require an intact CCAAT box; whereas, very strong, articulate TATA-containing promoters, with several sites for powerful activators are somewhat less critically dependent on the CCAAT box. Hence, the mobility-shift experiments described above and the luciferase data implicate that the CCAAT box may be playing an important role in regulating *Sox9* transcription.

Other studies show that NFY is an essential molecule involved in the spatially specific expression of the Hox gene, *Hoxb4* (Gilthorpe et al., 2002). Members of the *Hox* gene family display spatially restricted expression patterns along the anterior-posterior (AP) axis of the developing embryo, which requires tightly regulated transcription of genes. These observations indicate that the CCAAT motif may play an important role within the *Sox9* proximal promoter and may also control the temporal and spatial regulation of *Sox9*. Although the regulatory elements controlling *Sox9* expression are scattered over 1 Mb of sequence in humans, these present studies resulted in the localization and identification of a 120 bp region in the proximal promoter necessary for transcription. Furthermore, the CCAAT motif was found within this sequence that binds a NFY DNA-binding complex and this has recently been shown by another group (Colter et al., 2005).

Promoter under various contexts: Presence of enhancer/repressor elements

With 430 million years of evolution between mammals and *Fugu*, comparative analysis has been a useful tool in identifying genes and gene regulatory elements (Baxendale et al., 1995; Mason et al., 1995; Venkatesh and Brenner, 1995). The *Fugu* genome is very similar to that of human but is 7.5 times smaller thereby containing a reduced number of introns and a greater density of genes (Brenner et al., 1993).

We analyzed the most proximal promoter of *Sox9* in *Fugu*. Luciferase data indicates that the most proximal 4 kb of the promoter drove the highest expression as compared to the most proximal 2 kb region. Furthermore, three regions distal to the promoter of approximately 5 kb in length were sub-cloned upstream of the 4 kb proximal promoter. These regions exhibited differential luciferase activity, suggesting the presence of activators and repressors regulating *Sox9* expression. Further comparative analysis and sequence alignments between the mouse and *Fugu Sox9* promoter should be used to analyze the activity of these potential distal regulatory

regions. Also, a more thorough examination of the sequences of these regions should be preformed in order to target specific transcriptional modulators of *Sox9* activity.

Five conserved elements (E1-E5) were identified upstream of *Sox9*, using comparative analysis between human, mouse and *Fugu* promoter sequences (Bagheri-Fam et al., 2006; Bagheri-Fam et al., 2001). Interestingly, the majority of CD translocation breakpoints separate elements E3-E5 from *Sox9* and transgenic experiments have indicated that skeletal-specific elements may lie within this region. Luciferase reporter activity of proximal promoter constructs containing elements E1-E5, alone or in combination, suggests that there may be enhancer regulatory elements in E1 and E2 of the *Sox9* promoter, and repressor elements in E4 and E5. In particular, E4 exhibited highly repressive luciferase activity of the mouse 2kb *Sox9* proximal promoter and contains putative binding sites for the transcription factors, KLF-4 and MSX-1.

Krüppel-like factors are a family of transcription factors containing a zinc finger DNA binding domain that has been shown to play a critical role in a number of cellular processes throughout embryogenesis, including the coordination of differentiation in chondrogenesis of the developing limb bud (Schuh et al., 1986; Segre et al., 1999). Microarray data from our lab reveals a dynamic expression pattern of *Klf-4* throughout chondrogenesis. *Klf-4* expression further increases upon treatment of primary mesenchymal limb cultures with BMP4, indicating that it is a putative downstream target of BMP signalling and may play an important role in chondrogenesis. Furthermore, KLF-4 may interact with the E4 region of *Sox9* promoter, since it increases luciferase activity of this region by ~ 5-fold upon transient transfection into primary mesenchymal limb cultures. It has also been shown that homeobox-containing genes, *Msx* genes, control a number of cellular processes during embryogenesis (Bendall and Abate-Shen, 2000). The progressive increase of *Msx-1* gene expression throughout the chondrogenic program along with the modulation of its expression by BMP4, suggests that *Msx-1* may play an important role in regulating chondroblast differentiation. MSX-1 may also interact with the E4

region of the *Sox9* promoter, since it increases mouse *Sox9* proximal promoter activity. Furthermore, the binding site for MSX-1 located within the element E4, seems to be specific to MSX-1 since co-transfection of *Msx-2* has no effect on proximal promoter activity.

Reporter constructs containing conserved elements E1-E5 can identify potential regulatory elements in the distal region of the promoter, since both proximal and distal elements may be involved in the tissue-specific regulation of *Sox9.* However, there is also a need to investigate 3' control of the gene since *cis*-regulatory elements have been located in 3' sequences. Recently, a 30-bp element in the first intron of *Sox9* was shown to act as an enhancer in the embryonic carcinoma-derived chondrogenic cells, ATDC5 (Morishita et al., 2001). Secondly, sequence comparison between human and *Fugu* genomes identified 3 short conserved elements, E6-E8, 3' to *Sox9*. When enhancer elements are accurately identified, transgenic mice harbouring these promoter constructs will be valuable in examining their role in regulating *Sox9* expression *in vivo*.

Further comparative analysis and sequence alignments between the mouse, human and *Fugu Sox9* promoters will be needed to identify other transcription factors regulating conserved regulatory regions of the distal *Sox9* promoter. Tissue-specific expression could be addressed using transient transgenesis in mice with constructs harbouring the 2 kb mouse *Sox9* proximal promoter coupled to the *E. coli β-galactosidase* (*β-gal*) gene and various elements (E1-E5) (Bagheri-Fam et al., 2006). The expression of *β-gal* should be followed in E10.5 to E13.5 mouse embryos and compared to the endogenous *Sox9* expression pattern (as determined by *in situ* hybridization). Most importantly, we will focus on identifying those constructs that drive expression to the developing limb cartilages. Furthermore, a more extensive multi-species alignment will be carried out on the *Sox9* gene and we will incorporate newly identified elements into these analyses. Nonetheless, it will be difficult to reconstruct *Sox9* expression from isolated

elements as there may be additional elements required. The context of the elements within these large intergenic regions may also be important for appropriate expression of *Sox9*.

The precise expression of genes during development is determined in part by cellular and environmental signals that control the activity of a myriad of transcription factors. Many of these transcription factors bind to *cis*-regulatory DNA elements located in the promoter regions of their target genes. Elucidating the complex molecular networks that define the transcriptional activity of certain genes is imperative in gaining knowledge to elaborate developmental processes. In particular, our focus has been on delineating the molecular mechanisms underlying chondrogenesis, a multi-step process critical for proper skeletal development. To this end, we have characterized *Sox9* proximal promoter activity in primary mesenchymal cultures and have identified a region required for maximal trans-activation. We have also described the potential regulatory effects of the distal elements, E1-E5.

Our results convincingly demonstrate the complex organization of *Sox9* regulatory regions and the necessity of both proximal and distal elements within the *Sox9* promoter region. Nonetheless, the molecular networks regulating *Sox9* expression are far from being entirely defined; however, our studies presented a comprehensive analysis that will make proficient future experiments possible. Hence, future studies with the promoter constructs described in these experiments will allow us to identify critical regulatory elements involved in the regulation of *Sox9* expression during skeletogenesis.

4.5 Figures

Figure 4.1. Promoter analysis using transient transfections of mouse *Sox9* promoter constructs. (A) Schematic diagram of the mouse *Sox9* proximal promoter shows the location ofthe restriction sites used to make deletion constructs. (B) Various 5'-deletion constructs of mouse genomic *Sox9* DNA, with a common 3' end (+107 bp), except pGL3-MmS-5, were made. Transient transfections were performed in primary mesenchymal cells isolated from the limbs of 11.5 d.p.c. mouse embryos at a density of 2×10^7 cells/ml. Luciferase activity was normalized to *Renilla* luciferase to control for transfection efficiency. Minimal luciferase activity was observed upon transfection of the 3 kb (pGL3-MmS-3) and 5 kb (pGL3-MmS-5) *Sox9* promoter constructs. However, upon transfection of the 2 kb promoter construct (pGL3-MmS-2), there was a significant increase in luciferase activity. Both the addition of 1 kb upstream and deletion of the region between the *Sac*I (-193 bp) and *Nhe*I (-73 bp) abolished nearly all luciferase activity. An even smaller promoter construct pGL3-MmS-(-44/+107) still produced some weak luciferase activity. (C) The 2 kb *Sox9* promoter fragment was sub-cloned into pGL3-NXBH in both forward and reverse orientations and transient transfections were performed in primary mesenchymal cultures, C3H10T1/2 cells and COS-P7 cells. Only the forward, 5' to 3' oriented, fragment exhibits significantly increased luciferase activity compared to the reverse constructs. Furthermore, the pGL3-MmS-2 promoter activity is significantly higher in primary mesenchymal cultures and in C3H10T1/2 cells in comparison to COS-P7 cells (data displayed as a ratio between pGL3-MmS-2-forward: pGL3-MmS-2-reverse). (D) Upon transient transfection of different promoter constructs in other cell lines (C3H10T1/2), luciferase activity was completely abolished with the construct which does not contain the 120 bp region between *Sac*I (-193 bp) (pGL3-MmS-*Sac*I) and *Nhe*I (-73 bp) (pGL3-MmS-*Nhe*I).

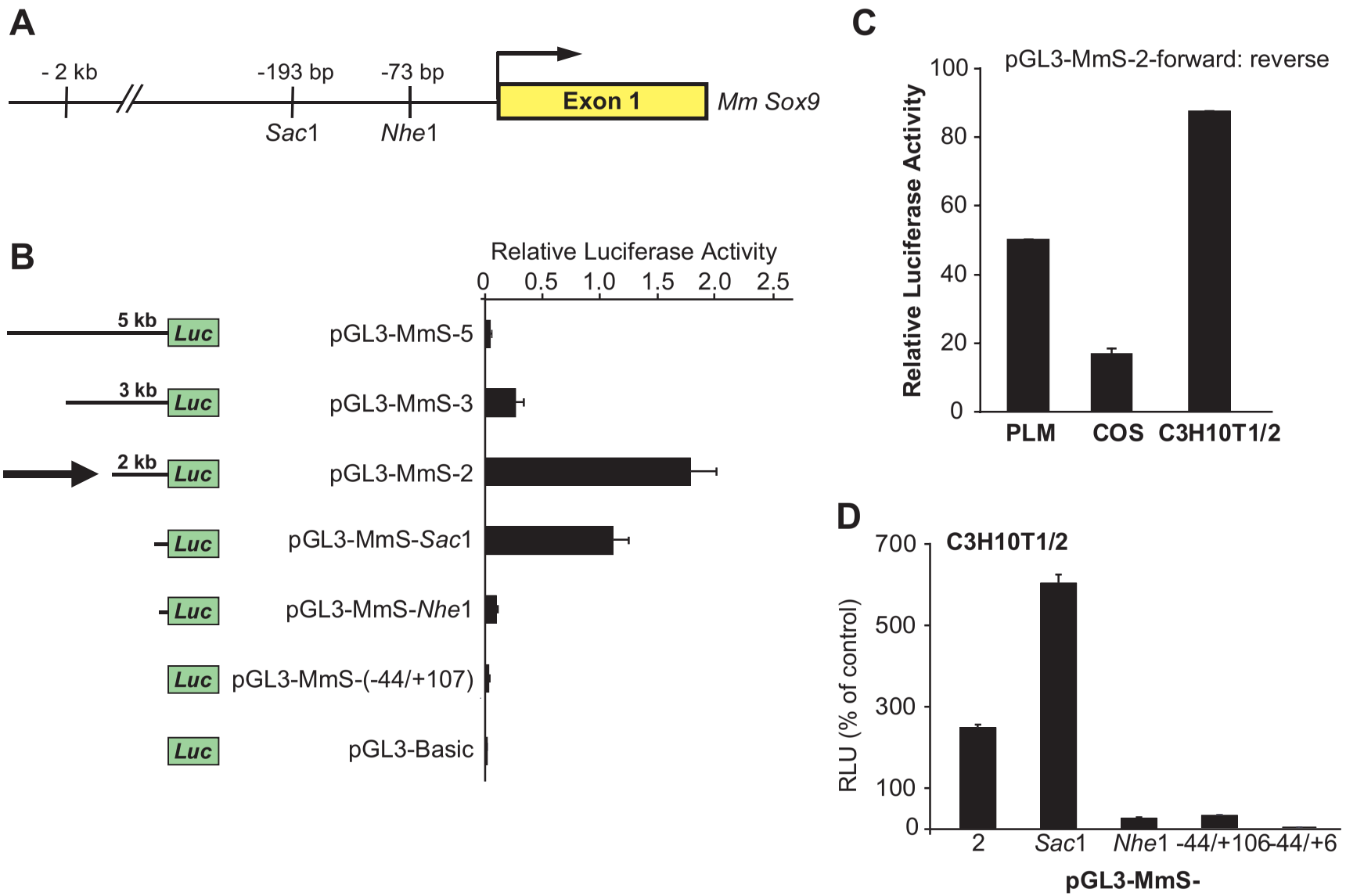
A
- 2 kb
-193 bp
-73 bp
Exon 1
Mm Sox9
Sac1
Nhe1
B
Relative Luciferase Activity
0
0.5
1.0
1.5
2.0
2.5
5 kb
3 kb
2 kb
Luc
pGL3-MmS-5
pGL3-MmS-3
pGL3-MmS-2
pGL3-MmS-Sac1
pGL3-MmS-Nhe1
pGL3-MmS-(-44/+107)
pGL3-Basic
C
pGL3-MmS-2-forward: reverse
Relative Luciferase Activity
100
80
60
40
20
0
PLM
COS
C3H10T1/2
D
C3H10T1/2
RLU (% of control)
700
500
300
100
0
2
Sac1
Nhe1
-44/+106
-44/+6
pGL3-MmS-

Figure 4.2. Sequence alignment of the *Sox9* proximal promoter region between human, mouse and *F. rubripes*. The putative CCAAT boxes are highlighted in red. The transcriptional start site is indicated by an arrow (orange). The ATG translational start site for all three species is also highlighted in bold. *Sac*I and *Nhe*I restriction sites are highlighted in green. Conserved nucleotides are marked with an *.

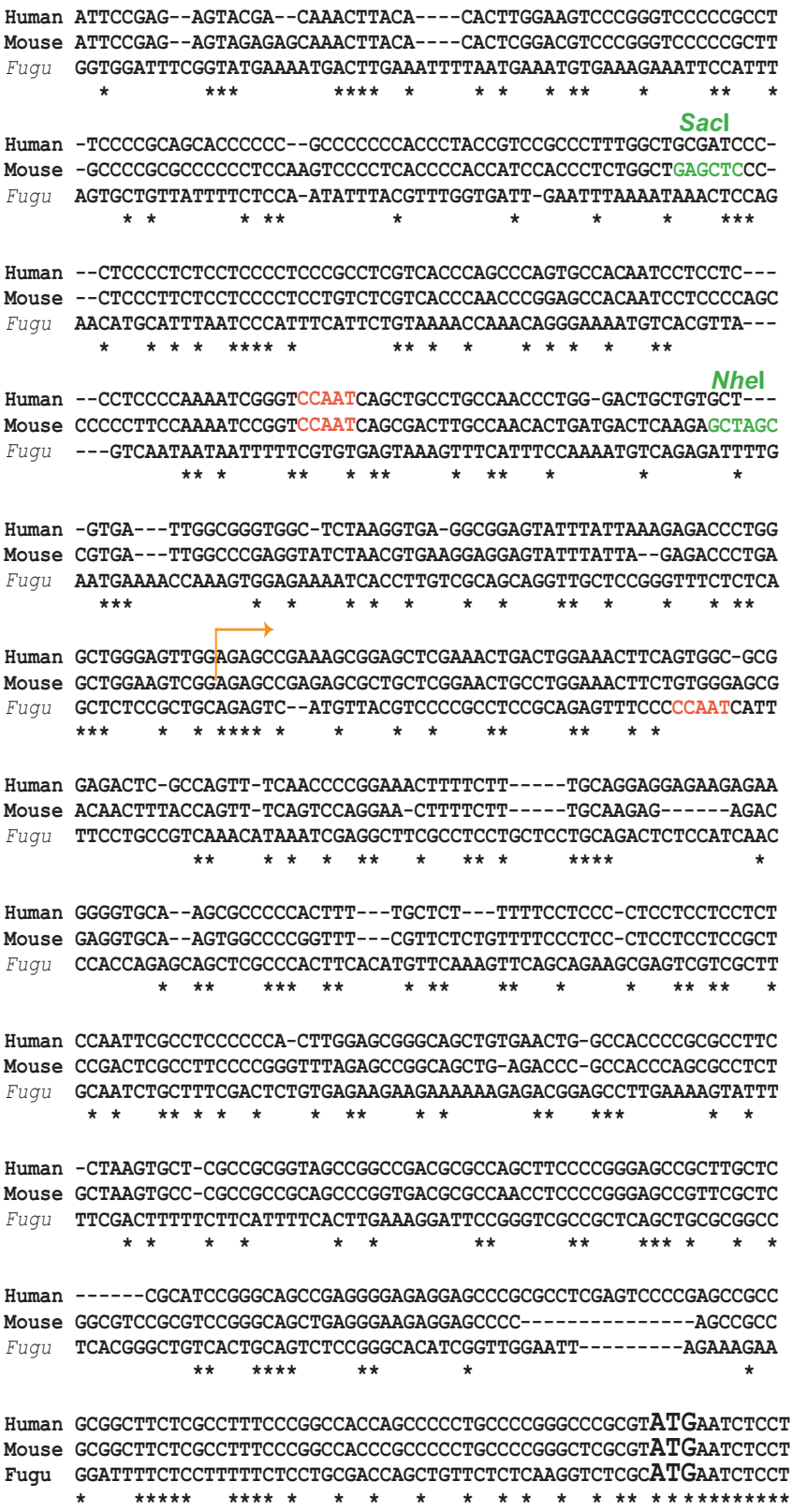
Human ATTCCGAG--AGTACGA--CAAACTTACA----CACTTGGAAGTCCCGGGTCCCCCGCCT
Mouse ATTCCGAG--AGTAGAGAGCAAACTTACA----CACTCGGACGTCCCGGGTCCCCCGCTT
Fugu GGTGGATTTCGGTATGAAAATGACTTGAAATTTTAATGAAATGTGAAAGAAATTCCATTT
SacI
Human -TCCCCGCAGCACCCCCC--GCCCCCCCACCCTACCGTCCGCCCTTTGGCTGCGATCCC-
Mouse -GCCCCGCGCCCCCCTCCAAGTCCCCTCACCCCACCATCCACCCTCTGGCTGAGCTCC-
Fugu AGTGCTGTTATTTTCTCCA-ATATTTACGTTTGGTGATT-GAATTTAAAATAAACTCCAG
Human --CTCCCCTCTCCTCCCCTCCCGCCTCGTCACCCAGCCCAGTGCCACAATCCTCCTC---
Mouse --CTCCCTTCTCCTCCCCTCCTGTCTCGTCACCCAACCCGGAGCCACAATCCTCCCCAGC
Fugu AACATGCATTTAATCCCATTTCATTCTGTAAAACCAAACAGGGAAAATGTCACGTTA---
NheI
Human --CCTCCCCAAAATCGGGTCCAATCAGCTGCCTGCCAACCCTGG-GACTGCTGTGCT---
Mouse CCCCCTTCCAAAATCCGGTCCAATCAGCGACTTGCCAACACTGATGACTCAAGAGCTAGC
Fugu ---GTCAATAATAATTTTTCGTGTGAGTAAAGTTTCATTTCCAAAATGTCAGAGATTTTG
Human -GTGA---TTGGCGGGTGGC-TCTAAGGTGA-GGCGGAGTATTTATTAAAGAGACCCTGG
Mouse CGTGA---TTGGCCCGAGGTATCTAACGTGAAGGAGGAGTATTTATTA--GAGACCCTGA
Fugu AATGAAAACCAAAGTGGAGAAAATCACCTTGTCGCAGCAGGTTGCTCCGGGTTTCTCTCA
Human GCTGGGAGTTGGAGAGCCGAAAGCGGAGCTCGAAACTGACTGGAAACTTCAGTGGC-GCG
Mouse GCTGGAAGTCGGAGAGCCGAGAGCGCTGCTCGGAACTGCCTGGAAACTTCTGTGGGAGCG
Fugu GCTCTCCGCTGCAGAGTC--ATGTTACGTCCCCGCCTCCGCAGAGTTTCCCCCAATCATT
Human GAGACTC-GCCAGTT-TCAACCCCGGAAACTTTTCTT-----TGCAGGAGGAGAAGAGAA
Mouse ACAACTTTACCAGTT-TCAGTCCAGGAA-CTTTTCTT-----TGCAAGAG------AGAC
Fugu TTCCTGCCGTCAAACATAAATCGAGGCTTCGCCTCCTGCTCCTGCAGACTCTCCATCAAC
Human GGGGTGCA--AGCGCCCCCACTTT---TGCTCT---TTTTCCTCCC-CTCCTCCTCCTCT
Mouse GAGGTGCA--AGTGGCCCCGGTTT---CGTTCTCTGTTTTCCCTCC-CTCCTCCTCCGCT
Fugu CCACCAGAGCAGCTCGCCCACTTCACATGTTCAAAGTTCAGCAGAAGCGAGTCGTCGCTT
Human CCAATTCGCCTCCCCCCA-CTTGGAGCGGGCAGCTGTGAACTG-GCCACCCCGCGCCTTC
Mouse CCGACTCGCCTTCCCCGGGTTTAGAGCCGGCAGCTG-AGACCC-GCCACCCAGCGCCTCT
Fugu GCAATCTGCTTTCGACTCTGTGAGAAGAAGAAAAAAGAGACGGAGCCTTGAAAAGTATTT
Human -CTAAGTGCT-CGCCGCGGTAGCCGGCCGACGCGCCAGCTTCCCCGGGAGCCGCTTGCTC
Mouse GCTAAGTGCC-CGCCGCCGCAGCCCGGTGACGCGCCAACCTCCCCGGGAGCCGTTCGCTC
Fugu TTCGACTTTTTCTTCATTTTCACTTGAAAGGATTCCGGGTCGCCGCTCAGCTGCGCGGCC
Human ------CGCATCCGGGCAGCCGAGGGGAGAGGAGCCCGCGCCTCGAGTCCCCGAGCCGCC
Mouse GGCGTCCGCGTCCGGGCAGCTGAGGGAAGAGGAGCCCC--------------AGCCGCC
Fugu TCACGGGCTGTCACTGCAGTCTCCGGGCACATCGGTTGGAATT---------AGAAAGAA
Human GCGGCTTCTCGCCTTTCCCGGCCACCAGCCCCCTGCCCCGGGCCCGCGTATGAATCTCCT
Mouse GCGGCTTCTCGCCTTTCCCGGCCACCCGCCCCCTGCCCCGGGCTCGCGTATGAATCTCCT
Fugu GGATTTTCTCCTTTTTCTCCTGCGACCAGCTGTTCTCTCAAGGTCTCGCATGAATCTCCT

Figure 4.3. Characterization of the 120 bp region of the mouse *Sox9* proximal promoter with EMSAs. (A) Two 60 bp probes (A and B) were made which span the 120 bp *Sox9* promoter region between *Sac*I (-193 bp) and *Nhe*I (-73 bp) restriction sites. (B) EMSAs were performed in the presence of either probe, ^{32}P-labeled, with nuclear extracts from various cells (1 – COS-P7, 2 – RCS, 3 – primary mesenchymal cultures, 4 – MC3T3). A specific DNA-protein complex was formed upon incubation of nuclear extracts with probe B (as indicated by the arrow). (C) An oligonucleotide that replaced a CCAAT-binding site with TTAAT was made and used in competitive EMSAs. Competitive EMSAs were performed in the presence of either ^{32}P-labeled probe B or mutant probe B (B*) and increasing amounts of cold probe B* and probe B, respectively (50-, 100-, 200-molar excess). Increasing amounts of cold probe B* could not dissociate the labeled probe B/nuclear protein complex. Moreover, labeled probe B* incubated with the same nuclear extracts could not form the specific complex. (D) To identify the protein complex, an anti-NFY-B antibody was incubated with RCS nuclear extracts and labeled probe B. The antibody was able to supershift the probe B/protein complex as indicated by the arrow. (E) Oligonucleotides for this 60 bp region were also designed and sub-cloned upstream of the luciferase gene in the modified pGL3-NXBH vector. Transient transfections of the mutated CCAAT motif (pGL3-B*) in primary mesenchymal cultures results in decreased luciferase activity in comparison to luciferase activity observed for the vector alone (pGL3-NXBH) or the wild-type construct (pGL3-B). Furthermore, co-transfection of pGL3-B with *Nfy-a*, *-b*, *-c* in primary mesenchymal cultures results in a further increase in luciferase activity compared to control; whereas there is no change in luciferase activity upon co-transfection of pGL3-B* with *Nfy-a*, *-b*, *-c*.

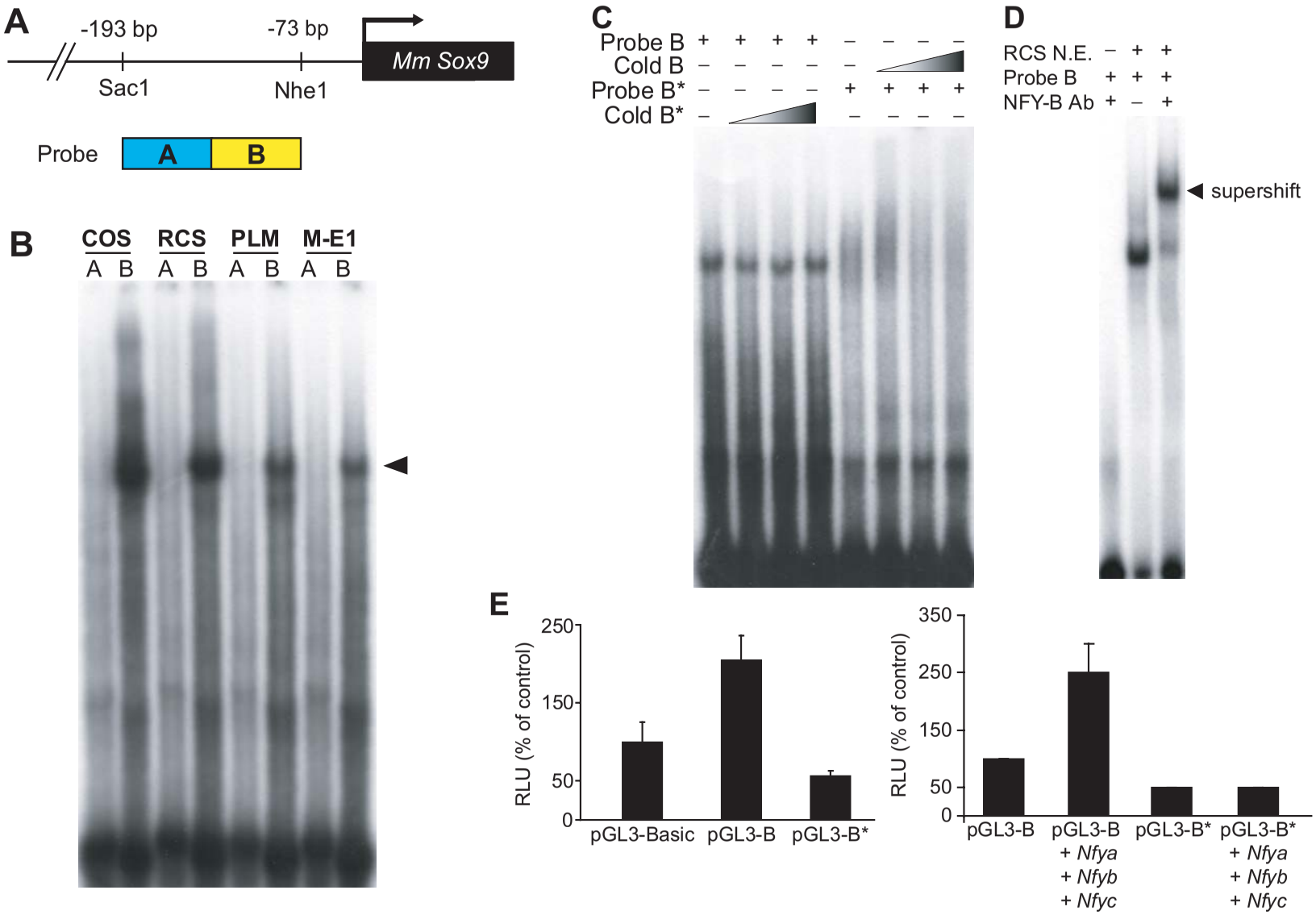
A
-193 bp
-73 bp
Mm Sox9
Sac1
Nhe1
Probe
A
B
B
COS
RCS
PLM
M-E1
A B A B A B A B
C
Probe B + + + + – – – –
Cold B – – – – – –
Probe B* – – – – + + + +
Cold B* – – – –
D
RCS N.E. – + +
Probe B + + +
NFY-B Ab + – +
supershift
E
RLU (% of control)
250
150
50
0
pGL3-Basic
pGL3-B
pGL3-B*
RLU (% of control)
350
250
150
50
0
pGL3-B
pGL3-B + Nfya + Nfyb + Nfyc
pGL3-B*
pGL3-B* + Nfya + Nfyb + Nfyc

Figure 4.4. Highly conserved distal elements affect mouse *Sox9* proximal promoter activity. (A) Using PCR, elements E1-E5 (Bagheri-Fam et al., 2006; Bagheri-Fam et al., 2001) were amplified, sub-cloned (alone or in combination) upstream of the pGL3-MmS-2 promoter construct and transiently transfected into primary mesenchymal cultures. (B) The element E4, significantly decreases proximal promoter luciferase activity compared to the control construct, whereas E1/E2 slightly increases proximal promoter activity.

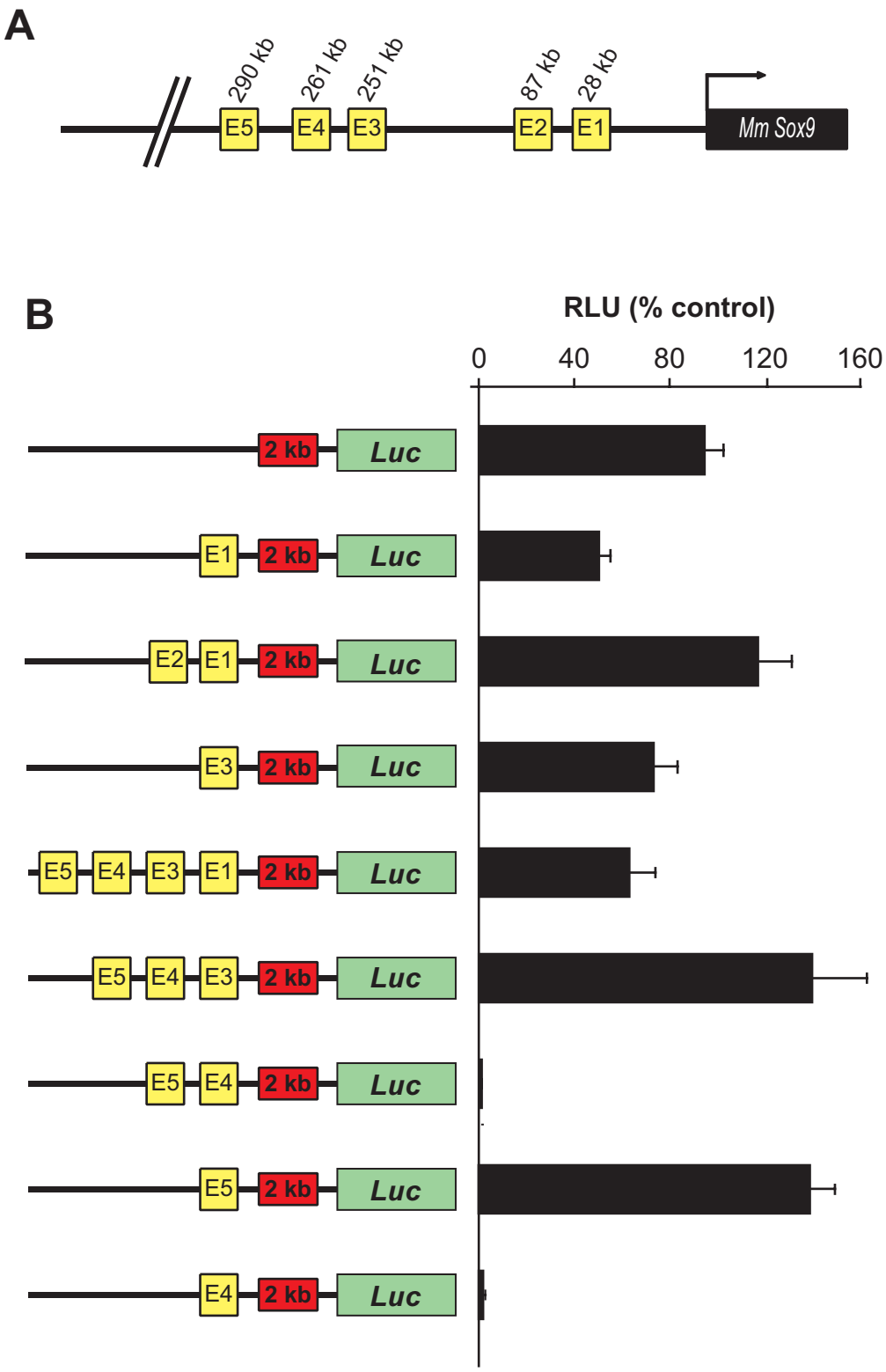
A
290 kb
261 kb
251 kb
87 kb
28 kb
E5
E4
E3
E2
E1
Mm Sox9
B
RLU (% control)
0
40
80
120
160
2 kb
Luc
E1
E2
E3
E5
E4

Figure 4.5. Characterization of the *Sox9* proximal promoter in *F. rubripes*. (A) The 4 kb *Sox9* PCR fragment was sub-cloned into pGL3-NXBH (pGL3-FrS-4) in both forward and reverse orientations. Upon transient transfection of these constructs into primary mesenchymal cultures, highest luciferase activity is observed with the 4 kb promoter construct (pGL3-FrS-4) compared to the 2 kb promoter construct (pGL3-FrS-2); whereas there was minimal luciferase activity observed with a construct containing the 4 kb promoter fragment in the reverse orientation. Furthermore, the pGL3-FrS-4 construct exhibits decreased luciferase activity in C3H10T1/2 cells, which express *Sox9* weakly and in COS-P7 cells, which do not express *Sox9*, compared to primary mesenchymal cultures. The pGL3-FrS-2 promoter activity is also notably higher in primary mesenchymal cultures and C3H10T1/2 cells compared to COS-P7 cells. (B) The remainder of the *F. rubripes Sox9* promoter was divided into three fragments approximately 5 kb in length (A, B, C). Each of these fragments was amplified using PCR and sub-cloned (alone or in combination) upstream of the 4 kb proximal promoter construct (pGL3-FrS-4). (C) Transient transfections of 4 kb promoter constructs containing regions A and B in primary mesenchymal cultures results in increased luciferase activity. Conversely, transfection of the promoter construct containing region C results in little to no change in luciferase activity. Transfection of all of these promoter constructs in C3H10T1/2 and COS-P7 cells results in decreased proximal promoter activity.

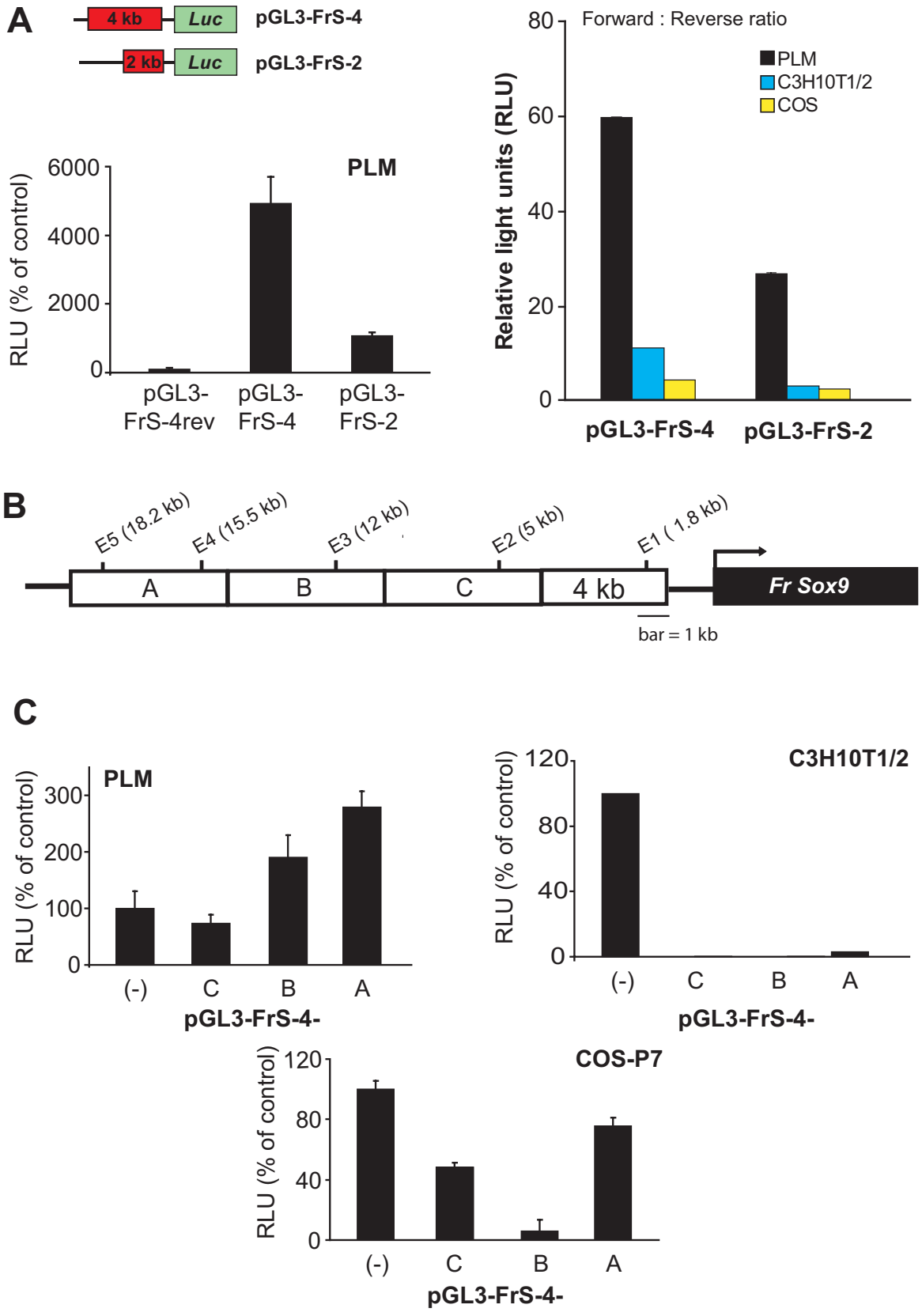
A
4 kb
Luc
pGL3-FrS-4
2 kb
Luc
pGL3-FrS-2
PLM
RLU (% of control)
6000
4000
2000
0
pGL3-FrS-4rev
pGL3-FrS-4
pGL3-FrS-2
Forward : Reverse ratio
PLM
C3H10T1/2
COS
Relative light units (RLU)
80
60
40
20
0
pGL3-FrS-4
pGL3-FrS-2
B
E5 (18.2 kb)
E4 (15.5 kb)
E3 (12 kb)
E2 (5 kb)
E1 (1.8 kb)
A
B
C
4 kb
Fr Sox9
bar = 1 kb
C
PLM
RLU (% of control)
300
200
100
0
(-)
C
B
A
pGL3-FrS-4-
C3H10T1/2
RLU (% of control)
120
80
40
0
(-)
C
B
A
pGL3-FrS-4-
COS-P7
RLU (% of control)
120
80
40
0
(-)
C
B
A
pGL3-FrS-4-

Figure 4.6. Transcriptional regulation of the element E4 of the *Sox9* promoter. (A) Previously in the lab, transcriptional profiling with Affymetrix U74 v2 chips A and B was performed to identify differential gene expression in E11.5 primary mesenchymal cultures in response to BMP4 (20 ng/ml) (Hoffman et al., 2006). This microarray data demonstrates a progressive increase in *Klf-4* and *Msx-1* throughout the chondrogenic program (Figure 4.6A). Microarray data shows that there is a progressive increase in *Klf-4* and *Msx-1* throughout the chondrogenic program. (B) Transient transfection of the mouse *Sox9* proximal promoter containing the element E4 exhibits a significant decrease in luciferase activity in primary mesenchymal cells. The element E4 was analyzed using both rVISTA and TFSEARCH against the TRANSFAC database. Results indicate that this region contains putative KLF-4 and MSX-1 binding sites. Co-transfection of both *Klf-4* and *Msx-1* with pGL3-MmS-2-E4 exhibits increased luciferase activity; however, co-transfection of *Msx-2* does not alter pGL3-MmS-2-E4 promoter activity. (C) Co-transfection of *Klf-4*, *Msx-1* or *Msx-2* with pGL3-FrS-4A does not affect promoter activity. (D) Microarray data shows that the expression of both *Klf-4* and *Msx-1* is modulated by BMP4. (E) Co-transfection of *Klf-4* with a SOX9-responsive reporter (pGL3-4x48) results in decreased reporter gene activity, even when primary mesenchymal cultures are treated with BMP4. (F) Co-transfection of *Msx-1* with the SOX9-reporter gene (pGL3-4x48) results in decreased reporter gene activity, even in the presence of BMP4. (G) However, co-transfection of *Msx-2* has no effect on pGL3-Mm2-E4 reporter activity.

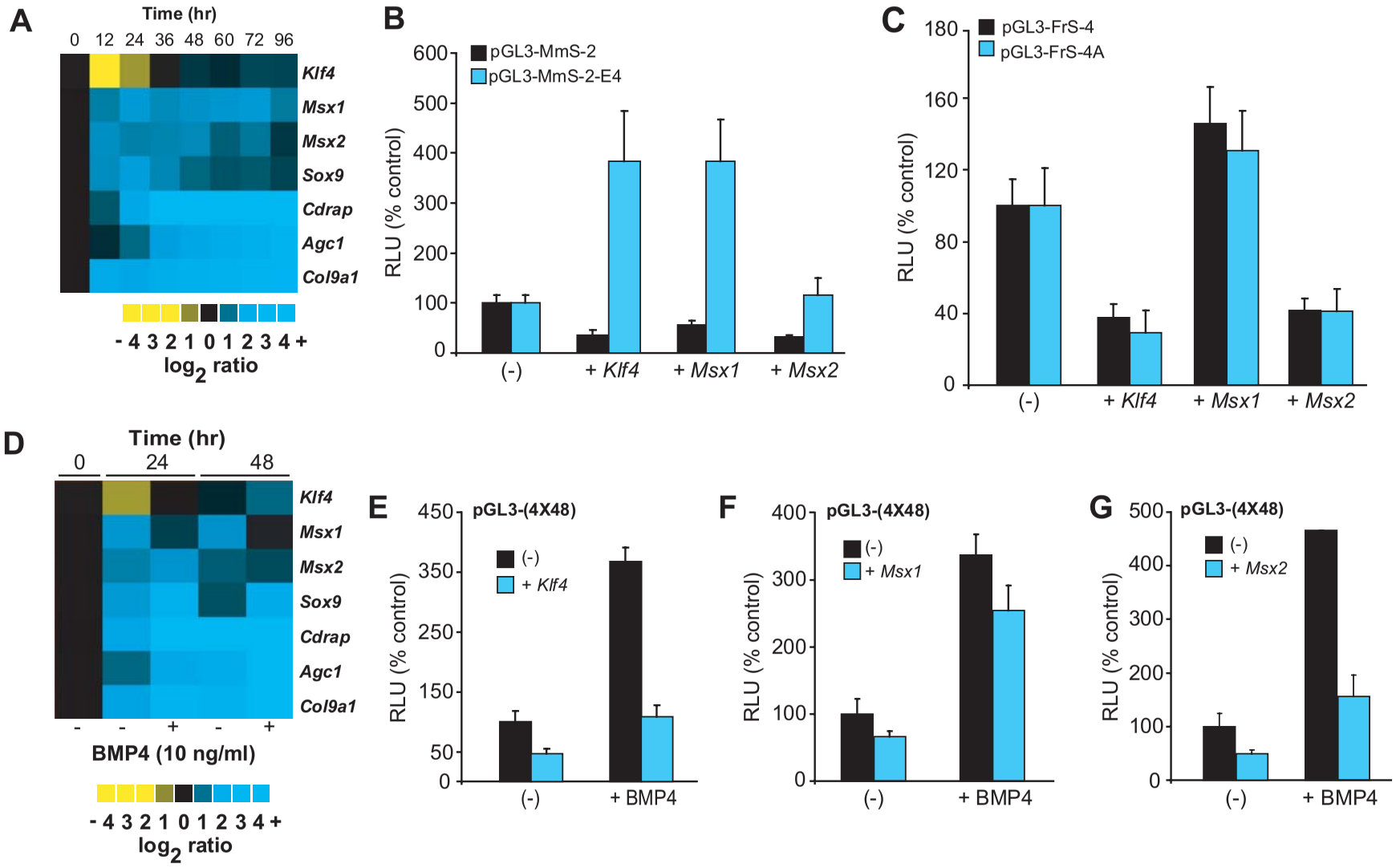
A
Time (hr)
0 12 24 36 48 60 72 96
Klf4
Msx1
Msx2
Sox9
Cdrap
Agc1
Col9a1
- 4 3 2 1 0 1 2 3 4 +
log2 ratio
B
pGL3-MmS-2
pGL3-MmS-2-E4
RLU (% control)
600
500
400
300
200
100
0
(-)
+ Klf4
+ Msx1
+ Msx2
C
pGL3-FrS-4
pGL3-FrS-4A
RLU (% control)
180
160
120
80
40
0
(-)
+ Klf4
+ Msx1
+ Msx2
D
Time (hr)
0
24
48
Klf4
Msx1
Msx2
Sox9
Cdrap
Agc1
Col9a1
- - + - +
BMP4 (10 ng/ml)
- 4 3 2 1 0 1 2 3 4 +
log2 ratio
E
pGL3-(4X48)
(-)
+ Klf4
RLU (% control)
450
350
250
150
50
0
(-)
+ BMP4
F
pGL3-(4X48)
(-)
+ Msx1
RLU (% control)
400
300
200
100
0
(-)
+ BMP4
G
pGL3-(4X48)
(-)
+ Msx2
RLU (% control)
500
400
300
200
100
0
(-)
+ BMP4

Table 4.1. PCR primers for *Sox9* genomic DNA (GenBank #AC040983)

Fragment	Position on mouse *Sox9*	Primers
2 kb	-1852/+107	sense: 5'-CCAGAACATAGAAGTGCGTC-3' antisense: 5'-CTTGCACCTCGTCTCTCTTG-3'
3 kb	-2952/+107	sense: 5'-GGATCCTCTAGTGCCTCTGTGTTGG-3' antisense: 5'-CTTGCACCTCGTCTCTCTTG-3'
5 kb	-2952/+1750	sense: 5'-GGATCCTCTAGTGCCTCTGTGTTGG-3' antisense: 5'-AGAAGAGATGTGAGTCTGTTCCGTG-3'

Table 4.2. PCR primers for Sox9 fragments containing elements E1-E5

GenBank Accession #	Size (bp)	Primers
AC040983	258	sense: 5'-TAACGCGTGTCTACCTGTCTGTCCACCT-3' antisense: 5'-TACGCGTGAATTCGGTAATTCCTTCTTGGCTGC-3'
AC053470	341	sense: 5'-TAGAATTCATGTCTCTGGAGCTCACTGG-3' antisense: 5'-TAGAATTCCCTTCTCCTACCTCTTCTCT-3'
AC005144	424	sense: 5'-TAGCTGCAGTAGCCACACTGCATTGTCTC-3' antisense: 5'-TAGCTGCAGCTCTCTGGTCAAGCACTCAT-3'
AC005144	411	sense: 5'-TAGACTAGTACTCCACACCATGCCTATGA-3' antisense: 5'-TAGACTAGTCTGCAGCCATTGAGCACTGACCTTC-3'
AC005144	546	sense: 5'-TAGCTCGAGAACATCCAGGAGAGGTGCTT-3' antisense: 5'-TAGCTCGAGACTAGTGCATGTACACACACACCT-3'

Table 4.3. PCR primers for *Sox9* genomic DNA (GenBank #AF329945)

Fragment	Position on *Fugu rubripes Sox9*	Primers
4 kb	-3722/+211	sense: 5'-TGCGCAGGTTAAGGTGGAGG-3' antisense: 5'-GATGGAGAGTCTGCAGGAGC-3'

Table 4.4. PCR primers for *Sox9* genomic DNA (GenBank #AF329945)

Fragment (~ 5 kb)	Primers
A	sense: 5'-ATAAGCTTATCTAGAGTCATGTCTACATACGGAGG-3' antisense: 5'-TAAAGCTTTTCTCGTGGCTTCTGTGTGG-3'
B	sense: 5'-ATTCTAGAAGGCGCGCCCCACTTGGCACGTCTCAGGA-3' antisense: 5'- TATCTAGATCGCAGCAGCTCAGCACAAT-3'
C	sense: 5'-ATGGCGCGCCACCTGCAGAACATGTGTTGG-3' antisense: 5'- TAGGCGCGCCCCAACAGTTGGCGGTCAGAT-3'

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CHAPTER V: Discussion

In recent years, specific factors have been identified and shown to mediate the initiation of limb bud formation and the subsequent development and patterning of the vertebrate limb (Ahn et al., 2001; Barrow et al., 2003). Extensive analysis of these molecules has revealed complex interactions between signaling pathways operated by secreted factors of a number of families, including members of the WNT and BMP families (Ahn et al., 2001; Kawakami et al., 1999). These signaling molecules may impact a specific transcription factor or a network of transcription factors to regulate the chondrogenic program. *Sox9* is one example of a transcription factor that has essential, non-redundant roles in specifying the commitment and differentiation of mesenchymal cells to the chondrocytic lineage in developing skeletal elements (Akiyama et al., 2002; Bi et al., 1999). Hence, identification of factors that function both distantly and upstream of *Sox9*, along with other transcription factors, would be useful in delineating the molecular mechanisms that regulate the chondrogenic program.

5.1 Identification of elusive regulatory elements that drive *Sox9* expression in cartilage

We have shown that the first 2 kb of the mouse *Sox9* promoter drives the highest reporter expression as compared to other deletion constructs in primary mesenchymal cultures. Further characterization of this fragment and nested deletions thereof, in conjunction with electrophoretic mobility shift analysis (EMSA), has revealed that *Sox9* transcription requires a CCAAT box that binds a CCAAT binding factor (NFY) (Figure 5.1). Interestingly, CCAAT boxes have been identified in a number of genes and reports have found that the NFY complex may play a direct role in tissue-specific gene expression (Gilthorpe et al., 2002). Moreover, comparative analysis between human, mouse and *Fugu* promoter sequences (Bagheri-Fam et al., 2006; Bagheri-Fam et al., 2001) has revealed five conserved elements (E1-E5) within the *Sox9* promoter (Figure 5.1). Interestingly, the majority of CD translocation breakpoints separate E3-E5 from *Sox9* and transgenic experiments have indicated that skeletal-specific elements may lie within this region (Bagheri-Fam et al., 2001). Proximal promoter constructs containing elements E1-E5 suggests

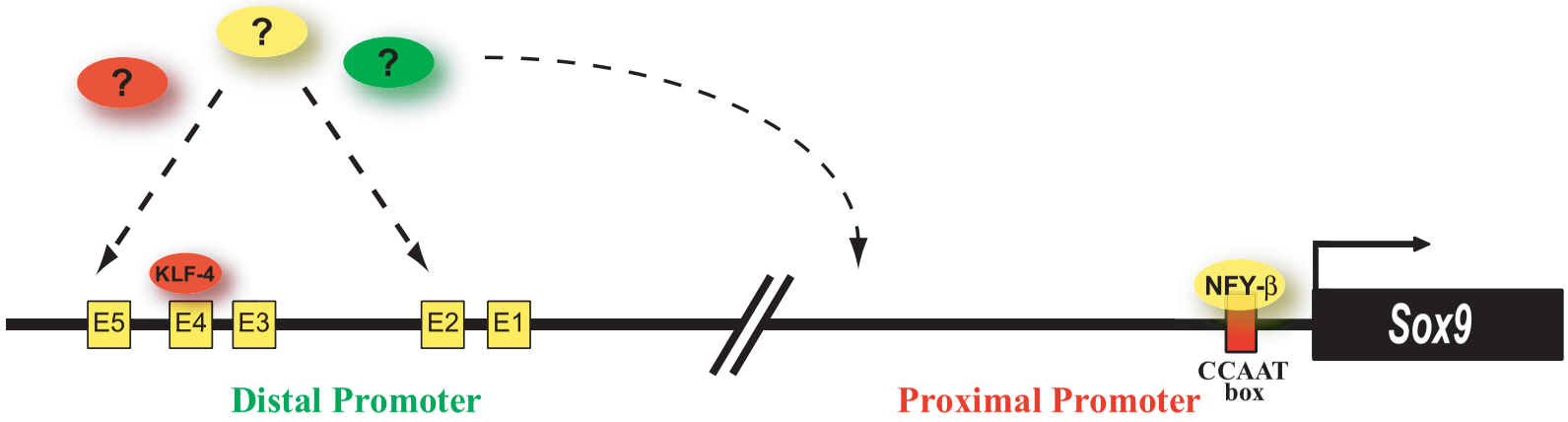


Figure 5.1: The proximal promoter along with tissue-specific distal regulatory elements modulate *Sox9* expression. Deletion constructs of the mouse *Sox9* promoter revealed a proximal promoter spanning - 2 kb from the transcriptional start site, while mobility shift assays demonstrated that a CCAAT motif was involved in the transactivation of *Sox9*. In *Fugu rubripes*, deletion constructs of the *Sox9* promoter revealed a proximal promoter spanning - 4 kb from the transcriptional start site, and potential regulatory regions spanning the remainder of the promoter. Moreover, comparison of the mammalian *Sox9* upstream intergenic sequence to that of *Fugu* has further identified 5 short highly conserved sequences that are contained within 18 kb of upstream *Fugu* sequence (Bagheri-Fam et al., 2006; Bagheri-Fam et al., 2001). Analysis of the transcriptional activity of these conserved sequences in primary mesenchymal cells has led to the identification of positively and negatively-acting regulatory regions, such that transient transfections revealed potential enhancer and repressor elements within the distal *Sox9* promoter of both mouse and *Fugu*. More specifically, the E4 element was analyzed using both rVISTA and the TRANSFAC database and transcription factor binding sites for KLF-4 and MSX-1 were identified.

that there may be enhancer regulatory elements in E2 of the *Sox9* promoter, and repressor elements in E4. In particular, E4 exhibited highly repressive luciferase activity of the mouse 2 kb *Sox9* proximal promoter and contains putative binding sites for the transcription factors KLF-4 and MSX-1. It will also be important to use comparative analysis and sequence alignments between the mouse, human and *Fugu Sox9* promoters to identify transcription factors in addition to KLF-4, MSX-1 and TCF that may regulate *Sox9*.

Previous studies in our lab have shown that there are number of potential SOX binding sites within the 2 kb *Sox9* proximal promoter (TRANFAC database). Surprisingly, SOX9 decreases transcriptional activity of the 2 kb proximal promoter when transiently transfected into primary mesenchymal cultures (data not shown). These results suggest that *Sox9* may be acting to repress its own transcription either by directly binding to the promoter or by acting in an indirect fashion. Other transcription factors including *Pax1*, *Pax9*, *Nkx3.1*, *Nkx3.2* and *Barx2* have also been shown to control the level of *Sox9* (Lefebvre and Smits, 2005). Further studies are required to determine the mechanism by which *Sox9*, along with other transcription factors, regulate *Sox9* transcription.

Previous studies have assayed the regulatory potential of elements E1 to E7 in transgenic mice (Bagheri-Fam et al., 2006) however no skeletal expression was detected with any of the elements tested (Bagheri-Fam et al., 2006). Therefore, regulatory elements controlling *Sox9* expression in cartilage have not yet been identified. Our 2 kb proximal promoter fragment appears to have more promise of driving cartilage specific expression. This fragment gave the highest reporter activity in our cultures of limb bud mesenchyme. As we have shown before these cells contain a large proportion of skeletal progenitor cells.

If skeletal expression is not detected from transgenics harbouring our 2 kb promoter fragment, a more extensive multi-species alignment should also be carried out on the *Sox9* promoter and newly identified elements should be incorporated into these analyses. Nonetheless,

it important to recall that single elements may not work as well as combinations of elements. The context of the elements within these large intergenic regions may also be important for appropriate expression of *Sox9*. It is also important to investigate 3' control of *Sox9* since cis-regulatory elements have been located in sequences located downstream of the *Sox9* promoter. Sequence comparison between the human and *Fugu* genomes have already identified short conserved elements, E6-E8, located 3' to *Sox9* (Bagheri-Fam et al., 2006; Bagheri-Fam et al., 2001) that could be more carefully investigated.

5.2 Signaling molecules that regulate *Sox9* expression

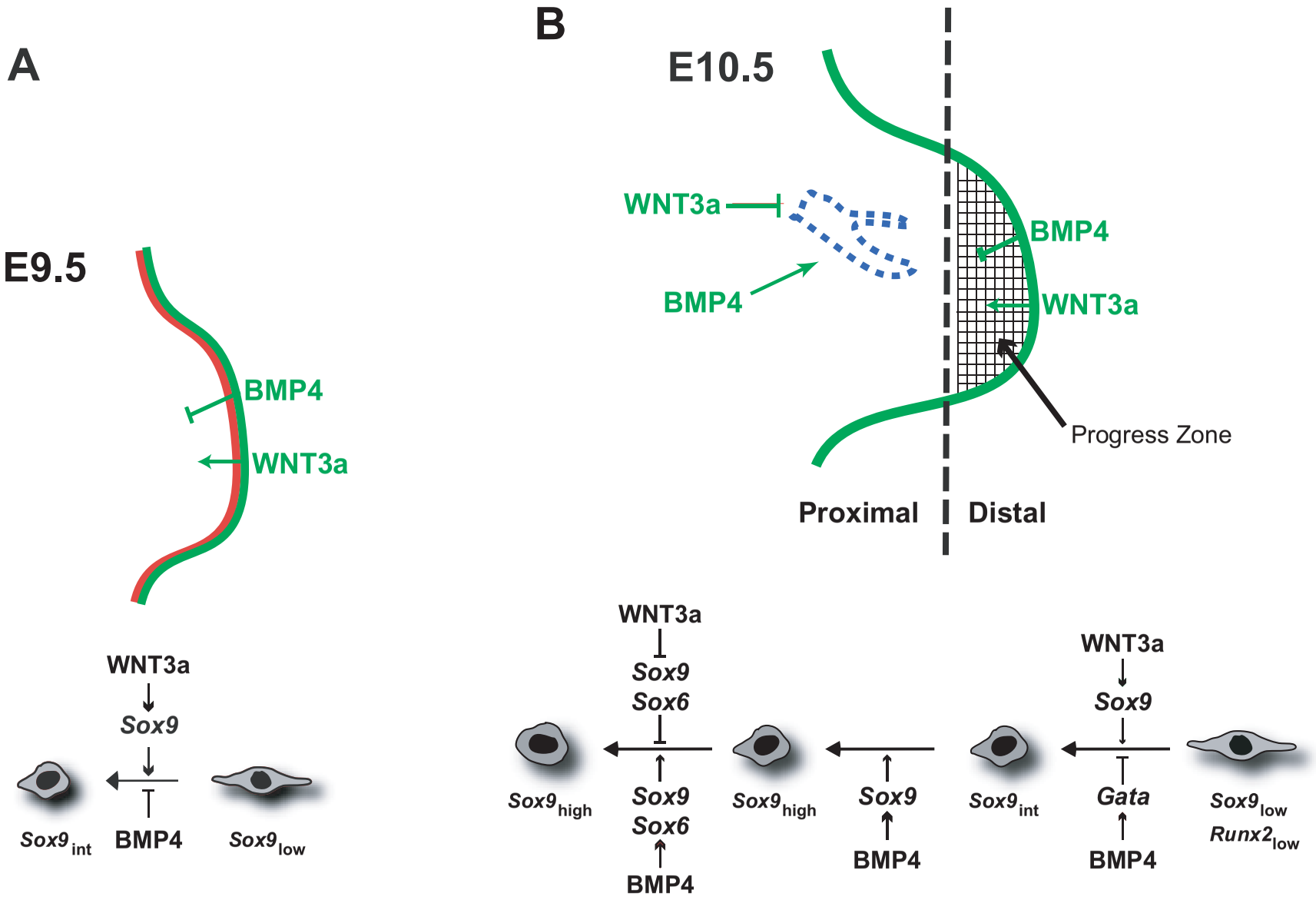
Sox9 expression and/or activity may be a target of signaling molecules that are known to influence the chondrogenic program. In fact, it has been proposed that *Sox9* is regulated by the hedgehog (Hh) family of intercellular ligands. Specifically, sonic hedgehog (SHH) has been shown to transiently activate *Sox9* expression in chick pre-somitic tissue and this activation is more pronounced in the presence of BMP (Chimal-Monroy et al., 2003; Zeng et al., 2002). Several studies indicate that BMP signaling regulates *Sox9* expression (Semba et al., 2000; Zehentner et al., 1999). For example, inactivation of both *Bmpr1a* and *Bmpr1b* in mice results in a lack of *Sox9* expression (Yoon et al., 2005). Other studies have also demonstrated that *Sox9* expression is induced soon after TGF-β-bead implantation in the developing chick limb bud interdigital region (Chimal-Monroy et al., 2003). These studies suggest that TGF-β and BMP activity may regulate *Sox9* expression during chondrogenesis and downstream effectors of these pathways may control chondrogenic-specific enhancers of the *Sox9* gene. Our studies have also shown that BMP addition to primary mesenchymal cultures modulates *Sox9* expression.

We have also shown that WNT3a modulates *Sox9* expression in primary mesenchymal cultures. Interestingly, highly conserved Tcf/Lef1 binding sites within the E3 enhancer element of the *Sox9* promoter have been identified; therefore, direct regulation of *Sox9* by WNTs is possible (Bagheri-Fam et al., 2006). There is evidence from our own data and that of others that

Wnts are involved in one of the primary functions of *Sox9*, the determination of cartilage. When *β-catenin*, a required effector of canonical Wnt signaling, is inactivated in mesenchymal progenitor cells, they form chondrocytes but not osteoblasts (Day et al., 2005). Thus, in normal development, β-catenin may not be required to initiate the chondrogenic program. This contrasts with our experiments in which exogenous WNT3a does stimulate the earliest phase of chondrogenesis in E9.5 limb buds (Figure 5.2). Thus, we have shown for the first time that WNTs are sufficient to activate chondrogenesis. As the limb develops (E10.5, E11.5), WNT3a continues to promote chondrogenesis in those cells derived from regions directly beneath the AER but negatively regulates chondrogenesis in cultures derived from more proximal regions of developing limb. These effects of WNT3a suggest that the *Sox9* promoter may have several WNT responsive elements that could act in repressor or activator roles depending on their context. As I pointed out earlier, the transgenic mice made with the E3 enhancer containing the TCF binding sites (Bagheri-Fam et al., 2006) did not have expression in cartilage. However, these experiments do not rule out the possibility that E3 could function in combination with other enhancer elements to direct *Sox9* expression in chondrocytes. It is also possible that the E3 enhancer operates within a select window of the chondrogenic program (perhaps early and not late) and this would have been missed in the aforementioned study. Further, the studies described herein have revealed stage-specific activities of canonical WNTs in regulating chondrogenesis, and in this regard it might be useful to determine if these elements also function in a stage-dependent manner. Identification of other conserved Tcf/Lef1 binding sites within the *Sox9* promoter would also help determine whether WNT signaling is in fact directly regulating *Sox9* expression.

Further analysis is important for determining whether BMPs and WNTs influence the same, distinct or multiple regulatory regions within the *Sox9* promoter. It is expected that some of the factors that enhance SOX9 reporter gene activity in our studies will also positively

Figure 5.2. Schematic representation of limb development in the mouse. (A) We have shown that WNT3a exhibits pro-chondrogenic effects including increased cartilage formation and increased *Sox9* expression early in limb development (E9.5). Conversely, BMP4 appears to inhibit the chondrogenic program in the early limb mesenchyme. (B) As the limb develops (E10.5), WNT3a continues to promote chondrogenesis in those cells derived from regions directly beneath the AER (progress zone), but negatively regulates chondrogenesis in cultures derived from more proximal regions of the developing limb (decreased *Sox6* and *Sox9* expression). Conversely, our studies have shown that BMP4 inhibits chondrogenesis in the early limb mesenchyme which appears to be mediated through the expression of *Gata* transcription factors, but exhibits potent pro-chondrogenic activity at later stages of development accompanied with increased *Sox6* and *Sox9* expression.


A
E9.5
BMP4
WNT3a
WNT3a
Sox9
Sox9 int
BMP4
Sox9 low
B
E10.5
WNT3a
BMP4
BMP4
WNT3a
Progress Zone
Proximal
Distal
WNT3a
Sox9
Sox6
Sox9 high
Sox9
Sox6
BMP4
Sox9 high
Sox9
BMP4
Sox9 int
WNT3a
Sox9
Gata
BMP4
Sox9 low
Runx2 low

regulate *Sox9* transcription. More importantly, do these factors operate through a similar network to affect the same regulatory element(s) in the *Sox9* promoter, or do they operate through distinct networks to affect separate sites within the promoter at specific stages of development?

5.3 Downstream targets of WNTs, in addition to *Sox9*

Classical studies carried out in the chick embryo over two decades ago established an important role for the ectoderm in promoting cartilage formation in the early underlying mesenchyme. Interestingly, at slightly later stages the ectoderm inhibited cartilage formation (Gumpel-Pinot, 1980; Solursh and Reiter, 1988). Shortly thereafter, additional studies revealed that a "diffusible factor" appeared to be released from the ectoderm that stimulated chondrogenesis in a stage-dependent manner (Solursh and Reiter, 1988). However, the nature of this factor has remained elusive. We have shown for the first time that canonical WNTs exhibit the properties consistent with this "factor". The canonical WNT, WNT3a, promotes chondrogenesis in more immature chondrogenic cells, while inhibiting chondroblast differentiation in more advanced chondrogenic cells. Further, several *Wnt*s have been reported to be expressed in the early limb ectoderm and activation of the canonical pathway has been detected in the subjacent mesenchyme.

Consistent with the chondrogenic modulatory activity of WNTs, numerous genes implicated in skeletal development are impacted by WNT signaling according to our microarray analysis, including: *Alx3*, *Pax1*, *Pax9*, *Meox2*, *Foxc1*, *Foxc2*, *Runx2* and *Dlx2*. *Pax1* was previously shown to be down-regulated following attenuation of β-catenin signaling (Hill et al., 2006). Consistent with this, WNT3a increases the expression of *Pax1*, in addition to *Pax9* (Figure 5.2, Figure 5.3). Furthermore, *Pax1* and *9* are expressed in limb precartilaginous condensations, and are subsequently down-regulated during chondroblast differentiation (LeClair et al., 1999). *Pax1* and *9* are differentially induced and/or repressed in the various E10.5

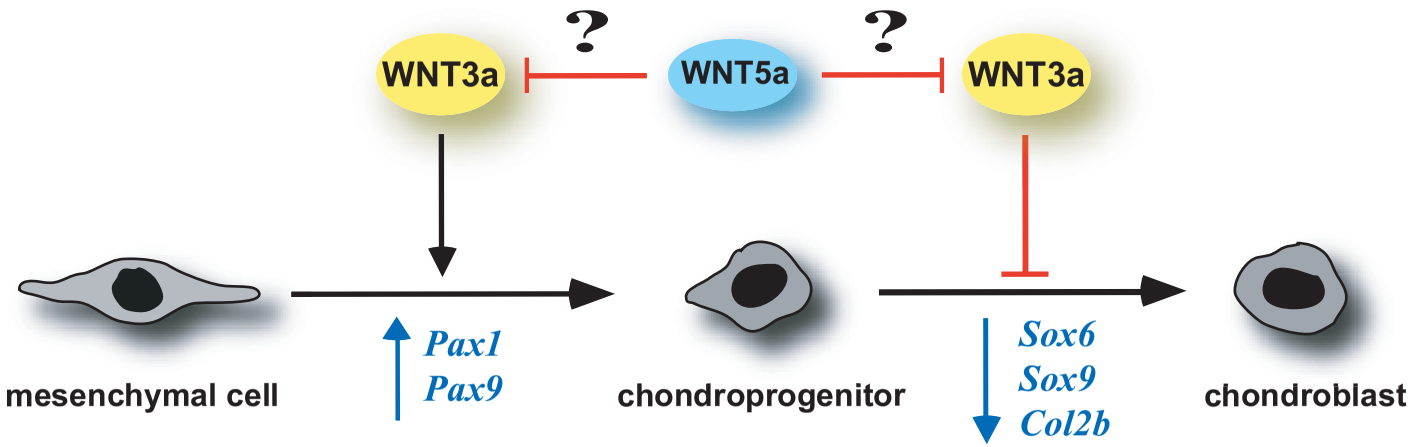


Figure 5.3: WNT signaling exhibits stage-dependent actions in chondrogenesis. Specifically, the canonical WNT, WNT3a, promotes commitment of mesenchymal cells to the chondrocytic lineage and increases the appearance of precartilaginous condensations but inhibits chondroblast differentiation. Furthermore, genes involved in the formation of precartilaginous condensations (*Pax1*, *Pax9*) are significantly up-regulated upon treatment of early (distal) limb mesenchymal cultures with WNT3a. However, WNT3a inhibits chondroblast differentiation in those cultures derived at later stages of limb development (proximal limb mesenchymal cultures). In these cultures, WNT3a dramatically decreases the expression of genes essential to the chondrogenic program, including *Sox6*, *Sox9* and *Col2b*. Moreover, the non-canonical, WNT5a, which is abundantly expressed in the distal underlying mesenchyme, may inhibit canonical WNT signaling, thereby providing conditions conducive to chondrogenesis.

mesenchymal populations, in response to WNT3a addition, with the DM cultures exhibiting the greatest increase in *Pax9* expression, in some instances > 100 fold in 24 hrs. *Pax9* knockouts present with limb defects, characterized by a supernumary pre-axial digit (Peters et al., 1998), whereas *Pax1* null mutants exhibit scapular abnormalities (Wilm et al., 1998). The capacity of WNT3a to stimulate the expression of these pre-chondrogenic markers is consistent with their proposed action in promoting precartilaginous condensation.

5.4 SOX9 targets

Previous studies have shown that SOX9 binds to and activates chondrocyte-specific enhancer elements in *Col2a1* (Lefebvre et al., 1997). Specifically, SOX9 binds to a site in the *Col2a1* enhancer which is essential for enhancer activity in chondrocytes. This site is highly homologous to the consensus binding site for other SOX and HMG proteins (Lefebvre et al., 1997). Functional SOX binding sites are also found in other cartilage-specific cis-acting elements, including *CD-RAP* (Xie et al., 1999), *Col11a2* (Bridgewater et al., 1998), *Agc1* (Sekiya et al., 2000) and *Cartm* (Rentsendorj et al., 2005). These studies demonstrated the ability of SOX9 to bind to and activate regulatory elements within these genes which potentially involves the cooperation of L-SOX5, SOX6 and/or other co-factors yet to be discovered.

The canonical WNT pathway mediated by β-catenin/TCF complexes may be a downstream target of SOX9 (Huelsken and Birchmeier, 2001; Moon et al., 2002) . Studies have shown that SOX9 and Tcf/Lef1 compete for overlapping sites within β-catenin (Akiyama et al., 2004) to regulate its expression. Other evidence that *Wnt*s are targets of SOX9 is that *Wnt* pathway genes are expressed in some of the same cell types as *Sox9*. For example, β-catenin is highly expressed in mesenchymal cells committed to the chondrocytic lineage (Ryu et al., 2002).

5.5 WNT and BMP interactions

Based on microarray data from our lab, treatment of primary mesenchymal limb cultures with WNT3a results in little or no change in *Noggin* or *Bmp* expression. In addition, treatment

of primary cultures with BMP4 does not affect the expression of *Wnts* or negative regulators of the WNT signaling pathway, including *Nkd2* and *Axin2*, whose expression is reflective of activation of the canonical WNT signaling pathway. Our experimental design removes the epithelium so we would not have detected *Wnt3a* or *Wnt7a* expression on our chips. Nonetheless, this suggests that at least in our experimental model and these early stages of limb development the two pathways appear to be operating independently with little or no direct crosstalk.

There is still the possibility that the two signals we have studied act sequentially to initiate chondrogenesis. Our studies have shown that BMP4 inhibits chondrogenesis in the early limb mesenchyme which may be mediated through the expression of *Gata* transcription factors, but exhibits potent pro-chondrogenic activity at slightly later stages of development (Figure 5.2). Thus, BMP4 appears to promote chondrogenesis following the specification of mesenchymal cells to the chondrocytic lineage by WNT3a signaling. The idea that the Wnt/β-catenin signaling lies upstream of the BMP signaling pathway is also supported, in that, mutants lacking *Wnt/β-catenin* signaling in the limb ectoderm lack expression of ventrally expressed *Bmp* ligands (Barrow et al., 2003).

5.6 Interactions between BMPs and SHH in chondrogenesis

In the axial skeleton, it has been shown that SHH induces early sclerotomal markers and is sufficient to elicit chondrogenesis (Fan and Tessier-Lavigne, 1994; Munsterberg et al., 1995). Chondrogenesis induced by SHH requires active BMP signaling, as also observed in the mesoderm, suggesting that there is a common program for chondrocyte differentiation in both axial and appendicular cartilage. In the axial skeleton, BMPs only promote chondrogenesis of somitic mesoderm after exposure to SHH, suggesting the role of SHH in patterning the somite is to induce responsiveness to BMP signals. We show that cultures derived from the developing

appendicular skeleton respond similarly, in that, those mesenchymal cultures derived from the early limb mesenchyme are not responsive to BMP.

5.7 Downstream targets of BMPs mediate temporal specificity

Interestingly, pro-chondrogenic responses to BMP4 in early mesenchymal cultures (ie. E10.5 HL-DM) can be acquired by simple short-term culture indicating that these programs are either established in the limb and their manifestation is time-dependent or the absence of ectodermal-derived signals promotes this transition. The fact that distal mesenchymal cultures from the forelimb and hindlimb exhibit differing responses to BMPs suggests that competency is not entirely dependent on the ectoderm, but that an intrinsic timing mechanism may be operating that governs BMP competency.

Gata transcription factors could potentially play a role in regulating chondrogenic competency within the limb mesenchyme. Specifically, the observed disparate activities seem to be correlated with *Gata* expression. When BMP4 strongly induces *Gata* expression, chondrogenesis is severely inhibited and this is accompanied by decreased expression and activity of *Sox9* (Figure 5.2, Figure 5.4). However, in cultures derived at later stages (from proximal regions), *Gata* expression exhibits little change in response to BMP4 addition, further emphasizing that the induction of *Gata* gene expression by BMP4 is characteristic of the early limb mesenchyme. As determined by quantitative real-time PCR, multiple *Gatas*, particularly *Gata5*, are expressed in the early limb mesenchyme and their expression quickly decreases as development ensues. *Gata6* has been reported within sites of chondrogenesis in the sclerotome (Brewer et al., 2002) and more recent studies have demonstrated that *Gata6* is expressed in cartilaginous condensations of the developing limb of E12.5 mice (Alexandrovich et al., 2007); however the expression of *Gata*s early in limb development has not been addressed. Hence, *in situ* hybridization of *Gata*s in the early limb (E9.5 - E12.5) should be performed to the clearly define the spatiotemporal expression pattern of these factors early in the developing limb.

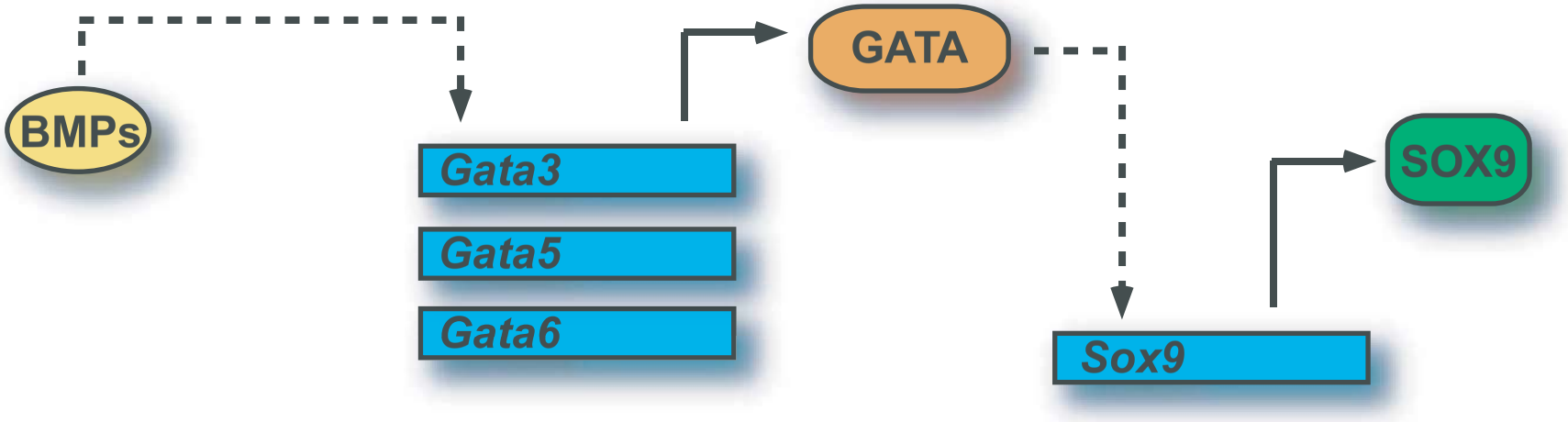


Figure 5.4. Manipulation of *Gata* factors affects SOX9 activity. *Gatas* can act as either transcriptional activators or repressors which is dictated by associated factors and the promoter context. We have observed dose-dependent effects of *Gatas*, with higher expression being associated with a reduction in SOX9 activity. This is consistent with the activity of BMPs, as under conditions where *Gatas* are highly induced, chondrogenesis is inhibited.

Moreover, null mutants have been generated for different *Gata*s; however, chondrogenic phenotypes are difficult to assess due to early embryonic lethality of these animals (Koutsourakis et al., 1999). Also since, at least, 4 *Gata*s are expressed in the early limb and these factors do exhibit functional redundancy it may be necessary to eliminate more than one *Gata* to have a functional consequence. Conditional *Gata* knock-outs in the limb would be beneficial in establishing the functional requirement of *Gata*s in chondrogenesis *in vivo*. Since *Gata5* exhibits the most robust and dynamic expression early, mice conditionally null for *Gata5* in the limb mesenchyme, crossed with heterozygous Prx1-Cre mice [Cre is directed to the limb mesenchyme (Logan et al., 2002)] may help elucidate the specific role of *Gata5* in limb development. Furthermore, a number of studies have examined the expression of a number of genes upon inactivation of *Bmps* or their receptors (*Bmpr1a*); however, the expression of *Gata*s has not been addressed (Ovchinnikov et al., 2006). It would be beneficial to examine *Gata* expression in these knockouts as well as in knockouts of specific BMP antagonists such as *Noggin*. Of course, *Gatas* are likely only part of the story, as the microarray data has revealed changes in the expression of a number of genes, it will be important to systematically evaluate these and in this regard, the SOX9 reporter gene assays would be a good place to initially evaluate their SOX9-modulatory activity.

5.8 Future experiments

The ability to generate transgenic and knockout mice has greatly facilitated the analysis and physiological functions of various components of the BMP and WNT signaling pathways; however, early embryonic lethality sometimes precludes analyses of a role of specific molecules at later stages of development. Our results have demonstrated that many molecules involved in BMP and WNT signaling (especially β-catenin) operate in a transient stage- and context-dependent fashion which compromises *in vivo* analysis of these molecules. Transient pharmacological administration of BMP or WNT inhibitors/activators *in vivo* and/or the use of

more sophisticated knockout or knockin models with temporal control of gene expression (inducible CRE, or tet-inducible system, etc.)), seems to be the only alternative for precisely defining the biological function of these molecules *in vivo*.

To more accurately distinguish prechondrogenic cells we can use transgenic embryos that contain a 6.3-kb portion of the *Co2a1* promoter driving expression of EGFP (Grant et al., 2000). Control cultures exhibit weak transgene expression within 24 h, and by 96 h there is an abundance of EGFP-positive cartilage nodules in micromass cultures (Hoffman et al., 2006). Hence, the ability of WNT3a to modulate this transgene expression, or cartilage nodule formation, will be investigated early in limb development (E10.5). Moreover, we have previously shown that the implantation of BMP4-soaked beads into limb organ cultures stimulates ectopic Col2-EGFP expression (Hoffman et al., 2006). Implantation of WNT3a-soaked beads into limb organ cultures of *Col2*-EGFP mice (E10.5) will also be performed. Additional experiments will be carried out *in ovo*, in chick limbs, in the presence and absence of the ectoderm (source of WNT3) to further investigate the *in vivo* role of canonical WNT signaling early in limb development. If canonical WNT signals are important in early chondrogenesis, then it is expected that cartilage formation will be significantly enhanced in cartilage-deficient early primary limb mesenchymal cultures and ectoderm-less limbs.

Herein we have identified a potentially interesting and novel role for GATA factors in the early limb. However, at present very little is known about these factors may be functioning to regulate skeletogenesis, an important first step in this investigation would be to identify the downstream targets of GATA factors in the early limb. The advent of chromatin immunoprecipitation (ChIP) on chip (ChIP-on-chip) methodologies has greatly accelerated the identification of potential binding sites occupied by transcription factors within the genome. ChIP-on-chip is a powerful technique that combines chromatin immunoprecipitation (ChIP) with microarray technology (chip) and is used to investigate the interactions between proteins and

DNA *in vivo*. Briefly, protein-DNA complexes are cross-linked, immuno-precipitated, purified, amplified, labeled and then allowed to hybridize to high resolution arrays. ChIP on chip data could be combined with our microarray data to identify those GATA target genes that change in response to BMP treatment or changes in *Gata* expression. Furthermore, nuclear extracts prepared from primary mesenchymal cells or whole limbs could be used in EMSAs to confirm GATA binding to identified sequences. Together, these studies will lead to the identification of direct downstream target genes of the GATA factors and provide critical insights into GATA function in early limb development.

Expression profiling of microarray data has yielded a large number of potentially interesting genes. There are a number of genes which change more than 10 fold upon treatment with WNT3a or BMP4. Even more surprisingly, quantitative real-time PCR reveals that a number of genes change significantly within 3 hours of plating and treatment of primary mesenchymal limb cultures. Analysis of these genes within a specific time frame will allow us to specifically identify direct targets of WNT3a or BMP4. Another important component of this assay is that the functions of combinations of genes can be easily assessed by using a number of reporter genes, including the SOX9-reporter gene and previously described *Sox9* promoter constructs (both mouse and *Fugu*). This aspect is critical if the networks underlying the chondrogenic program are to be determined. Hypothetically, if gene X lies upstream of gene Y in a regulatory network, we would test this possibility by examining the ability of gene Y to rescue inhibition of gene X, and for the activity of gene X to be inhibited by interfering with gene Y. Furthermore, phenotypic profiling and microarray data will be imported into Spotfire to identify genes that cluster together and likely form networks or pathways.

Another approach to investigate epistatic relationships of genes involved in the chondrogenic program will be to combine siRNA knockdown of specific genes with quantitative real-time PCR, to investigate how specific genes of interest respond to the silencing of a specific

gene of interest. We have successfully used these strategies to investigate the relationship of the BMP pathway to other signaling pathways in chondrogenesis (Hoffman et al., 2006). Genes identified will be tested for functional relevancy throughout the chondrogenic program in primary mesenchymal limb cultures. Loss and gain-of-function approaches including siRNA knock-down and over-expression will be used to manipulate the activity of identified genes and their effects on different reporter genes will be evaluated. Luciferase-based reporters, fluorescent-based reporters and a number of other techniques will be used to study the effects of newly identified genes on a number of cellular processes including cell proliferation, motility, differentiation and morphology.

In the lab, we have also developed a novel high-throughput functional assay that relies on transient co-transfection of primary mesenchymal cultures with expression plasmid(s) and reporter genes allowing us to perform hundreds (400-800) of transfections per week. These types of assays allow us to investigate the functions of combinations of genes which is critical in delineating the molecular networks underlying the chondrogenic program. Those genes confirmed to exhibit differential expression and which previous chondrogenic roles have not been defined will be further analyzed using *in situ* hybridization to define their spatiotemporal expression pattern in the developing limb. Ultimately we hope to identify the exact mechanism(s) underlying the regulation of genes modulated by BMP and WNT signaling pathways.

5.9 Health impact of the research

Great progress has been made in recent years in identifying specific molecules and transcription factors which are critical to the chondrogenic program. The large scale analysis of gene expression and function described herein will undoubtedly lead to the identification of new targets, which will enable development of regenerative strategies to enhance cartilage repair for the treatment of skeletal trauma and musculoskeletal diseases such as osteoarthritis.

5.10 References

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