ROLE OF HEDGEHOG SIGNALING AND BONE MORPHOGENETIC PROTEINS DURING INTERVERTEBRAL DISC AND LIMB DEVELOPMENT

By

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To my wife and family

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LIST OF ABBREVIATIONS

AER	apical ectodermal ridge
Bmp	bone morphogenetic protein
Bmpr	bone morphogenetic protein receptor
BrdU	5-bromo-2'-deoxyuridine
DNA	deoxyribonucleic acid
E	embryonic day
En1	engrailed1
Fgf	fibroblast growth factor
GFP	green fluorescent protein
Ihh	Indian hedgehog
IP	intraperitoneal
P	postnatal day
Ptch1	patched1
RNA	ribonucleic acid
Shh	sonic hedgehog
Smo	smoothened
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
ZPA	zone of polarizing activity

Abstract of Dissertation Presented to the Graduate School
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Degeneration of the intervertebral discs, in particular the nucleus pulposus in the center of the disc, occurs as a person ages and can lead to intense pain. A classically identified “notochordal” cell population in the nucleus pulposus is thought to regulate disc homeostasis. In this study, we provide the first direct evidence that all cell types in the adult mouse nucleus pulposus are derived from the embryonic notochord. Additionally, rare isolated embryonic notochord cells were found to remain in the vertebral column and resembled notochordal remnants. The development and characterization of a mouse model that can be used to fate map nucleus pulposus precursor cells in any mutant background will be useful for uncovering the cellular and molecular mechanisms of disc degeneration.

The vertebrae notochord is a transient rod-like structure that produces secreted factors that are responsible for patterning surrounding tissues. Here we demonstrate that hedgehog signaling is required for formation of the intervertebral discs. Removal of hedgehog signaling in the notochord resulted in the formation of an aberrant notochord sheath. In the absence of the notochord sheath, small nuclei pulposi were formed with most notochord cells dispersed throughout the vertebral bodies. Our data suggests that

the formation of the notochord sheath requires hedgehog signaling and the notochord sheath functions as a “wrapper” around the notochord to constrain these cells along the vertebral column.

In addition to our studies investigating the role of hedgehog signaling, we investigated the role of bone morphogenetic proteins 2 and 4 (BMP2 and BMP4) in the apical ectodermal ridge (AER). To investigate the role BMP ligands expressed in the AER play in limb development we selectively inactivated both *Bmp2* and *Bmp4* in this tissue. The autopods of mice lacking both of these genes contained extra digits, digit bifurcations and interdigital webbing due to a decrease in programmed cell death and an increase in cell proliferation in the underlying mesoderm. However, removal of both *Bmp2* and *Bmp4* in this tissue did not result in defects in proximal-distal patterning. Our data suggests that AER expression of *Bmp2* and *Bmp4* are required for digit patterning but not for limb outgrowth.

CHAPTER 1
INTERVERTEBRAL DISC DEVELOPMENT

Low Back Pain

Low back pain will affect most people over the age of 65 in industrialized countries. In the U.S., treatment of low back pain is estimated to cost 50-100 billion dollars per year (reviewed in (Katz, 2006)). For the majority of people, bed rest will relieve most symptoms of back pain but in a small population of patients the condition will become chronic. Both the severity and incidence of back pain increase as people age. Most back pain is thought to originate from the degeneration of the intervertebral discs or through physical damage to the disc. This leads either to herniation of the middle part of the disc called the nucleus pulposus into the vertebral column and/or tears, bulging, and rupture of the annulus fibrosus, which surrounds the nucleus pulposus. Reduction in the thickness of the discs results in the compression of the vertebral facets, which then exert pressure on the nerve roots, leading to back pain (reviewed in (Hunter et al., 2003a)).

Structure of the Intervertebral Disc

The intervertebral discs connect two adjacent vertebrae and provide shock absorption and structural stability and flexibility to the spinal column. The intervertebral disc has three major components: the nucleus pulposus, the gelatinous inner core of the intervertebral discs; the annulus fibrosus, a fibrous capsule that surrounds the nucleus pulposus; and the cartilaginous end plates, which are situated at the articular surfaces of the intervertebral disc and the adjacent vertebrae (Humzah and Soames, 1988). The nuclei pulposi, which originate from the embryonic notochord (Walmsley, 1953; Choi et al., 2008) are composed primarily of proteoglycan, water and collagen type II and are

located in the middle of each intervertebral disc (reviewed in (Adams and Roughley, 2006)). It is the nucleus pulposus that is thought to be required for the generation and maintenance of the disc's structural integrity (Setton and Chen, 2006). Damage to or loss of the nucleus pulposus as a person ages often leads to disc disease and back pain (Hunter et al., 2003a). As the discs age or are damaged, the nucleus pulposus is dramatically altered. Proteoglycan and water content decreases in the nucleus pulposus and collagen type II is replaced by type I collagen so that nuclei pulposi become more fibrous and contain less water.

The annulus fibrosus is thought to originate from the sclerotome and surrounds the mature nucleus pulposus (Bagnall et al., 1988; Huang et al., 2000). The annulus fibrosus is composed of concentric lamellae of collagen fibers (Adams and Roughley, 2006). With increased age, the annulus fibrosus stiffens and microstructural clefts or tears commonly occur in the tissue. Two thin cartilage endplates are located between each intervertebral disc and vertebral body, which allows nutrients to diffuse from the vertebral body into the nucleus pulposus (Urban et al., 2004).

Development of the Intervertebral Disc

In higher vertebrates, the notochord is a transient rod-like structure that is located along the midline. In low vertebrates and in primitive fish the notochord persists throughout development and provides the structural integrity required for locomotion (reviewed in (Stemple, 2005)). Signals from the notochord serve as a signaling center to form the floor plate and induce the differentiation of ventral somites into the sclerotome. After the induction of sclerotome, cells of the sclerotome migrate and condense around the notochord to form an unsegmented perichordal tube. After formation of the perichordal tube the axial mesenchyme exhibits a metameric pattern of

more condensed and less condensed regions. More condensed regions give rise to inner annulus fibrosus and outer annulus fibrosus and less condensed regions develop into vertebral bodies (Aszodi et al., 1998; Smits and Lefebvre, 2003).

During annulus fibrosus formation, notochord cells are simultaneously removed from regions of the embryo containing the vertebral bodies and relocated to the intervertebral mesenchyme to form the nucleus pulposus through an unknown mechanism (Walmsley, 1953; Choi et al., 2008). Interestingly, mouse mutants that formed aberrant cartilage around the vertebral column (but still had a normal notochord and notochord sheath) failed to remove notochord cells from the vertebral bodies resulting in malformed intervertebral discs (Wallin et al., 1994; Aszodi et al., 1998; Lettice et al., 1999; Peters et al., 1999). These data support the hypothesis that a mechanical force driven by the forming vertebral bodies is responsible for pushing notochord cells into the intervertebral discs (Theiler, 1988; Rufai et al., 1995; Aszodi et al., 1998; Smits and Lefebvre, 2003).

Molecular Signaling in Notochord and Intervertebral Disc Development

During embryogenesis the notochord produces signaling molecules to induce cell differentiation and to pattern the somites. *Foxa2* is a forkhead/ winged-helix transcription factor and is required for specifying the node and notochord. *Foxa2* mutant mice did not form node and lacked notochord cells (Ang and Rossant, 1994; Weinstein et al., 1994). *Brachyury (T)*, a T-box transcription factor, is expressed in the notochord and is required for cell differentiation and survival. *Brachyury* heterozygous mutants contained abnormal vertebrae posterior to the sacrum, which resulted in decreased tail length. *Brachyury* homozygote mutant embryos died at E10 due to defects in mesoderm formation and notochord differentiation (Herrmann et al., 1990).

Sonic hedgehog (SHH) is secreted from the notochord and patterns the somites. In addition, secreted SHH induces Shh mRNA expression in the floor plate which results in propagate of the hedgehog signaling in neural tube (reviewed in (Placzek, 1995)). Interestingly, in *Shh* null mice the notochord is formed but is quickly dismantled during early development (Chiang et al., 1996); thus, Shh is not required for initiation of the notochord but is required for maintenance of notochord structure. *Shh* is also expressed in nuclei pulposi in both prenatal and early postnatal intervertebral discs (DiPaola et al., 2005; Dahia et al., 2009); however, the role of *Shh* in transforming the notochord into nuclei pulposi and in postnatal intervertebral discs is still unknown.

Pax genes encode paired box transcription factors and are required for normal development of the axial skeleton. *Pax1* is expressed in the sclerotome at E8.5 onward and *Pax1* expression is restricted to the anlagen of the annulus fibrosus in later development (Smits and Lefebvre, 2003). *Pax1* homozygous mutants have defects in the formation of vertebral bodies and intervertebral discs. In these mutants, the notochord was not removed from vertebral bodies and remained as a rod-like structure between vertebrae.

Collagen type II is mostly expressed in cartilage. In collagen type II mutant mice, the vertebral column was severely malformed and the notochord persists until birth (Aszodi et al., 1998). The analysis of *Pax* and Collagen mutants suggest that the formation of nuclei pulposi is indirectly affected by vertebral column formation.

Sox5, Sox6 are expressed in the notochord and sclerotome-derived tissues (Smits and Lefebvre, 2003). *Sox5* and *Sox6* double mutant mice exhibited severe malformation of the axial skeleton and nuclei pulposi. In these mice, the notochord sheath failed to

develop and the notochord underwent cell death (Smits and Lefebvre, 2003). It has been suggested that the notochord sheath supports structural integrity of the notochord and resists the internal pressure of cells located in the notochord and the outer pressure produced from vertebral column. However, the origin and molecular mechanism of notochord sheath formation are still unknown.

Postnatal Changes in the Mature Nucleus Pulposus

Changes in the structure of the nucleus pulposus are a distinctive feature of disc degeneration. At birth, the nucleus pulposus is mostly composed of cells that are morphologically similar to the embryonic notochord and these cells have been referred to 'notochordal cells'. The notochordal cells are large (25- 85 μm in diameter) and are highly vacuolated (Hunter et al., 2003a; Hunter et al., 2003b). The vacuoles appear to contain glycoaminoglycans to control osmotic swelling pressure (Adams et al., 1990). With increased age, these notochordal cells decrease in number and the population of chondrocyte-like cells increases in a mature nucleus pulposus. These cells are called 'chondrocyte-like cells' because of their similar morphology to cartilage chondrocytes. Chondrocyte-like cells are smaller (~ 10μm in diameter) than notochordal cells and lack intercellular vacuoles (Hunter et al., 2003a; Hunter et al., 2003b).

In a number of species, notochordal cells have been observed to gradually disappear during adult life, and depletion of this cell population correlates temporally with the onset of disc degeneration (Maldonado and Oegema, 1992; Stevens et al., 2000; Hunter et al., 2003a). Some studies suggested that notochordal cells in needle punctured mouse models sequentially transformed into chondrocyte-like cells (Yang et al., 2009a). In addition, co-culture studies of notochordal cells and chondrocyte-like

cells demonstrated that notochordal cells interact with chondrocyte-like cells and stimulate proteoglycan production (Aguiar et al., 1999). These data suggest that notochordal cells are progenitor cells of mature nucleus pulposus cells and may stimulate other cells in the nucleus pulposus to maintain structure, integrity, and homeostasis of the disc.

CHAPTER 2
LIMB DEVELOPMENT

The Role of Sonic Hedgehog (Shh) in Limb Development

During early limb development, a number of molecular pathways establish the three axes of the limb. The secreted protein SHH plays an important role in limb pattering. *Shh* is expressed in a small domain of the posterior limb bud called the Zone of Polarizing Activity (ZPA). SHH protein is secreted from cells located in the ZPA and generates a posterior to anterior concentration gradient (Riddle et al., 1993). Cells exposed to a high concentration of SHH for long periods of time generate more posterior like digits, while cells exposed to lower concentrations of SHH for shorter amounts of time generate more anterior like digits (Yang et al., 1997; Harfe et al., 2004). In *Shh* null mice, no digits were formed in the forelimb and only digit 1 was formed in the hindlimb. These data demonstrate that digit 1 formation in the hindlimb is independent of *Shh* signaling (Chiang et al., 2001). *Shh* is important during early digit patterning; however, *Shh* is no longer expressed after digit condensation begins (Marti et al., 1995). Hence, it has been proposed *Shh* activates secondary signals that are responsible for patterning the forming digits. One candidate for the secondary signal functioning downstream of *Shh* are the Bmp protein family.

The Role of Bone Morphogenetic Protein (Bmp) in the Limb

Bmp2 is expressed in the posterior domain of the limb when *Shh* is expressed. Ectopic *Shh* expression in the anterior of the limb bud can induce anterior *Bmp2* expression. These data suggest that *Bmp2* is downstream of *Shh* signaling (Yang et al., 1997). However, the function of *Bmp2* in digit pattering cannot be studied using the null allele since *Bmp2* null mice die before digits form (Zhang and Bradley, 1996).

Another member of the *Bmp* family, *Bmp7*, has also been proposed to function downstream of *Shh* although *Bmp7* expression does not overlap with *Bmp2* in the early limb bud (Yang et al., 1997). Mice that lack *Bmp7* survive to birth and have partially penetrant preaxial polydactyly in the hindlimb. However, *Bmp7* null mice did not contain a digit pattering defect (Dudley and Robertson, 1997). It has been suggested that *Bmp7* is not required for digit patterning or that other BMP protein expressed in the limb can compensate for the function of *Bmp7* (Dudley and Robertson, 1997).

Bmp4 shares a high degree of homology with *Bmp2*. In the early limb bud, *Bmp4* is expressed in both the anterior and posterior mesoderm. The role Bmp4 plays in limb development cannot be investigated using a null allele since mice homozygous null for *Bmp4* die before digit formation (Winnier et al., 1995). A recent report demonstrated that mesodermally expressed *Bmp2* and *Bmp4* together are required for completion of osteogenesis (Bandyopadhyay et al., 2006). *Bmp2* has been shown to play an essential role in the signaling cascade that is required for repairing bone fractures (Tsuji et al., 2006). These data indicate that BMP proteins can compensate for each other but suggests that their functions do not overlapped completely.

Bmp2, Bmp4, and *Bmp7* in addition to being expressed in the limb mesoderm are also expressed in the AER in developing limbs. The functions of these proteins in this region of the limb are unclear but recent data have suggested that BMPs expressed in the AER have different functions from mesodermally expressed Bmps (Wang et al., 2004; Bandyopadhyay et al., 2006; Pajni-Underwood et al., 2007; Maatouk et al., 2009).

Examination of the role BMPs play in the AER have been done by misexpression of the Bmp antagonist, noggin, or by conditional removal of the Bmp receptor *Bmpr1a*.

Noggin overexpression in the AER resulted in syndactyly, postaxial polydactyly and ventralization of dorsal limb structures (Wang et al., 2004). A recent report demonstrated that expression of *Bmpr1a* in the AER was required for AER induction and Fgf-mediated cell survival (Pajni-Underwood et al., 2007).

Shh-Fgf **Positive Feedback Loop**

Vertebrate limb outgrowth is controlled by the *Shh-Fgf* positive feedback loop (Niswander et al., 1994). *Shh* expression in the ZPA upregulates *Gremlin* expression in the adjacent mesenchyme which maintains *Fgf4* expression in the AER. *Gremlin* is a Bmp antagonist that prevents Bmps from downregulating *Fgf4*. When *Fgf4* and *Gremlin* expression decreases, *Shh* expression is downregulated and the rate of cell proliferation decreases (Scherz et al., 2004).

In the limb, cells that have one time expressed SHH, expand anteriorly (Harfe et al., 2004). Former *Shh* expressing cells cannot express *Gremlin* (Scherz et al., 2004). As a result, the expansion of *Shh* descendants provides a barrier between *Shh* descendants and cells competent to express *Gremlin*. The *Shh-Fgf* feedback loop continues until SHH can no longer activate *Gremlin*. The length of time this feedback loop is active in the limb may influence digit length (Scherz et al., 2004).

The Role of ***En1, Wnt7a,*** **and** ***Lmx1b*** **in Limb Development**

During formation of the vertebrate limb, dorsoventral identity is controlled by signals emanating from the limb ectoderm. *Engrailed 1 (En1)*, a homeodomain transcription factor, is expressed in the ventral ectoderm and ventral side of the AER (Loomis et al., 1996). *En1* represses *Wnt7a* expression in the ventral ectoderm, and restricts its expression to the dorsal ectoderm. WNT7A activates LIM homeodomain 1 (*lmx1*) expression in the dorsal mesenchyme and *Lmx1* specifies dorsal limb fates.

Ectopic expression of *Lmx1b* induces the dorsalization of the limb (Riddle et al., 1995; Vogel et al., 1995). Mice in which *Wnt7a* has been removed contain duplicated ventral structures in dorsal limb as a result of expression of *En1* in the ventral structure (Parr and McMahon, 1995). *Lmx1b* homozygote mutant mice showed dorsal-to ventral transformation (Chen et al., 1998). Bmps have been proposed to function upstream of *En1* during dorsoventral patterning (Soshnikova et al., 2003). Misexpression of the BMP antagonist Noggin resulted in lack of *En1* expression in the ventral ectoderm (Pizette et al., 2001) suggesting that, loss of Bmp signaling results in a dorsalized limb.

CHAPTER 3
IDENTIFICATION OF NUCLEUS PULPOSUS PRECURSOR CELLS AND NOTOCHORDAL REMNANTS IN THE MOUSE: IMPLICATIONS FOR DISC DEGENERATION AND CHORDOMA FORMATION

Introduction

It has been estimated that two-thirds of Americans will experience at least one episode of back pain in their lifetimes. The majority will recover within a month, however 4.5 million people a year will become disabled from back pain at a cost of $16 billion a year (health care costs and lost work time)(Praemer, 1992; Pope, 1997). Both the severity and incidence of back pain increase as people age.

The most common cause of back pain is degeneration of the intervertebral discs (reviewed in (Hunter et al., 2003a)). This usually manifests itself in one of two ways; either through herniation of disc material into the vertebral column or through the reduction of disc thickness. Reduction in the thickness of the discs results in the compression of the vertebral facets, which then exert pressure on the nerve roots, leading to back pain. In a normal vertebral column, the intervertebral discs join adjacent vertebral bodies where they provide shock absorption and facilitate mobility of the spine (Urban and McMullin, 1988; Hunter et al., 2003a).

Each disc has three major components; the nucleus pulposus, the gelatinous inner core of the intervertebral discs; the annulus fibrosus, a fibrous capsule that surrounds the nucleus pulposus and consists of concentric lamellae of collagen fibers; and the superior and inferior cartilaginous end plates, which are situated at the articular surfaces of the intervertebral disc and the adjacent vertebrae (Humzah and Soames, 1988). It is the nucleus pulposus that is thought to be required for the generation and maintenance of the disc's structural integrity (Setton and Chen, 2006). Damage to or loss of the

nucleus pulposus as a person ages often leads to disc disease and back pain (Hunter et al., 2003a).

In humans, cells found in the adult nucleus pulposus are primarily small, chondrocyte-like cells. In juvenile and adults, a second population of cells in the nucleus pulposus has been proposed to function in disc renewal and homeostasis. These cells are much larger than the chondrocyte-like cells and, although their cell lineage is unclear, classical histological studies described them as being of "notochordal" origin (Walmsley, 1953). The coincident loss of the notochordal population of cells and the onset of disc degeneration during the life of many mammalian species suggests that this cell population may be involved in maintenance and/or repair of the disc (Hunter et al., 2003a).

Over 60 years ago, the embryonic nucleus pulposus was postulated to form from the embryonic notochord (Walmsley, 1953) and studies in rat have supported this hypothesis (Rufai et al., 1995). However, a number of more recent reports have suggested that the adult nucleus pulposus is only partially formed from the embryonic notochord or has a different origin entirely (Kim et al., 2003; Vujovic et al., 2006). The ability to identify in vivo the precursor cells of the nucleus pulposus would greatly aid in developing and characterizing mouse models of disc degeneration.

In humans, "notochordal remnant" cells have been proposed to transform into a rare type of tumor called a chordoma through an unknown mechanism (Yamaguchi et al., 2004; Yamaguchi et al., 2005). It has been proposed that notochordal remnants are derived from the embryonic notochord since notochordal remnants are similar in size and shape to notochord cells and reside in the region of the embryo in which the

embryonic notochord was present (Yamaguchi et al., 2004; Yamaguchi et al., 2005). In mice, notochordal remnants have not been described, which has made it very difficult to create a mouse model for chordoma.

Despite the clinical importance of nucleus pulposus cells, their embryonic origin has never been demonstrated by cell lineage analysis. In order to investigate whether the embryonic notochord gives rise to the entire nucleus pulposus, we genetically marked these cells during early mouse embryogenesis and followed their lineage into adulthood. In order to generate this fate map, we took advantage of the *Shhcre* and *ShhcreERT*^{2} mouse alleles we created previously (Harfe et al., 2004). In mice containing the *Shhcre* or *ShhcreERT*^{2} alleles, CRE is expressed in the notochord and activates expression of CRE-inducible reporter alleles in this tissue. Using these alleles we obtained direct evidence that, in the mouse model system, the embryonic notochord directly gives rise to all cell types present in the nucleus pulposus of the intervertebral discs. A small number of notochord cells were also found to reside in the vertebrae between the intervertebral discs. These cells are the elusive mouse notochordal remnants.

Results

Shh-expressing Cells in the Mouse Embryo Form the Nucleus Pulposus of the Intervertebral Discs.

To determine if the nucleus pulposus was derived from *Shh*-expressing cells we took advantage of the *Shhcre* allele we had created previously (Harfe et al., 2004) to fate map *Shh*-expressing cells, including those that reside in the notochord. The *Shhcre* allele was created by knocking into the *Sonic hedgehog* (*Shh*) gene the site-specific recombinase gene *cre*. Using this allele, CRE protein was expressed

everywhere that *Shh* was normally expressed, including the embryonic notochord (Harfe et al., 2004). Mice containing the *Shhcre* allele were crossed to the CRE reporter lines R26R or R26R:EYFP (Soriano, 1999; Srinivas et al., 2001).

In mice containing both the *Shhcre* and a reporter allele, CRE recombinase driven from the *Shh* promoter instigated activation of the reporter allele. Importantly, once reporter expression was activated, it continued to be expressed in cells in which the recombination event occurred, in this case all *Shh*-expressing cells including the notochord, and in all descendants of these cells throughout the life of the animal (Fig. 3-1).

In embryos that contained the *Shhcre* allele and either the *LacZ* (R26R allele) or *EYFP* reporter alleles, we observed reporter gene expression in the notochord at embryonic day (E) 10.5 (Harfe et al., 2004; Choi and Harfe, 2011). At E12.5, the notochord had begun to segregate along the anteroposterior axis, and bulges of labeled cells were observed at the positions where the intervertebral discs will form (Fig. 3-1A).

By E15.5, cells of the notochord had aggregated in areas where the nucleus pulposi were forming, and the vertebral bodies were mostly devoid of *Shhcre* descendant cells (Fig. 3-1B). Interestingly, at E15.5 we observed small streams of labeled cells in the developing vertebral bodies (Fig. 3-1B). The majority of these cells were not present one day later (Fig. 3-1C). However, a small number of these cells remained between nucleus pulposi and could be visualized using the R26R reporter, which is more sensitive than the ROSA:EYFP reporter (see Fig. 3-3C). These data suggest that some notochordal cells do not end up residing in the nucleus pulposi but instead remain between the intervertebral discs.

By E16.5, cells that expressed *Shhcre* had formed disc-shaped condensations between the vertebrae (Fig. 3-1C). Expression of the reporter in *Shhcre* cells was observed as intense staining throughout the entire nucleus pulposus in newborns (Fig. 3-1D and Fig. 3-2A). By contrast, the annulus fibrosis, which surrounds the nucleus pulposus in the intervertebral discs, was found to be composed almost entirely of cells that had never expressed *Shh* (Fig. 3-2A).

The Tamoxifen-Inducible Allele *ShhcreERT^2* Identifies the Embryonic Notochord as the Source of Nucleus Pulposus Cells.

Since *Shh* is expressed in both the notochord and the nucleus pulposus until birth (DiPaola et al., 2005), the *Shhcre* allele has the potential to activate reporter genes at early stages in the notochord, and at later stages in nucleus pulposus cells, irrespective of their embryonic origin. Thus, using the *Shhcre* allele, we could not exclude the possibility that notochord cells marked in E11.5 embryos may be eliminated by cell death, and that CRE is then re-expressed in the fully formed nucleus pulposus. To test directly whether the notochord gives rise to all cell types in the nucleus pulposus, we used a temporally controlled *Cre*, the tamoxifen-inducible *ShhcreERT^2* allele (Harfe et al., 2004), to pulse-label cells residing in the notochord but not the *Shh*-expressing cells found during later development in the nucleus pulposus. The *ShhcreERT^2* allele is identical to the *Shhcre* allele used in our initial fate map studies, except that CRE protein can be activated at discrete stages of embryonic development by injecting pregnant mothers with a single does of tamoxifen. Pregnant mothers carrying E8.0 pups were injected intraperitoneally with tamoxifen and the pups were examined at E13.5 to determine the fate of the notochord. At E13.5 all cells of the nucleus pulposus

were labeled (Fig. 3-2B and data not shown), indicating that the entire nucleus pulposus is descended from the notochord.

To verify that the injected tamoxifen was cleared from the embryo by E13.5, which is after notochord formation but prior to the formation of intervertebral discs, we analyzed reporter expression in external genitalia, in which the preputial glands are known to activate *Shh* at E13.5 (Perriton et al., 2002; Seifert et al., 2008). Tamoxifen injections at E8.0 did not label the preputial glands, indicating that the tamoxifen was cleared from the mouse prior to E13.5, which is consistent with published reports that Cre activity is undetectable ~48 hours after tamoxifen injection (Hayashi and McMahon, 2002) (Fig. 3-2C, D). These findings exclude the possibility that reporter gene expression is re-activated in intervertebral discs after E13.5.

The Adult Nuclei Pulposi is Composed Entirely of *Shh* Descendant Cells.

To determine if the adult nucleus pulposus was composed entirely of cells that had at one time expressed *Shh*, we examined adult discs from animals containing the *Shhcre* and *R26R* LacZ reporter alleles. In these $\geq$19 month-old animals, all cells of the nucleus pulposus appeared to be labeled, suggesting that this tissue is derived entirely from cells that have expressed *Shh* (Fig. 3-3A, B). The nucleus pulposus appeared to be a homogeneous population of *Shhcre* descendant cells (i.e., non-labeled cells could not be detected).

Conversely, the annulus fibrosus, cartilaginous end plates and the adjacent vertebrae were largely devoid of *Shhcre* descendant cells (see below for exception to this finding). Taken together with the finding that the majority of cells residing in the vertebral column and annulus fibrosus have never expressed *Shh* (they do not activate the *cre*-inducible *R26R* reporter construct nor has *Shh* expression ever been reported in

these tissues (DiPaola et al., 2005) and our unpublished data), these results suggest that that cells originating in the vertebral column and/or the annulus fibrosus most likely do not contribute to the mouse nucleus pulposus.

Notochord Cells That Do Not End Up Residing in the Nucleus Pulposus Form Notochordal Remnants in the Vertebral Column.

Although the majority of notochord cells ended up within the nucleus pulposus, a small number of cells were found to reside in the vertebral column, either in the vertebrae or, very rarely, in the annulus fibrosus (Fig. 3-2A and Fig. 3-3C). The location in which these cells were found was characteristic of the “notochordal remnants” that have been postulated to be present in humans but have never before been observed in mice. Notochordal remnants were found in all animals examined (n=12). These cells were first observed during embryonic nucleus pulposus formation and persisted throughout life, suggesting that notochordal remnants observed in adults arise during formation of the intervertebral discs. At all stages, notochordal remnants resided along the middle of each vertebra and were enriched on the ventral surface. Notochordal remnants were found along the entire length of the vertebral column.

Discussion

At least two distinct cell types have been demonstrated to reside in the nucleus pulposus in humans; chondrocyte-like cells and larger cells that have been referred to classically as “notochordal cells”. In addition to being larger than chondrocyte-like cells, notochordal cells have been reported to contain large vacuoles and express a number of proteins that are not found in chondrocyte cells (Maldonado and Oegema, 1992). In a number of species, notochordal cells have been observed to gradually disappear during adult life, and depletion of this cell population correlates temporally with the onset

of disc degeneration (Maldonado and Oegema, 1992; Stevens et al., 2000; Hunter et al., 2003a). These data have led to the proposal that notochordal cells may serve as nucleus pulposus stem cells.

The developmental origin of the two cell types found in the nucleus pulposus is currently unclear. For example, it has been proposed that both notochordal and chondrocyte-like cells are derived from the notochord (Walmsley, 1953; Hunter et al., 2003a), that only notochordal cells come from the notochord (Kim et al., 2003) or that neither of these cells types are notochord-derived (Vujovic et al., 2006). The above conclusions were derived primarily from histological examinations of intervertebral discs. In the experiments reported here, we used novel mouse alleles to fate map the embryonic notochord. Our experiments provide the first direct evidence that the notochord is the sole source of cells that form the entire nucleus pulposus of the mouse intervertebral discs.

In mice, it is unclear what the ratio of notochordal and chondrocyte-like cells is in the mature nucleus pulposus. An electron microscopic study suggested that in four month-old mice, the nucleus pulposus was composed of at least some notochordal cells (Higuchi et al., 1982). Based on the presence of numerous matrix proteins in the adult nucleus pulposus, chondrocyte-like cells are also likely present. Our finding that all cells examined in the adult nucleus pulposus are derived from *Shh*-expressing cells indicates that presumptive chondrocyte-like cells of the nucleus pulposus are derived from *Shh*-expressing notochord cells and, in contrast to previous suggestions, not from cells located in the surrounding *Shh*-negative mesenchyme (Kim et al., 2003). It is important to note that we cannot rule out the possibility that in organisms other then the

mouse, the nucleus pulposus may be derived, at least in part from non-*Shh* expressing tissues.

In our experiments we used both the CRE-inducible LacZ and EYFP reporters to mark the notochord and cells derived from this tissue. A number of mouse lines have been reported to undergo disc degeneration and/or premature aging (Kuro-o et al., 1997; Alini et al., 2008; Pignolo et al., 2008). However, the molecular defects underlying many of the abnormalities in these "aging" strains remain unclear. Using the *Shhcre* and *ShhcrERT*^{2} alleles described in this report it is now possible to conclusively determine the fate and function of the notochord, and during later development the nucleus pulposus, during disc degeneration in these mouse lines.

In addition to using the reagents described in this report to characterize disc degeneration in vivo, the ability to label notochord and intervertebral disc cells at any stage of mouse develop will allow for the purification of these cells (for example using fluorescence activated cell sorting). Purified cells could then be cultured *in vitro* and reintroduced into degenerating discs or used in microarray experiments to identify novel genes expressed at different stages of nucleus pulposus formation.

In humans, intraosseous benign notochordal cell tumors have been identified in 20% of a random sample of 100 vertebral columns examined during autopsy. These benign tumors have been proposed to form from the embryonic notochord (Yamaguchi et al., 2004). In this study, notochordal cell tumors were identified through microscopic examination and the smallest tumors identified were 1mm^{2}. The high incidence of notochordal cell tumors identified without the use of molecular markers suggests that the occurrence of these types of tumors in humans may be even higher. Our finding that

notochordal remnants were present throughout the vertebral column in all samples analyzed supports this hypothesis.

In humans, intraosseous benign notochordal cell tumors formed form notochordal remnants are postulated to very rarely transform into malignant tumors called chordomas (Mendenhall et al., 2005). In humans, it is rare for this type of tumor to occur in patients <40 years old (Enomoto et al., 1986). Interestingly, chordomas have been reported to occur at a much lower rate then intraosseous benign notochordal cell tumors have been observed (McMaster et al., 2001; Yamaguchi et al., 2004). The low occurrence of chordomas suggests that notochordal remnants lie dormant in most cases but may become malignant when stimulated, although the signals that initiate chordoma formation are unknown. Interestingly, the most prevalent DNA alteration in human chordomas has been reported to be an amplification of 7q36, which occurs in 69% of these types of tumors (Scheil et al., 2001). A similar chromosomal region has been proposed to contain a dominant oncogene in a family with familial chordomas (Kelley et al., 2001). In light of our findings that the *Shh*-expressing notochord forms notochordal remnants and all cell types in the mature nucleus pulposus, it is interesting that the region of chromosome 7 implicated in chordoma formation contains the gene *Shh* (Marigo et al., 1995).

Since the occurrence of notochordal remnants in humans is much higher than the reported incidence of chordomas, a second event, possibly a mutation or environmental insult during later life, must occur to cause notochordal cells to form tumors. Mutations resulting in constitutive activation of the *Shh* signaling pathway have been shown to result in the formation of numerous types of cancers in humans (McMahon et al., 2003)

and in mice, artificial overexpression of SHH in skin using a transgenic allele resulted in the development of a basal cell carcinoma-like tumor (Fan et al., 1997).

An enhancer element responsible for notochord expression has been identified in mice (Jeong and Epstein, 2003). If a similar enhancer element exists in humans, very rare activating mutations in this enhancer may result in overexpression (or sustained) expression of SHH in notochordal remnants in older patients. These findings raise the possibility that ectopic expression of SHH in notochordal remnants may cause chordomas by inducing these cells to behave like nucleus pulposus stem cells. In addition, *Brachyury* (Vujovic et al., 2006; Yang et al., 2009b), *Tsc1/2* (Lee-Jones et al., 2004) and *p16/CDKN2A* (Hallor et al., 2008) have been indirectly implicated in chordoma formation. The identification of mouse notochordal remnants raises the possibility of creating a mouse model of chordoma by altering expression of these genes in mouse notochordal remnants.

Materials and Methods

Strain Construction and Genotyping

The creation and genotyping of *Shhcre*, *ShhcreERT*^{2}, *R26R* and *R26R:EYFP* alleles have been described previously (Soriano, 1999; Srinivas et al., 2001; Harfe et al., 2004). *Shhcre* or *ShhcreERT*^{2} mice were mated to mice containing reporter alleles to create double heterozygous male animals. These animals were either mated to wild type females or analyzed directly. Animals were handled in accordance with the University of Florida Institutional Animal Care and Use Committee.

Detection of Report Activity

A single injection of tamoxifen (6mg/40g body weight) was intraperitoneal (IP) injected into pregnant dams. This dose has been shown to produce complete

recombination of floxed genes (Hayashi and McMahon, 2002). LacZ staining was performed as described previously. EYFP was detected using a Lecia MZ16 microscope and DFC300FX camera.

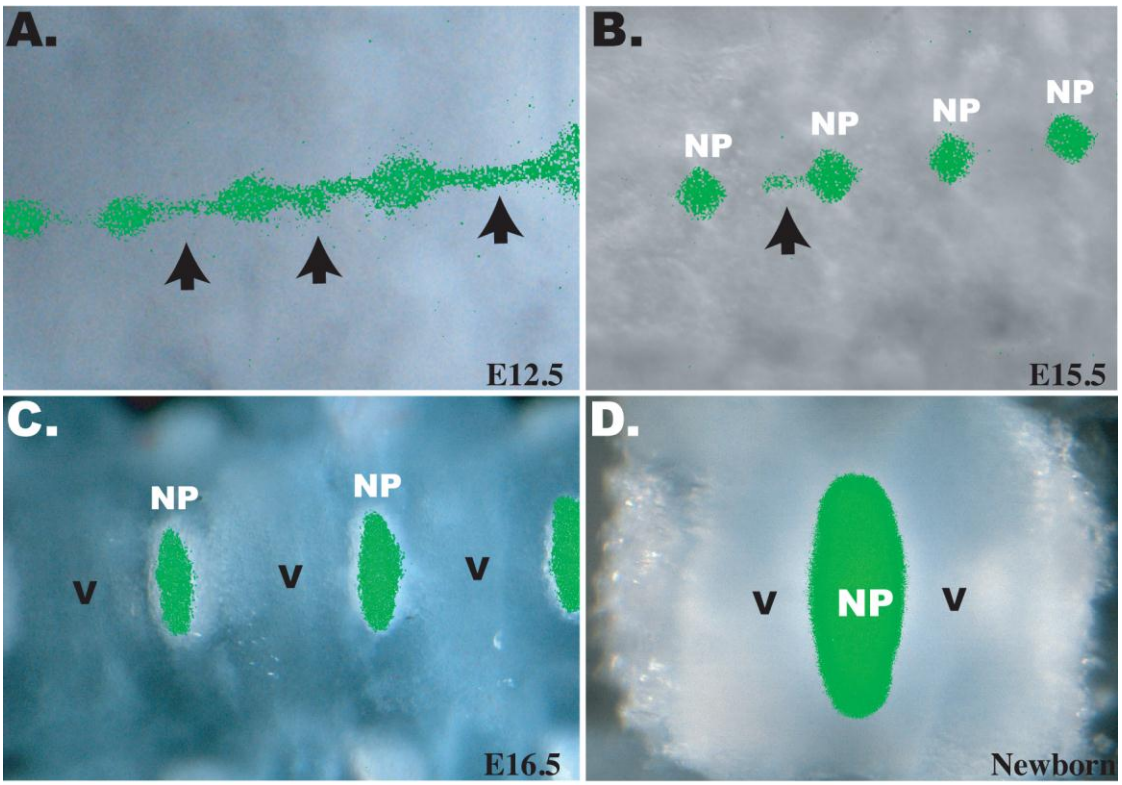

Figure 3-1. Fate mapping *Shh*-expressing cells in the axial skeleton using the *Shhcre* allele. The *Shhcre* allele was used to constitutively activate *R26R*::*EYFP* in the notochord. EYFP is observed as green. (A-D) Merged fluorescent and bright field images of the vertebral column. In all samples, a ventral view of the spinal column is shown. Embryos were harvested and all gut tissue was dissected away to visualize the underlying vertebral column. All images are of unfixed tissue. (A) At E12.5 the notochord begins to form "bulges" in locations where the nucleus pulposus of the intervertebral disc will form (the arrows denote the notochord). (B) By E15.5, clearly demarcated nuclei pulposi have formed from *Shhcre* expressing cells. Part of the notochord is still observed between the discs (arrow). (C, D) Cells that have expressed *Shhcre* are restricted to the nucleus pulposus and are mostly excluded from the vertebrae (v) at E16.5 (C) and at P0 (D). See Figure 3-2A and Figure 3-3B for exceptions. NP, nucleus pulposus; V, vertebral body. Reprinted with permission from *Identification of nucleus pulposus precursor cells and notochordal remnants in the mouse: implications for disk degeneration and chordoma formation*. Choi KS, Cohn MJ, Harfe BD; Copyright 2008 Dev Dyn.

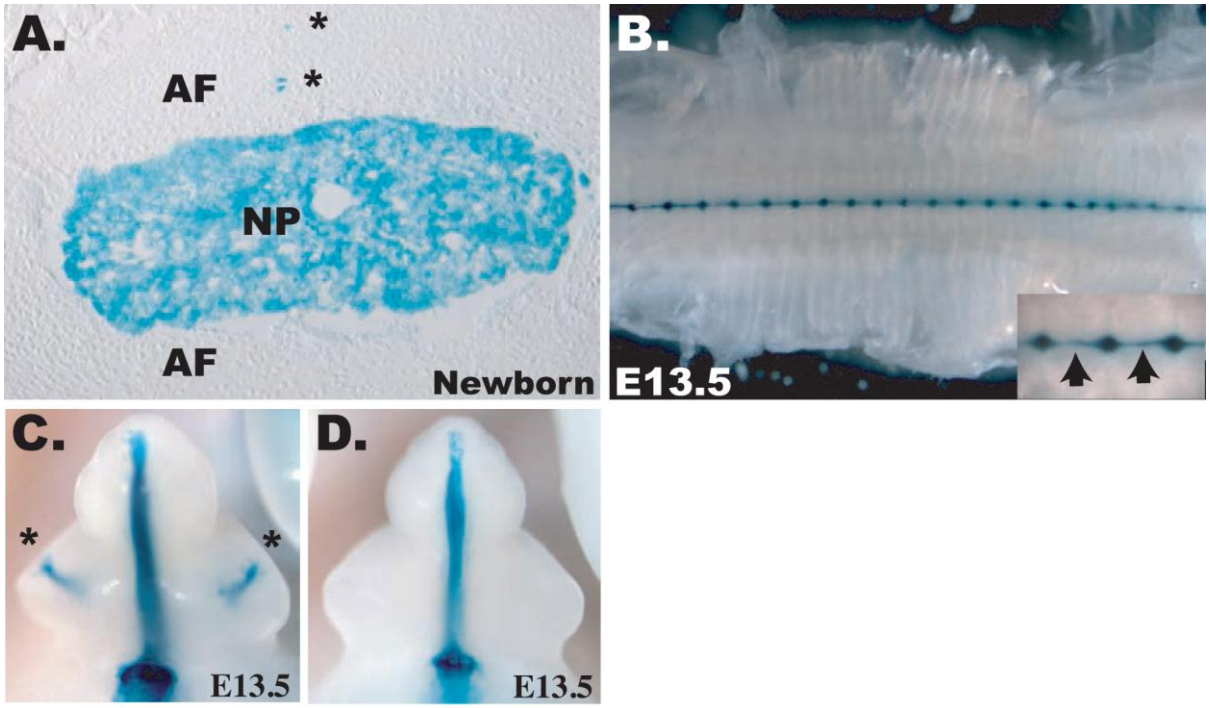

Figure 3-2. Fate-map of *Shh*-expressing cells using the *Shhcre* and the tamoxifen-inducible *ShhcreERT*^{2} alleles. (A) 10 µm transverse frozen section of the intervertebral disc of a *Shhcre*;*R26R* newborn mouse. Tissue was stained for the presence of β-galactosidase (blue). Note the entire nucleus pulposus is stained. Rare notochord descendants, denoted with an "*", are found in the annulus fibrosus (AF). (B-D) Distribution of *Shh* descendant cells in *ShhcreERT*^{2}*;R26R* mice pulse-labeled with tamoxifen at E8.0. Progeny were stained for β-galactosidase at E13.5. B, At E13.5 the nuclei pulposi of the intervertebral discs are forming. Ventral whole mount view is shown. All gut tissue has been dissected away so that the vertebral column is visible. The inset in (B) shows three intervertebral discs (ventral view). Note that some cells of the notochord (arrows) are still present between the forming discs. (C, D) Whole mount images of genital tubercles at E13.5. At this stage of development, male and female external genitalia are indistinguishable. The preputial glands (asterisks) are labeled in embryos constitutively expressing CRE in all *Shh*-producing cells (C). The absence of β-galactosidase in preputial glands of *ShhcreERT*^{2};*R26R* embryos exposed to tamoxifen at E8.0 (D) indicates that the tamoxifen has been cleared from these embryos prior to E13.5, when *Shh* expression is initiated in these glands (Perriton et al., 2002). The line of staining down the middle of the external genitalia in C and D is the urethra, which expresses *Shh* beginning at E9.75 (Perriton et al., 2002). Reprinted with permission from *Identification of nucleus pulposus precursor cells and notochordal remnants in the mouse: implications for disk degeneration and chordoma formation*. Choi KS, Cohn MJ, Harfe BD; Copyright 2008 Dev Dyn.

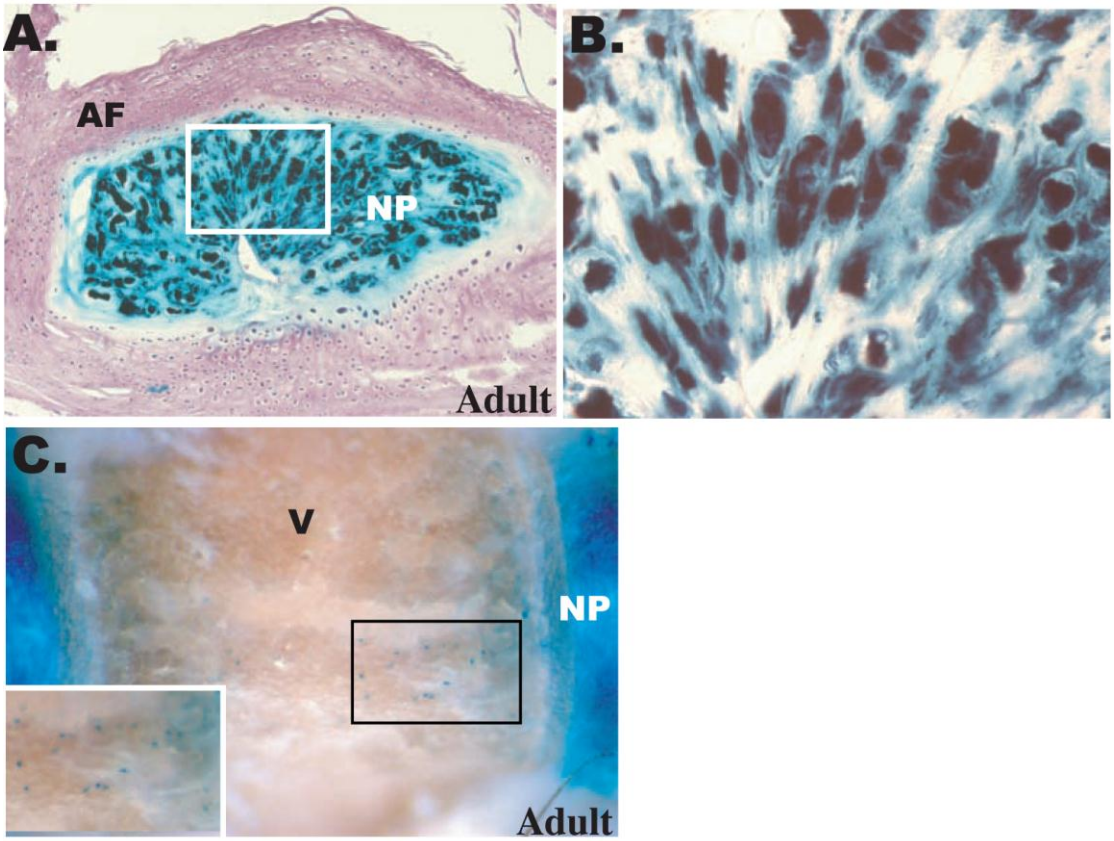

Figure 3-3. The nucleus pulposus and notochordal remnants in adult mice are composed of cells that have expressed *Shh*. (A) In a 19-month old *Shhcre*;*R26R* mouse, the nucleus pulposus is labeled and the annulus fibrosus is negative. 10 µm transverse paraffin section of an intervertebral disc stained for LacZ is shown. Cells were counterstained with nuclear fast red, which clearly marked the nuclei of cells. (B) 40X magnification of the boxed region in "A". In all samples examined (four discs from adult mice), all cells of the nucleus pulposus were stained blue indicating that the nucleus pulposus is composed of cells that have expressed *Shh*. (C) Notochordal remnants were found in adult vertebrae between each intervertebral disc in the *Shhgfpcre*;*R26R* mouse. Inset in (C) shows notochord remnants. Ventral view of the vertebral column is shown. Adult animals were stained in whole mount for LacZ and then dissected. Whole mount picture is shown. V, vertebrae between the discs; NP, nucleus pulposus; AF, annulus fibrosus. Reprinted with permission from *Identification of nucleus pulposus precursor cells and notochordal remnants in the mouse: implications for disk degeneration and chordoma formation*. Choi KS, Cohn MJ, Harfe BD; Copyright 2008 Dev Dyn.

CHAPTER 4
HEDGEHOG SIGNALING IS REQUIRED FOR FORMATION OF THE NOTOCHORD SHEATH AND PATTERNING OF NUCLEI PULPOSI WITHIN THE INTERVERTEBRAL DISCS

Introduction

Low back pain will affect most people over the age of 65 in industrialized countries. In the U.S., treatment of low back pain is estimated to cost 50-100 billion dollars per year (reviewed in (Katz, 2006)). For the majority of people, bed rest will relieve most symptoms of back pain but in a small population of patients the condition will become chronic. Most back pain is thought to originate from degeneration of the intervertebral discs or through physical damage to the disc. This leads either to herniation of the middle part of the disc called the nucleus pulposus and/or tears, bulging, and rupture of the annulus fibrosus, which surrounds the nucleus pulposus (reviewed in (Hunter et al., 2003a)).

The intervertebral discs connect two adjacent vertebrae and provide structural stability and flexibility to the spinal column. The nuclei pulposi, which originate from the embryonic notochord (Walmsley, 1953; Choi et al., 2008) are composed primarily of proteoglycan, water and collagen type II and are located in the middle of each intervertebral disc (reviewed in (Adams and Roughley, 2006)). As the discs age or are damaged, the nucleus pulposus is dramatically altered. Proteoglycan and water content decreases in the nucleus pulposus and collagen type II is replaced by type I collagen so that nuclei pulposi become fibrous and contain less water.

During mid-embryogenesis in both mice and humans the notochord, a transient rod-like structure that is located along the midline of embryos, becomes segmented and forms the intervertebral discs (Walmsley, 1953; Choi et al., 2008). In E12.5 to E15.5

mouse embryos, notochord cells are removed from regions of the embryo containing the vertebral bodies and are relocated to the intervertebral mesenchyme through an unknown mechanism. Interestingly, mouse mutants that formed aberrant cartilage around the vertebral column but a normal notochord and notochord sheath failed to remove notochord cells from the vertebral bodies resulting in malformed intervertebral discs (Wallin et al., 1994; Aszodi et al., 1998; Lettice et al., 1999; Peters et al., 1999). These data support the hypothesis that a mechanical force driven by the forming vertebral bodies is responsible for pushing notochord cells into the intervertebral discs (Theiler, 1988; Rufai et al., 1995; Aszodi et al., 1998; Smits and Lefebvre, 2003).

In numerous tissues, hedgehog signaling has been implicated in regulating pattern formation and cell proliferation and/or cell survival (Jensen and Wallace, 1997; Duprez et al., 1998; Britto et al., 2002). Previous studies have shown that *Sonic Hedgehog* (*Shh*) is expressed in nuclei pulposi in both prenatal and postnatal intervertebral discs (DiPaola et al., 2005; Dahia et al., 2009). In addition, *Indian Hedgehog* (*Ihh*) is expressed in condensing chondrocytes of the embryonic vertebral bodies and in the vertebral endplate during later development (DiPaola et al., 2005). Both *Shh* and *Ihh* produce secreted proteins that bind the transmembrane protein PATCHED1 (PTCH1) resulting in activation of the hedgehog signaling pathway (reviewed in (Ingham and McMahon, 2001)). In mice containing a null allele of *Shh*, the notochord formed but was quickly lost (Chiang et al., 1996). Since the notochord was quickly lost in *Shh* null animals the role of hedgehog signaling may play in transforming the notochord into nuclei pulposi remains unknown. *Ihh* is not expressed in nuclei pulposi (DiPaola et al., 2005), however, conditional removal of *Ihh* in chondrocytes of postnatal mice has been

shown to result in loss of the annulus fibrosus and enlargement of the nucleus pulposus. These data suggest that *Ihh* may be required within growth plates for intervertebral disc homeostasis (Maeda et al., 2007).

To directly examine the role hedgehog signaling plays in the formation of nuclei pulposi we conditionally removed *Smoothened* (*Smo*), which is required for all hedgehog signaling, in all *Shh*-expressing cells. In the vertebral column, *Smo* was removed from the mouse notochord and floor plate. Removal of *Smo* from the vertebral column, coupled with detailed fate-mapping and molecular analysis in this mutant background, allowed us to determine the role the hedgehog signaling pathway plays in transforming the notochord into nuclei pulposi.

Results

Removal of Hedgehog Signaling from the Mouse Notochord

Mouse embryos in which hedgehog signaling was removed from all cells die prior to formation of the intervertebral discs (Zhang et al., 2001). Previously, we had shown that the notochord forms the entire nucleus pulposus of each disc in the mouse vertebral column (Choi et al., 2008). To determine the role of hedgehog signaling during formation of nuclei pulposi, hedgehog signaling was removed from all *Shh*-expressing cells including notochord and floor plate in the vertebral column using a floxed mouse allele of *Smo* and the *Shhgfpcre* allele (Fig. 4-1A-D) (Long et al., 2001; Harfe et al., 2004).

Removal of Hedgehog Signaling from *Shh*-expressing Cells Results in Loss of Caudal Skeletal Elements

To confirm that hedgehog signaling was removed from the notochord and floor plate in embryos homozygous for the floxed *Smo* allele and that contained the

Shhgfpcre allele (*Smo*$^{f/f}$; *Shhgfpcre*), expression of *Ptch1* was analyzed using *Ptch1-lacZ* mice (Goodrich et al., 1997). PTCH1 is the receptor for SHH as well as a downstream target of hedgehog signaling. Using the *Ptch1-lacZ* allele we found that LacZ expression was absent in the caudal notochord and decreased in the neural tube in E9.5 *Smo*$^{f/f}$; *Shhgfpcre* embryos (Fig. 4-1D).

In mutant embryos in which hedgehog signaling was removed from *Shh*-expressing cells, severe defects in the caudal axial skeleton were observed. Beginning at E12.5, mutant embryos were observed to contain a truncated and thinner tail (Fig. 4-2B), which became more pronounced during postnatal development (Fig. 4-2D). In these mutant animals, skeleton analysis confirmed that no vertebrae formed caudal to the lumbar vertebra (Fig. 4-2F) and that numbers of axial cervical (C7) and thoracic (T13) vertebrae were not changed (n=3). To determine if loss of caudal skeleton elements in mutant embryos resulted from an increase in cell death, a lysotracker assay was performed on E11.5 embryos. An increase in cell death was detected in the caudal mutant somite and notochord in these embryos compared to normal controls (Fig. 4-2G-J). Since the notochord plays an important role in patterning the developing somites, which produce skeletal elements during later development (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994) we examined somitic expression of *Pax1* and *Pax3*. Consistent with the loss of caudal skeletal elements *Pax1* expression was diminished in tissue caudal to the hindlimbs (Fig. 4-3J). In contrast to the caudal region of the embryo, no increase in cell death was observed rostral to the hindlimb (see Fig. 4-6). Consistent with this observation, no apparent change in the expression pattern of *Pax1* and *Pax3* was observed in the rostral somites (Fig. 4-3A-H).

Loss of Caudal Structures upon Removal of Hedgehog Signaling Using the *Shhgfpcre* Allele

In all experiments in which *Smo* was removed using the *Shhgfpcre* allele, we observed a progressive rostral to caudal increase in severity of defects along the vertebral column. Caudal to the hindlimb, a drastic loss of skeletal elements was observed along with a decrease of *Pax1*. Previous work has suggested that the notochord is required to maintain *Pax1* expression in the developing somites and that loss of the notochord results in the dorsalization of the somites (Dietrich et al., 1993).

It is unclear why in our experiments all skeletal elements caudal to the hindlimbs are lost in mutant embryos. One possibility is that a burst of hedgehog activity, prior to inactivation of this pathway, occurred during very early development and was sufficient for formation of more rostral structures. For our analysis of the role hedgehog signaling plays in the formation of nuclei pulposi, regions of the embryo were examined in which *Pax1* and *Pax3* expression and skeletal elements were normal.

Removal of Hedgehog Signaling from *Shh*-expressing Cells Did Not Affect *Pax1* or *Pax3* Expression in Rostral Somites

The notochord plays an important role in patterning the developing somites (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994). To determine if defects in intervertebral disc development resulted from improper patterning of the somites, *Pax1* and *Pax3* expression was examined. *Pax1* and *Pax3* are expressed in presomitic mesoderm and become progressively restricted to sclerotome and dermomyotome, respectively, during somite development (Deutsch et al., 1988; Goulding et al., 1991).

Pax1 and *Pax3* RNA *in situ* hybridization were performed on control and mutant E10.5 and E11.5 embryos. No apparent change in the expression pattern of *Pax1 and Pax3* was observed in the rostral somites of E10.5 and E11.5 mutant embryos (Fig. 4-

3A-H). This was the same region of the embryo in which defects in nuclei pulposi formation were found upon removal of hedgehog signaling from the notochord (see Figs. 4-1 and 4-6). Interestingly, in E11.5 embryos *Pax1* expression was diminished in tissue caudal to the hindlimbs (Fig. 4-3J). This is the same region of the embryo that fails to form skeletal elements. In contrast, *Pax3* expression was not altered in the caudal somites of E11.5 mutant embryo upon removal of hedgehog signaling from the notochord (Fig. 4-3L).

Hedgehog Signaling is Required for Formation of Intervertebral Discs and Normal Cell Proliferation in the Notochord

Intervertebral discs are located between each vertebra in wild type mice. To determine the role hedgehog signaling played in formation of the intervertebral discs, newborn control and mutant vertebral columns were analyzed. Sections through mutant vertebral columns revealed that nuclei pulposi were smaller compared to control littermates (Fig. 4-4A-D). Mutant intervertebral discs contained an annulus fibrosus but this tissue appeared to have lost concentric lamellae within the annulus fibrosus, possibly as a result of the improper formation of nuclei pulposi in the center of the discs (Fig. 4-4E, F).

In a number of organs hedgehog signaling is required for cell proliferation and/or cell survival (Jensen and Wallace, 1997; Duprez et al., 1998; Britto et al., 2002). In these tissues, removal of hedgehog signaling often resulted in a decrease in cell proliferation and an increase in cell death. To determine whether removal of hedgehog signaling from the notochord resulted in a defect in cell proliferation, BrdU was administrated to pregnant dams three hours prior to harvest. BrdU assays were performed on the rostral regions of E11.5 wild type and mutant embryos. Using this

assay, notochord cells in E11.5 embryos were found to proliferate at a lower rate than controls (Fig. 4-5). These data suggest that the observed smaller nuclei pulposi located in the rostral vertebral column in mutant mice may partially result from a decrease in the rate of cell proliferation of notochord cells upon removal of hedgehog signaling.

Removal of Hedgehog Signaling Did Not Increase Cell Death in the Notochord

To determine if removal of hedgehog signaling from the notochord caused these cells to undergo cell death, TUNEL assays were performed on E12.5-14.5 mutant and wild type vertebral columns. At these stages of development, nuclei pulposi are forming from the notochord (Choi et al., 2008). In the notochord and forming discs of E12.5-14.5 wild type and mutant mice, aberrant cell death was not detected (Fig. 4-6). No ectopic cell death occurred in regions of mutant embryos that contained smaller nuclei pulposi indicating that cell death was not responsible for the formation of smaller nuclei pulposi upon removal of hedgehog signaling from the notochord.

Removal of Hedgehog Signaling Resulted in Aberrant Migration of Notochord Cells during Intervertebral Disc Formation

The mouse nucleus pulposus and annulus fibrosus are formed in highly condensed regions of intervertebral mesenchyme along the ventral midline of the embryo beginning at E12.5 (Smits and Lefebvre, 2003). Over the next 3 days the notochord forms the nuclei pulposi of the intervertebral discs. Notochord cells are normally excluded from regions of the vertebral column where vertebrae form. To determine if the aberrant nuclei pulposi found in mutant animals were due to defects in the transition of notochord cells into nuclei pulposi, the notochord in mutant animals was fate-mapped using the ROSA26 reporter allele (Soriano, 1999). In these animals, all

cells arising from the notochord were marked allowing for a detailed analysis of the fate of notochord cells throughout development.

At E12.5 a slightly thinner notochord was observed in mutant animals compared to controls (Fig. 4-7B), consistent with the observation that there is a decrease in cell proliferation in this tissue (see Fig. 4-5). In control E13.5 embryos, the notochord formed bulges between each vertebra where the future discs would form. In mutant embryos, this did not occur. Notochord cells continued to reside as a rod along the midline of the embryo. A small number of notochord cells were also found within the vertebral bodies (Fig. 4-7D). By E14.5 nuclei pulposi had formed from the notochord in control embryos with very few notochord cells still residing in vertebral bodies. However, in mutants few notochord cells were found to reside in the forming disc with most cells scattered throughout the vertebral column (Fig. 4-7F). In postnatal control animals, the nucleus pulposus was located inside the annulus fibrosus throughout the vertebral column. In contrast, mutant animals contained small nuclei pulposi with the majority of notochord cells dispersed throughout the vertebral column (Fig. 4-7H, J, L).

T (Brachyury) is Not Expressed in Notochord Cells That Reside Outside Nuclei Pulposi

To examine the cellular fate of mutant notochord cells we performed immunohistochemistry using an anti-T (Brachyury) antibody. In postnatal control embryos, notochord cells produced nuclei pulposi that expressed T (Brachyury) (Fig. 4-8C). Interestingly, while mutant notochord cells that resided within the discs did express T (Brachyury), cells outside the disc did not (Fig. 4-8D). These data suggest that mutant notochord cells that do not reside within a disc potentially lose nuclei pulposi/notochord-like characteristics.

Hedgehog Signaling is Required for Notochord Sheath Formation

The notochord sheath is composed of extracellular matrix proteins which surround the notochord in E10.0 embryos (Paavola et al., 1980). To determine if hedgehog signaling was required for notochord sheath formation, sections of E11.5 vertebral columns were stained with alcian blue to visualize the extracellular matrix composition surrounding the notochord. In control embryos the notochord sheath formed around the notochord in a rostral to caudal progression (Fig. 4-9A, C). Sheath formation occurred prior to cartilage condensation within the vertebral bodies. In mutant embryos a thin notochord sheath was observed in the rostral region of E11.5 embryos (Fig. 4-9B), however, no alcian blue staining was observed in the caudal region of mutant embryos (Fig. 4-9D). In addition, the caudal notochord of mutants was abnormally flatted (Fig. 4-9D, H, N). Laminin, a inner component of the notochord sheath, was found to surround the caudal notochord in both wild type and mutant embryos indicating that at least some components of the sheath are still present in the absence of hedgehog signaling within the caudal notochord (Fig. 4-9E-H).

To determine if the ultrastructure of the notochord sheath was affected by the removal of hedgehog signaling, transmission electron micrograph was performed on transverse section of E11.5 embryos. In wild type embryos, the notochord sheath consisted of a basal lamina layer and loosely organized collagen fibrils (Fig. 4-9I, K, M, O). In the rostral region of mutants, the notochord sheath consisted of basal lamina and a thin layer of collagen fibrils (Fig. 4-9J, L). In the caudal region of mutant embryos no electron dense material was observed (Fig. 4-9N, P).

Removal of Hedgehog Signaling after Formation of the Notochord Sheath Does Not Affect Nuclei Pulposi Patterning or Growth

Removal of hedgehog signaling prior to sheath formation resulted in a deformed sheath and aberrant formation of nuclei pulposi. These data suggested that proper formation of a notochord sheath was essential for normal patterning of nuclei pulposi along the vertebral column. However, it was possible that nuclei pulposi were not patterned correctly due to loss of hedgehog signaling and not due to the absence of a notochord sheath. To test this hypothesis, hedgehog signaling was removed after sheath formation using the tamoxifen inducible *Cre* allele *ShhcreERT*^{2} and the floxed *Shh* allele (Dassule et al., 2000; Harfe et al., 2004). Tamoxifen was administered at E8.5 (before sheath formation), E9.5 (during sheath formation), E10.5 or E11.5 (after the sheath formation; in a normal embryo, the sheath is first observed at E10.0 surrounding the notochord (Paavola et al., 1980)). To determine if hedgehog signaling was efficiently removed in *Shh*-expressing cells after tamoxifen exposure, we analyzed expression of *Shh* and *Ptch1* in E9.5 embryos that had been exposed to tamoxifen at E8.5 (Zhu et al., 2008). Both *Shh* and *Ptch1* expression were absent in the mutant notochord of treated embryos (Fig. 4-10B, D). Examination of the vertebral column of E11.5 control and *Shh*^{f}*/ShhcreERT*^{2} embryos in which hedgehog signaling had been removed after sheath formation occurred demonstrated that perdurance of the notochord sheath did not require hedgehog signaling (Fig. 4-11C, G). To determine if removal of hedgehog signaling from the notochord after sheath formation affected nuclei pulposi patterning, control and *Shh*^{f}*/ShhcreERT*^{2} notochords exposed to tamoxifen at E11.5 were fate-mapped using the *R26R* reporter allele (Soriano, 1999). In control and tamoxifen treated E11.5 embryos harvested at E18.5, no difference in

nuclei pulposi formation was observed rostral to sacral vertebrae (Fig. 4-10I, N, S) indicating that hedgehog signaling is not required for formation of nuclei pulposi after the sheath has formed.

Proper Formation of Vertebrae is Required for the Transition of the Notochord into Nuclei Pulposi

Removal of *Shh* from *Shh*-expressing cells at E8.5 or E9.5 resulted in the continued presence of the notochord throughout the vertebral column and an absence of nuclei pulposi throughout embryonic development (Fig. 4-10E, G, K, L, P, Q). Unlike when *Smo* was removed from *Shh*-expressing cells, notochord cells were not observed scattered throughout the vertebral column, even when the notochord sheath was abnormal. In addition, removal of *Shh* resulted in defects in formation of the vertebrae (Fig. 4-10K'-M' and P'-R'). An increasing severity in defective nuclei pulposi and vertebrae formation correlated with earlier removal of *Shh*. Removal of *Shh* at E10.5 resulted in formation of vertebrae but they lacked condensations (Fig. 4-10M'). In this experiment, nuclei pulposi began to form but notochord cells were still found to reside with the vertebral bodies (Fig. 4-10M). These data support the proposal that vertebrae may be responsible for forcing notochord cells into the forming intervertebral bodies (Fig. 4-12).

Discussion

Role of Hedgehog Signaling within the Mouse Notochord

During normal mouse development the notochord sheath surrounds the entire notochord beginning at E10.0 (Paavola et al., 1980). As the notochord begins to form visible nuclei pulposi at E12.5, the sheath remains around notochord cells. Our data directly addresses the role hedgehog signaling plays in formation of the notochord

sheath. In *Shh* null embryos, the notochord forms but then quickly disappears prior to sheath formation suggesting that hedgehog signaling is essential for maintaining a functional notochord (Chiang et al., 1996). Since *Shh* null embryos are defective in hedgehog signaling throughout the entire embryo it was not possible to determine if loss of the notochord in these mutant animals was an indirect consequence of loss of hedgehog signaling in other tissues. In our experiments, hedgehog signaling was removed from the notochord but was still present in tissues surrounding this structure. In these embryos, notochord cells persisted throughout embryonic and postnatal development. These data indicate that hedgehog signaling is not required for maintenance of this structure but instead is essential for normal formation of the notochord sheath that surrounds the embryonic notochord. Its is important to note that the *cre* allele used in these experiments, *Shhcre*, removes hedgehog signaling from the floor plate in addition to the notochord. While we currently have no evidence to support a role for hedgehog signaling within the floor plate in forming the notochord sheath, it is possible that signaling molecules within the floor plate that are downstream of the hedgehog signaling pathway may be important for forming at least some aspects of the notochord sheath.

Upon removal of all hedgehog activity, the sheath was disrupted but a ring of basal lamina was still found surrounding the notochord indicating that hedgehog signaling is not responsible for producing all components of the sheath. Laminin protein surrounding the hedgehog-null notochord could be produced from non-notochord cells, as suggested by experiments in zebrafish (Parsons et al., 2002). A second possibility is

that laminin is produced directly by notochord cells but does not require hedgehog signaling.

Role of the Notochord Sheath during Intervertebral Disc Formation

Although it is clear that a notochord sheath forms around the notochord in a number of different species including zebrafish, chick, mice and in humans the function this structure plays during development has remained elusive (Paavola et al., 1980; Camon et al., 1990; Gotz et al., 1995; Kimmel et al., 1995). During the transition of the notochord into nuclei pulposi, notochord cells have been proposed to be "squeezed" along the midline of the embryo by the condensing vertebra into the forming discs (Theiler, 1988; Rufai et al., 1995; Aszodi et al., 1998; Smits and Lefebvre, 2003). In embryos in which hedgehog signaling was removed from the notochord but contained normal vertebral bodies, notochord cells were observed to be scattered throughout the vertebral column. Mutant embryos that had a defective vertebral column, irrespective of if they had a normal notochord sheath, contained a rod-like notochord suggesting that vertebrae are needed to form normal discs.

We propose that a possible function of the notochord sheath may be to form a "wrapper" around the notochord (see Model in Fig. 4-12). Prior to the notochord forming nuclei pulposi, our model suggests that the sheath is required to maintain the rod-like structure of the notochord. In our experiments, loss of a functional sheath caused the notochord to flatten. During later wild type development, we propose that the sheath is flexible enough so that when the forming vertebrae exert swelling pressure the sheath expands but still constrains notochord cells to the dorsal midline of the embryo.

In regions of the embryo where the discs are forming, the notochord bulges outward and forms the nucleus pulposus of each intervertebral disc. We propose that in

the absence of a functional sheath, notochord cells are not constrained and become scattered throughout the vertebral column. Consistent with the proposed role for the notochord sheath in constraining notochord cells within the midline of the vertebrate embryo, an increase in the aberrant migration of notochord cells correlated with the observed increasing caudal severity of defects in sheath formation.

It is possible that abnormal nuclei pulposi formation observed upon removal of hedgehog signaling results from some other, non-sheath role for hedgehog signaling in this tissue. We cannot rule out the possibility that the hedgehog signaling pathway is responsible for activation of an unknown pathway(s) that is required for proper migration of notochord cells into the forming nuclei pulposi, independent of the presence of a notochord sheath. Mechanical removal of the notochord sheath from around the notochord in normal embryos could directly address this question, however, this experiment is technically challenging due to the inaccessibility of the notochord during vertebrate embryonic development.

Instead, we have taken a genetic approach to address this issue by removing hedgehog signaling after the notochord sheath has formed. In these embryos, the sheath was maintained and normal nuclei pulposi formation was observed. These data suggest that hedgehog signaling is required to specify formation of the notochord sheath but is not needed to maintain this structure during later embryogenesis. In wild type mice, the hedgehog signaling pathway remains present in nuclei pulposi throughout early postnatal life (Dahia et al., 2009). The role this signaling pathway plays in the postnatal intervertebral discs is unknown.

Materials and Methods

Mice

Mice containing the conditional floxed allele of *Smo*$^{f/f}$, *Shhgfpcre*, *ShhcreERT*^{2} , and Shh^{f}/^{f} have been described previously (Dassule et al., 2000; Long et al., 2001; Harfe et al., 2004). *Smo*$^{f/f}$;*Shhgfpcre* embryos were generated by crossing *Smo*$^{f/f}$ or *Smo*$^{f/+}$ females with a *Smo*$^{f/+}$;*Shhgfpcre* male. *Shh*^{f}/*ShcreERT*^{2} embryos were created by crossing *Shh*^{f}/^{f} or *Shh*^{f}/$^{+}$ females with *ShhcreERT*^{2}/+ males. For *Shh*^{f}/*ShcreERT*^{2} embryos, tamoxifen (Sigma) was gavaged at a concentration of 3mg/40g body weight in a pregnant female. In this report, *Smo*^{f}/^{f}; *Shhgfpcre* or *Shh*^{f}/*ShhcreERT*^{2} animals are referred to as mutant. Embryos that were either *Smo*$^{f/f}$, *Smo*$^{f/+}$, *Smo*$^{f/+}$;*Shhgfpcre,* *Shh*$^{f/+}$or *Shh*$^{f/f}$ were phenotypically wild type and were used as controls. In addition, embryos containing just the *Shhgfpcre* or *ShhcreERT*^{2} allele were indistinguishable from wild type. The *R26R* reporter allele was used in all fate mapping experiments (Soriano, 1999). All mouse strains were on a mixed genetic background. Animals were handled in accordance with the University of Florida Institutional Animal Care and Use Committee.

Histology and Immunohistochemistry

Embryos were then dehydrated through an ethanol series and embedded in paraffin (Fisher) or were incubated in 30% sucrose at 4^{o}C overnight and embedded in OCT (Sakura Finetek). Embryos were sectioned at 10 μm in rostral (forelimb level) and in caudal (hindlimb level) regions of the embryos and stained with hematoxylin (Fisher) and eosin (Fisher). For alcian blue staining, sections were stained with alcian blue (pH 2.5, Sigma) for 15 min, were washed in running tap water for 10 min and were then counterstained with nuclear fast red (Ricca chemical company). For picro-sirius red

staining, sections were stained with picro-sirius red (Poly scientific) for 45 min and then were washed in acidified water (0.025% acetic acid).

For immunohistochemistry, sections were stained with rabbit anti-laminin (DAKO) at 1:200, with rabbit anti-β-galactosidase (Invitrogen) at 1:500, with rabbit anti-T (Santa Cruz Biotechnology) at 1:200 or with rat anti- bromodeoxyuridine (BrdU, Accurate chemicals) at 1:200. A Cy2 or Cy3 conjugated secondary antibody (Jackson immuno research laboratories) was used at 1:200. Images were captured using a Leica DMRE microscope (Leica Microsystems Inc.) or Olympus BX61WI confocal microscope and were analyzed using Volocity 5.4 (PerkinElmer).

Cell Proliferation and Death Assay

To detect cell proliferation in E11.5 notochords, pregnant dams were injected with BrdU (50μg/g bodyweight) for 3 hours before harvest. Embryos were fixed and embedded in OCT (Sakura Finetek). Three wild type and three mutants were examined. The numbers of BrdU positive cells within the notochord were counted from five different 10 μm sections for each embryo. Unpaired student t-test was used to test significance.

For the TUNEL assay, embryos were fixed and embedded in paraffin (Fisher). Embryos were sectioned at 10 μm. The TMR-Red in situ cell death detection kit (Roche Diagnostics) was used following the manufacturers instruction. Cell death detection using Lysotracker (Molecular Probe 1:200) was performed following the manufacturers instruction.

Electron Microscopy

Fixed tissues were processed with the aid of a Pelco BioWave laboratory microwave (Ted Pella). The samples were washed in 0.1M sodium cacodylate buffer followed by 1% tannic acid. The samples were post fixed with 2% OsO_4, water washed and dehydrated in a graded ethanol series followed by 100% acetone. Dehydrated samples were infiltrated in graded acetone/Spurrs epoxy resin and cured at 60^{o}C (Ellis, 2006). Cured resin blocks were trimmed, thin sectioned and collected on formvar copper slot grids, post-stained with 2% aq. Uranyl acetate and Reynold's lead citrate. Sections were examined with a Hitachi H-7000 TEM (Hitachi High Technologies America, Inc.) and digital images acquired with a Veleta camera and iTEM software (Veleta- Olympus Soft-Imaging Solutions Corp.).

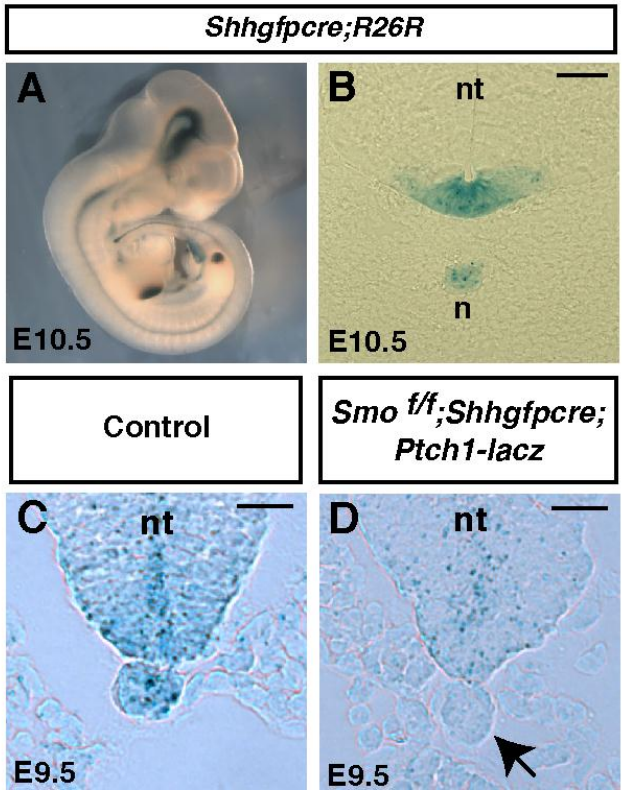

Figure 4-1. Removal of SMO in *Shh*-expressing cells. (A-B) Analysis of *Shhgfpcre* expression in an E10.5 embryo. Wholemount (A) and section (B) of a Xgal stained *Shhgfpcre;R26R* embryo. Note that staining is observed in the notochord (n) and floor plate of the neural tube (nt). (C-D) Analysis of *Ptch1:lacZ* expression revealed that hedgehog signaling was absent in mutant notochords. Section of *Ptch1:lacZ* expression of E9.5 control (*Smo*$^{f/f}$*; Ptch1:lacZ*)) and mutant (*Smo*$^{f/f}$;*Shhgfpcre*;*Ptch1:lacZ*) embryos. Note that *Ptch1:lacZ* expression was absent in the mutant caudal notochord (arrow). In the neural tube a decrease in *Ptch1:lacZ* was observed compared to controls. Lower expression may be due to the inability of floor plate cells to respond to SHH secreted from the notochord since the *Shhgfpcre* allele removed SMO from the floor plate in addition to the notochord. Scale bars in B is 50 μm, and C-D are 20 μm. notochord (n); neural tube (nt). Reprinted with permission from *Hedgehog signaling is required for formation of the notochord sheath and patterning of nuclei pulposi within the intervertebral discs*. Choi KS, Harfe BD; Copyright 2011 Proc Natl Acad Sci U S A.

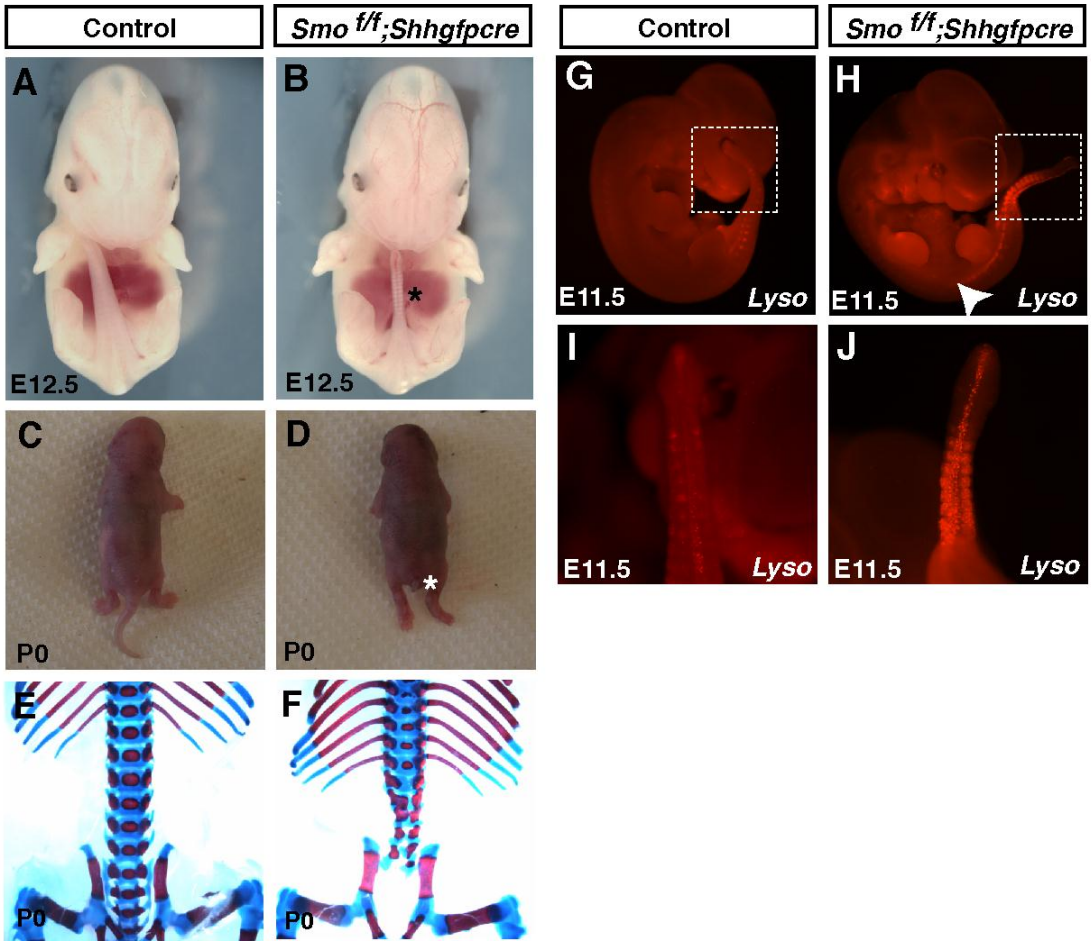

Figure 4-2. Removal of SMO in the notochord results in caudal truncation of vertebrae. (A-D) Bright field images of E12.5 and P0 wild type and mutant embryos in which *Smo* has been removed from *Shh*-expressing cells. An abnormally truncated and thinner tail (asterisk in B and D) was observed in the E12.5 and P0 mutant. (E, F) Skeleton preparation of control (E) and mutant (F) P0 mice. Note that there is no vertebrae formation caudal to lumbar vertebrae. (G-J) Lysotracker assay of control and mutant E11.5 embryos. I and J are higher magnification of the boxed regions shown in G and H. An increase in cell death occurred caudal to the lumbar vertebra (arrowhead in H). Reprinted with permission from *Hedgehog signaling is required for formation of the notochord sheath and patterning of nuclei pulposi within the intervertebral discs*. Choi KS, Harfe BD; Copyright 2011 Proc Natl Acad Sci U S A.

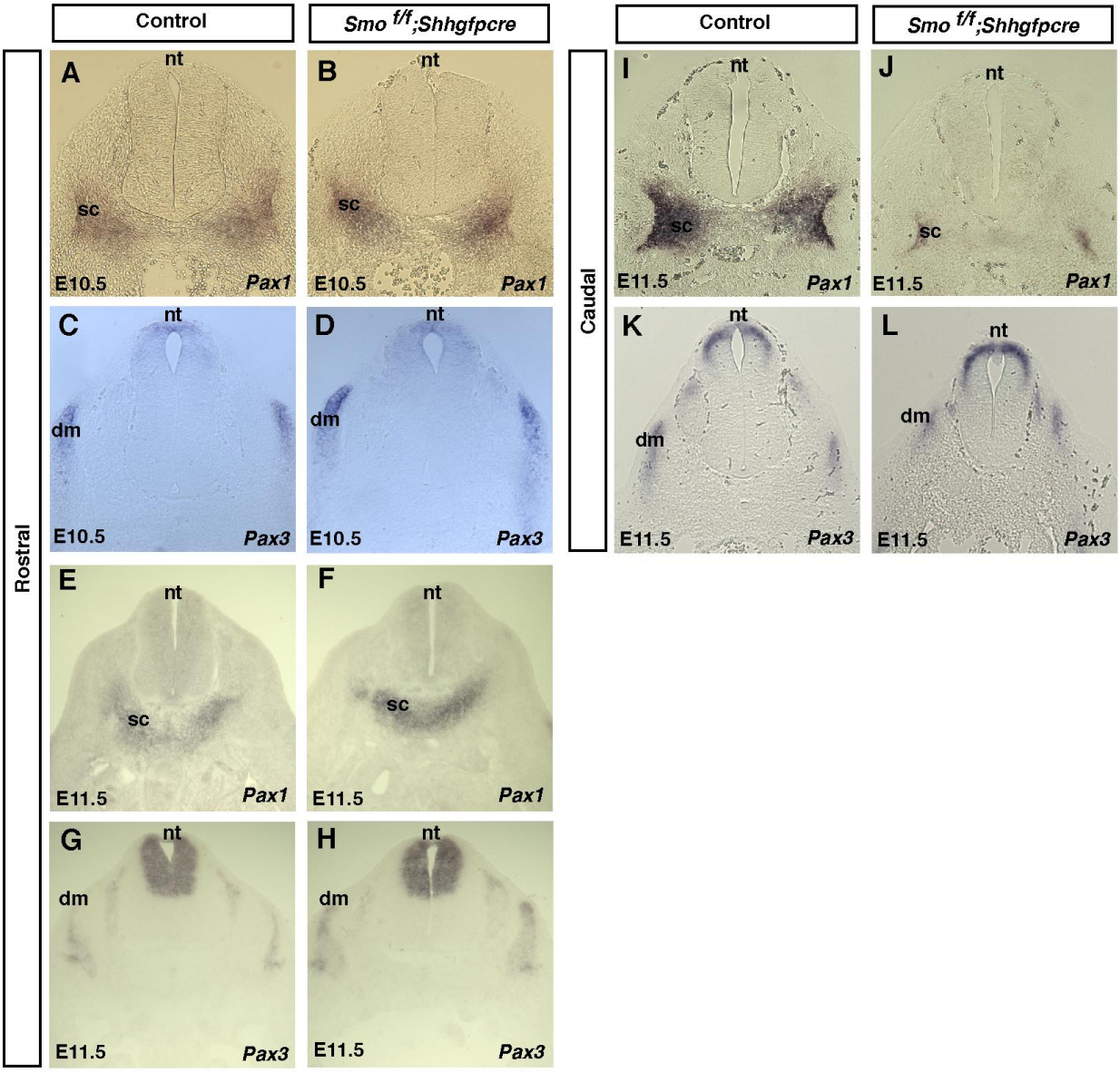

Figure 4-3. Expression of *Pax1* and *Pax3* in rostral somites was not affected by removal of hedgehog signaling in the notochord. (A-H) *Pax1* and *Pax3* RNA *in situ* hybridization in E10.5 and E11.5 rostral embryos. *Pax1* and *Pax3* expression in the somites of rostral mutant E10.5 and E11.5 embryos was not altered compared to age matched normal embryos. (I-L) *Pax1* and *Pax3* RNA *in situ* hybridization in E11.5 caudal embryos. *Pax1* expression was diminished in the somite of caudal mutant E11.5 embryos (J). *Pax3* expression was not changed in the caudal somite of control and mutant E11.5 embryos (K, L). sclerotome (sc); dermomyotome (dm); neural tube (nt). Reprinted with permission from *Hedgehog signaling is required for formation of the notochord sheath and patterning of nuclei pulposi within the intervertebral discs*. Choi KS, Harfe BD; Copyright 2011 Proc Natl Acad Sci U S A.

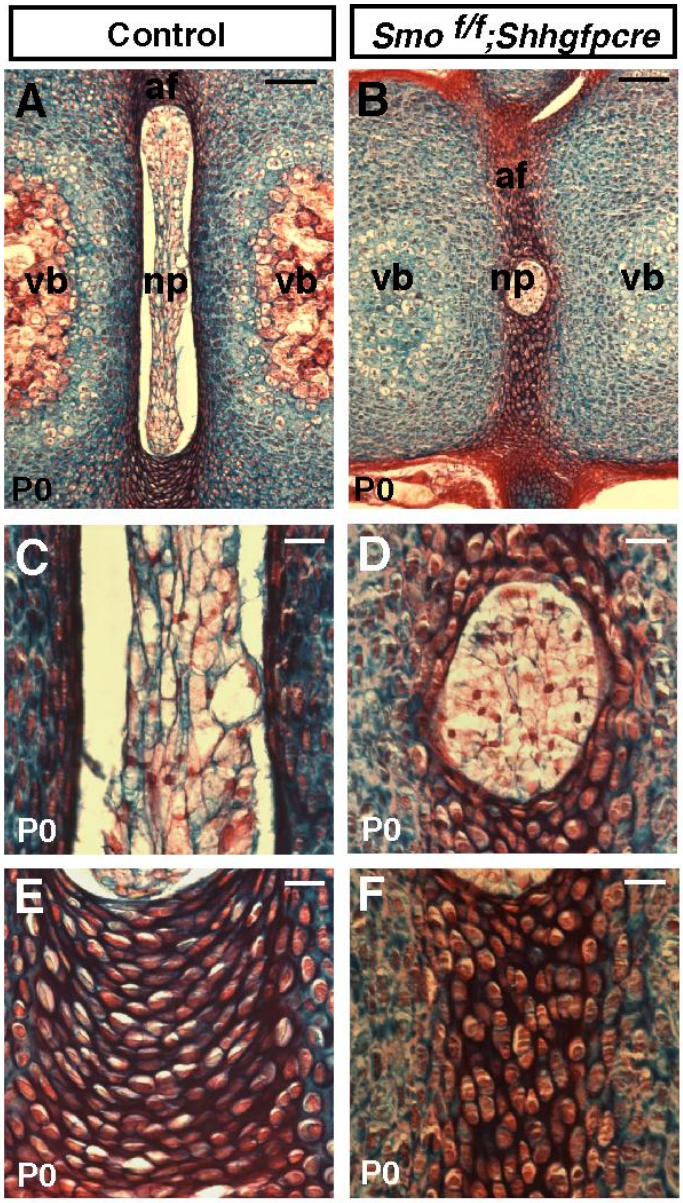

Figure 4-4. Removal of SMO in *Shh*-expressing cells results in abnormal development of the intervertebral discs. (A-F) Histological analysis of a sagittal section of the thoracic vertebral column using picro-sirius red and alcian blue staining. Close up view of nucleus pulposus (C, D) and annulus fibrosus (E, F). Mutant tissue contained a smaller nucleus pulposus than controls and concentric lamellae were absent in the annulus fibrosus. Scale bars in A-B are 100 μm, and C-F are 20 μm. vertebral body (vb); nucleus pulposus (np); annulus fibrosus (af). Reprinted with permission from *Hedgehog signaling is required for formation of the notochord sheath and patterning of nuclei pulposi within the intervertebral discs*. Choi KS, Harfe BD; Copyright 2011 Proc Natl Acad Sci U S A.

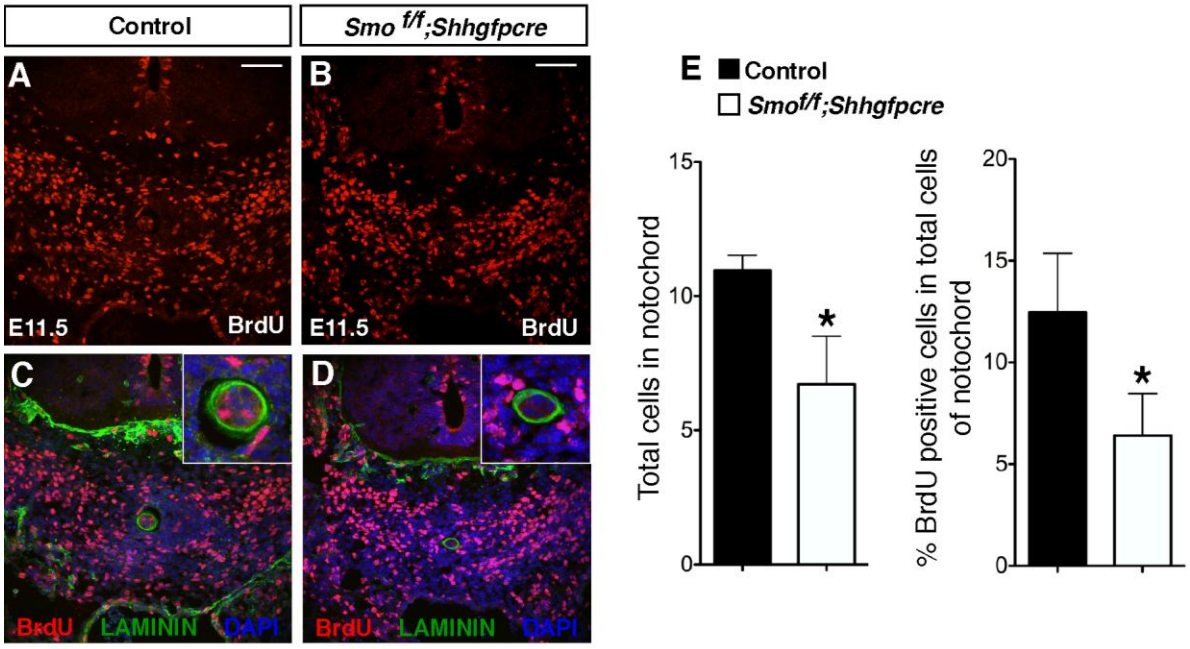

Figure 4-5. Removal of hedgehog signaling resulted in a decrease in cell proliferation in rostral mutant notochords. Representative transverse sections of the rostral vertebral column of E11.5 embryos are shown. (A-B) BrdU staining of control (A) and mutant (B) sections. (C-D) A merge picture of BrdU, DAPI, and Laminin (green). Laminin staining marked the inner layer of the notochord sheath and outlined the location of the notochord. At E11.5, the number of anti-BrdU positive cells in mutant notochords was decreased (D). (E) Quantification of the number of anti-BrdU positive cells/ total cells in the notochord demonstrated that the number of proliferating cells in mutant notochords was significantly decreased. Data are represented as means and the error bars represent the SD. A-B are 200 μm. * $p<0.05$. Reprinted with permission from *Hedgehog signaling is required for formation of the notochord sheath and patterning of nuclei pulposi within the intervertebral discs*. Choi KS, Harfe BD; Copyright 2011 Proc Natl Acad Sci U S A.

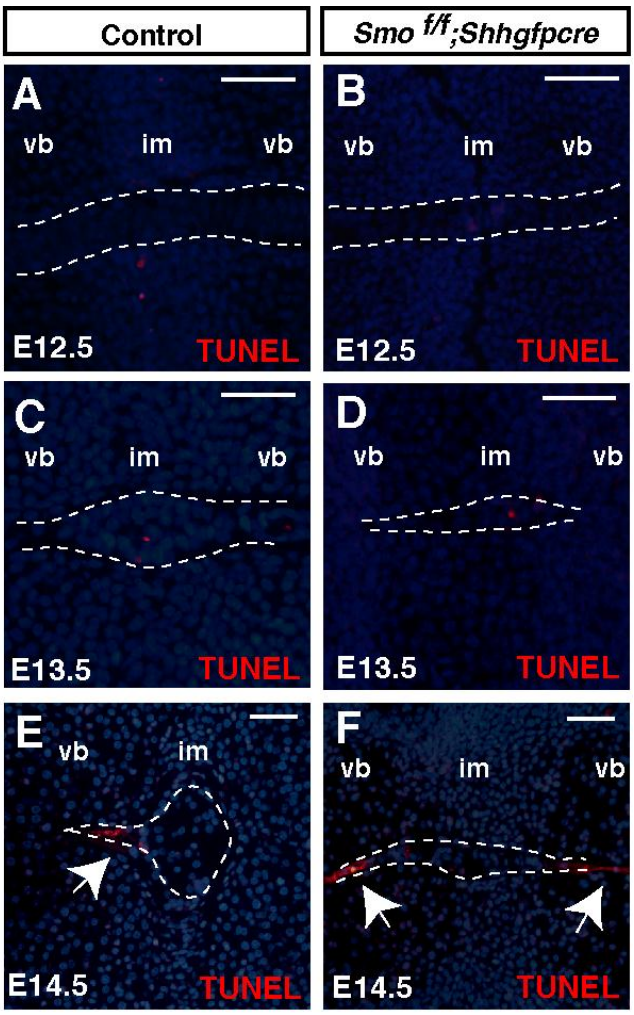

Figure 4-6. Aberrant cell death did not occur upon removal of hedgehog signaling in the notochord. (A-F) Sagittal sections of the rostral vertebral column of E12.5-14.5 embryos are shown. At E12.5-14.5, no ectopic cell death was detected in mutant notochords compared to controls upon removal of the hedgehog signaling pathway. In E14.5 embryos, notochord cells that did not reside in nuclei pulposi underwent cell death in both control (E) and mutant (F) embryos (arrows). Dotted lines outline the location of the notochord. Nuclei were counterstained using DAPI. Scale bars in A-F are 50 μm. vertebral body (vb); intervertebral mesenchyme (im). Reprinted with permission from *Hedgehog signaling is required for formation of the notochord sheath and patterning of nuclei pulposi within the intervertebral discs*. Choi KS, Harfe BD; Copyright 2011 Proc Natl Acad Sci U S A.

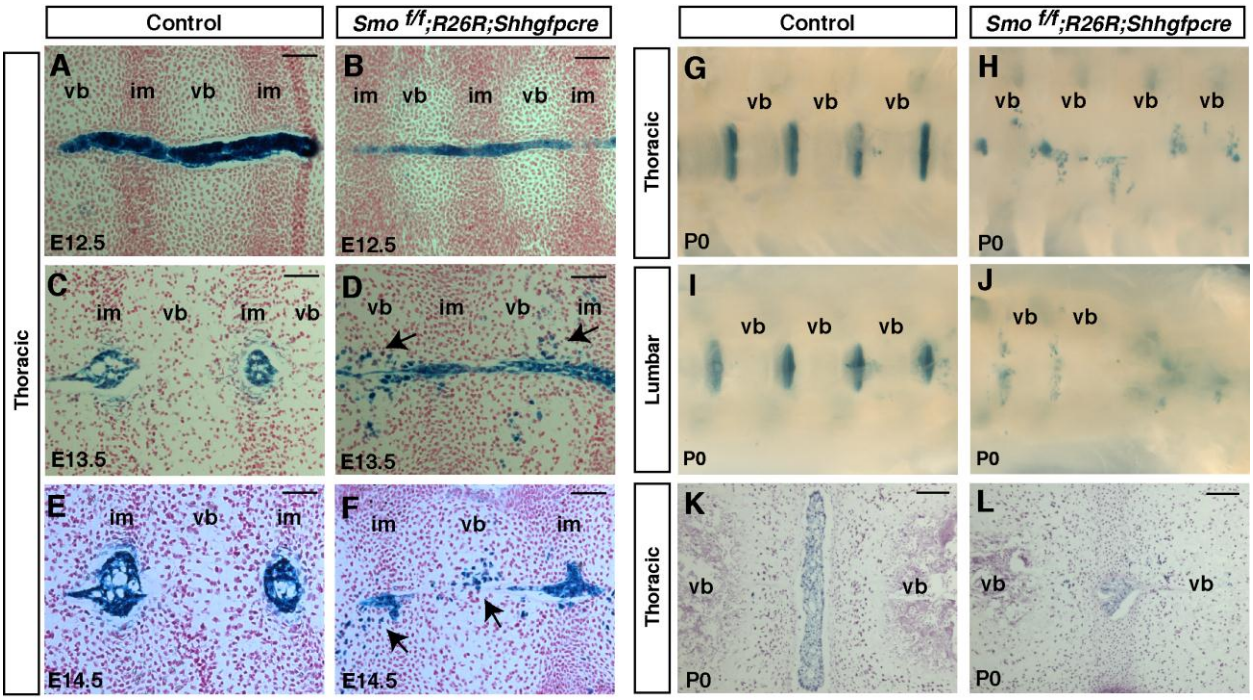

Figure 4-7. Aberrant migration of notochord cells throughout the vertebral column upon removal of hedgehog signaling in the notochord. (A-L) Cells were fate-mapped using the cre-inducible *R26R* allele. (A-B) Notochord (blue cells) formed a rod-like structure in both wild type and mutant animals until E12.5. In wild type E13.5 embryos (C) the notochord started to form a bulge between the vertebrae in regions where the intervertebral discs were forming. In mutants (D) the notochord remained as a rod-like structure and a few cells were found to reside outside the notochord (arrows). By E14.5, notochord cells had formed nuclei pulposi in wild type animals (E) but retained a rod-like structure in the mutant (F). A number of mutant notochord cells (arrows) resided outside the notochord. In P0 wild type animals (G, I), notochord cells had formed the nucleus pulposus of each intervertebral disc. In contrast, mutant notochord cells (H, J) were randomly found throughout the intervertebral mesenchyme and vertebrae. (K-L) Histological analysis of thoracic vertebrae from newborn mice demonstrated severe defects in nucleus pulposus structure and an increase in notochord cells residing in surrounding tissues. Scale bars in A-F are 50 μm and K-L are 100 μm. intervertebral mesenchyme (im); vertebral body (vb). Reprinted with permission from *Hedgehog signaling is required for formation of the notochord sheath and patterning of nuclei pulposi within the intervertebral discs*. Choi KS, Harfe BD; Copyright 2011 Proc Natl Acad Sci U S A.

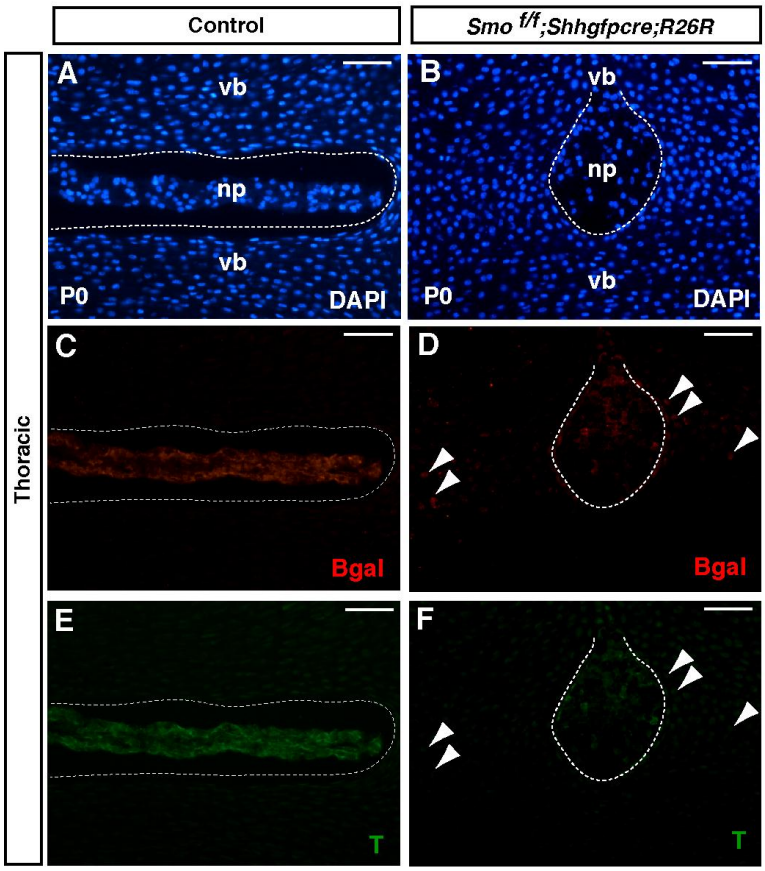

Figure 4-8. Notochord cells that did not reside within nuclei pulposi failed to express *T* (*Brachyury*). (A, C, E) In controls, most notochord cells (positive for β-galactosidase) gave rise to nuclei pulposi cells, outlined by dotted lines, and continued to express *T* (*Brachyury*). (B, D, F) In mutants, notochord cells were located throughout the vertebral column, annulus fibrosus and nucleus pulposus. *T* (*Brachyury*) expression was decreased in mutant notochord cells residing in forming nuclei pulposi. Notochord cells (arrowheads) that did not reside in nuclei pulposi did not express *T* (*Brachyury*). Scale bar = 50 μm. All sections were from P0 newborn mice. Reprinted with permission from *Hedgehog signaling is required for formation of the notochord sheath and patterning of nuclei pulposi within the intervertebral discs*. Choi KS, Harfe BD; Copyright 2011 Proc Natl Acad Sci U S A.

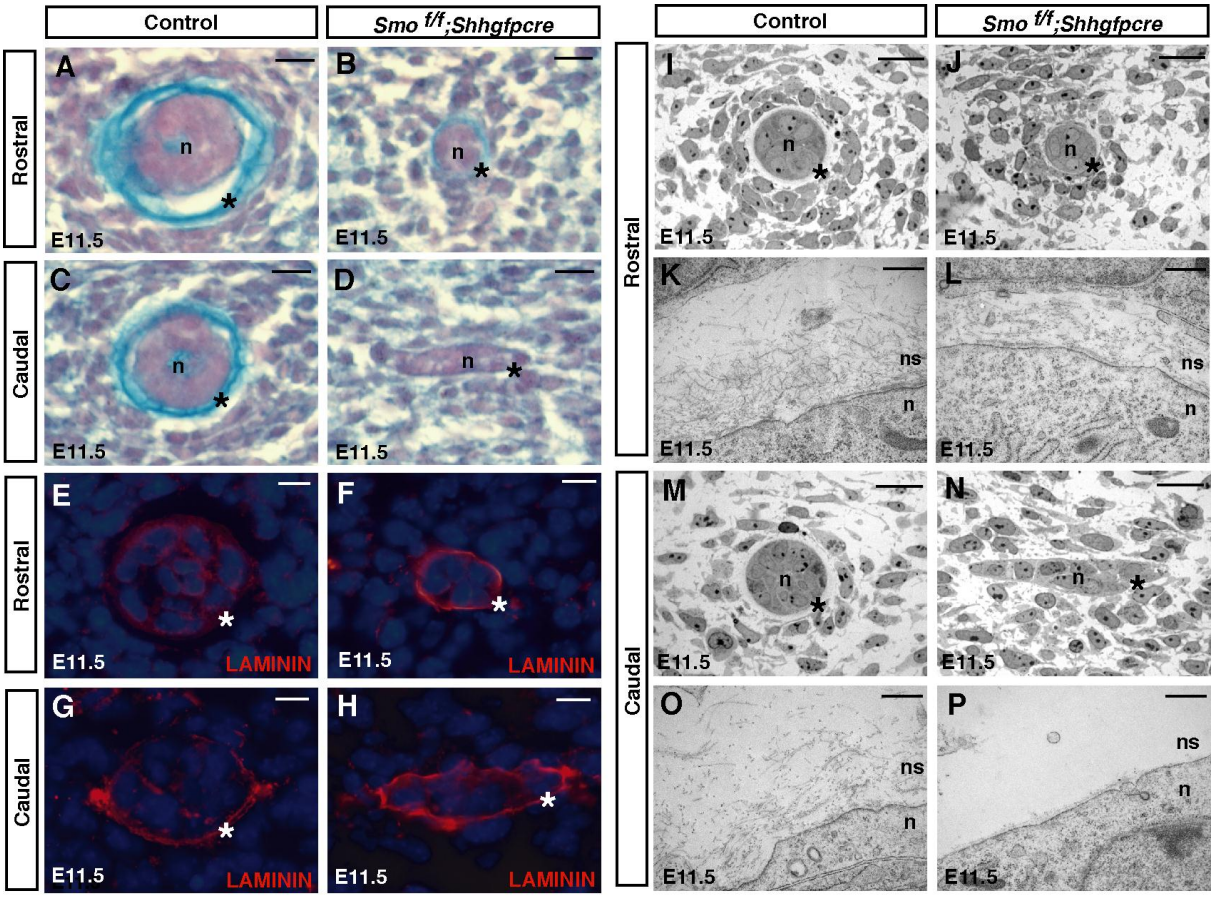

Figure 4-9. Hedgehog signaling is required for notochord sheath formation. Asterisks (*) indicate the location of the notochord sheath in all panels. (A-D) Histological analysis of transverse sections of rostral and caudal notochords from E11.5 embryos. Control (A, C) notochords were surrounded by the notochord sheath, which was visualized with alcian blue stain. In mutants, a thin layer of the notochord sheath was observed in the rostral notochord (B). In the mutant caudal notochord, the notochord sheath was absent (D). In both controls (E, G) and mutants (F, H) immnohistochemistry revealed that laminin surrounded the notochord. (I-P) Transmission electron micrograph of the notochord sheath. K, L, O, and P are higher magnification of the notochord sheath. The notochord sheath of controls contained basal lamina and collagen fibrils (K, O). In the mutant, basal lamina and a thinner layer of collagen fibrils formed in the rostral notochord (L) but the collagen fibrils appeared to be absent in the caudal notochord (P). Scale bars in A-H are10 μm, I, J, M and N are 20 μm, K, L, O and P are 0.5 μm. notochord (n); notochord sheath (ns). Reprinted with permission from *Hedgehog signaling is required for formation of the notochord sheath and patterning of nuclei pulposi within the intervertebral discs*. Choi KS, Harfe BD; Copyright 2011 Proc Natl Acad Sci U S A.

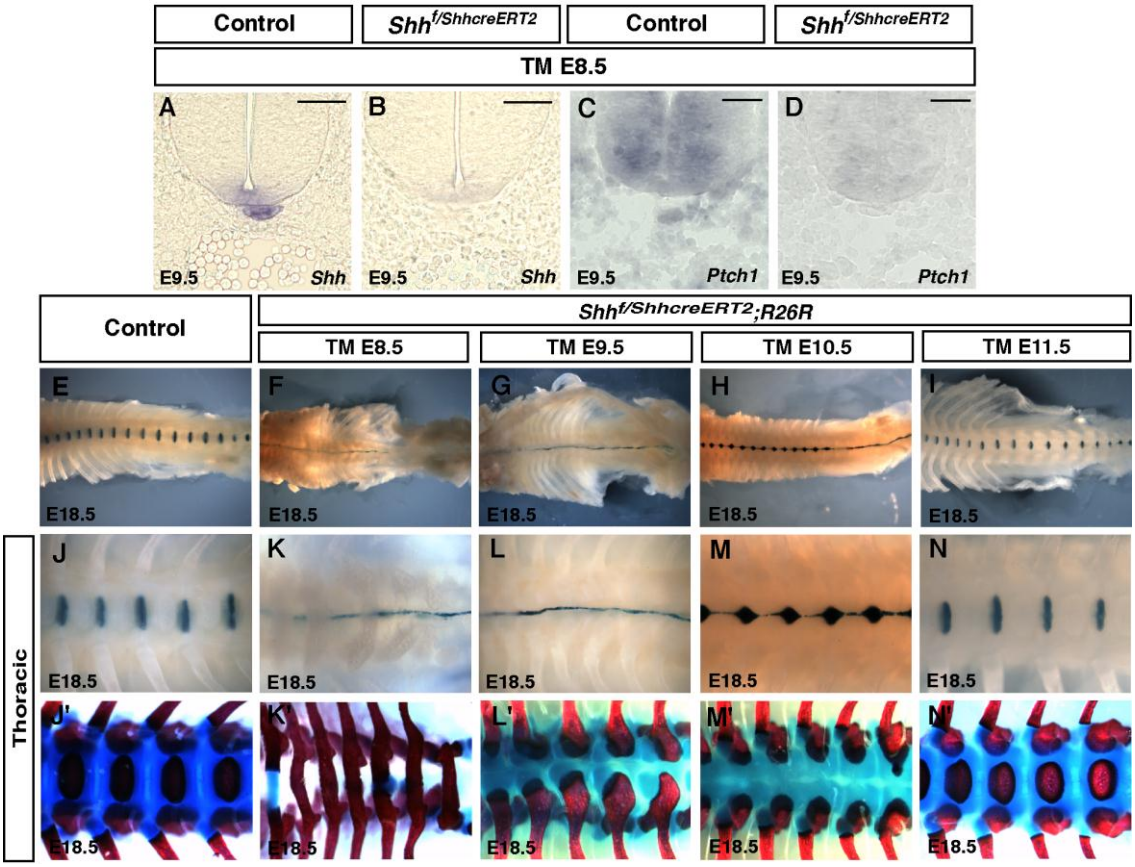

Figure 4-10. *Shh* is required for patterning the intervertebral discs. (A-D) Section RNA *in situ* hybridization of *Shh* (using a probe against the floxed exon 2) and *Ptch1* of E9.5 control (*ShhcreERT*^{2}) and mutant (*Shh*^{f}/*ShhcreERT*^{2}) embryos. *Shh* and *Ptch1* transcripts in E9.5 mutant embryos (B, D) were not detected 24 hours after tamoxifen (TM) injection. (E-S) Fate mapping of cells that have expressed *Shh* during intervertebral disc formation. Control (*ShhcreERT*^{2};R26R) and mutant (*Shh*^{f}/*ShhcreERT*^{2};R26R) embryos were harvested at E18.5 after a single TM injection at either E8.5, E9.5, E10.5 or 11.5. All images are ventral views of the vertebral column. (P) Loss of *Shh* in E8.5 embryos resulted in the truncation of the notochord in lumbar vertebrae (arrow). (L, Q, R) The notochord in mutants remained rod-like and failed to form nuclei pulposi upon removal of *Shh* from E9.5 or E10.5 embryos. (N, S) Nuclei pulposi formation was not affected by inactivation of *Shh* in E11.5 embryos. (J'-S') Skeleton preparation of thoracic and lumbar vertebrae. (K'-M', P'-R') Removal of *Shh* from E8.5 to E10.5 in *Shh*-expressing cells resulted in severe defects in formation of vertebral columns and lack of formation of ossification centers. (N', S') Removal of *Shh* from E11.5 embryos did not result in any phenotypic abnormalities in the vertebral regions rostral to the sacral vertebrae. Scale bar = 50 μm. Reprinted with permission from *Hedgehog signaling is required for formation of the notochord sheath and patterning of nuclei pulposi within the intervertebral discs*. Choi KS, Harfe BD; Copyright 2011 Proc Natl Acad Sci U S A.

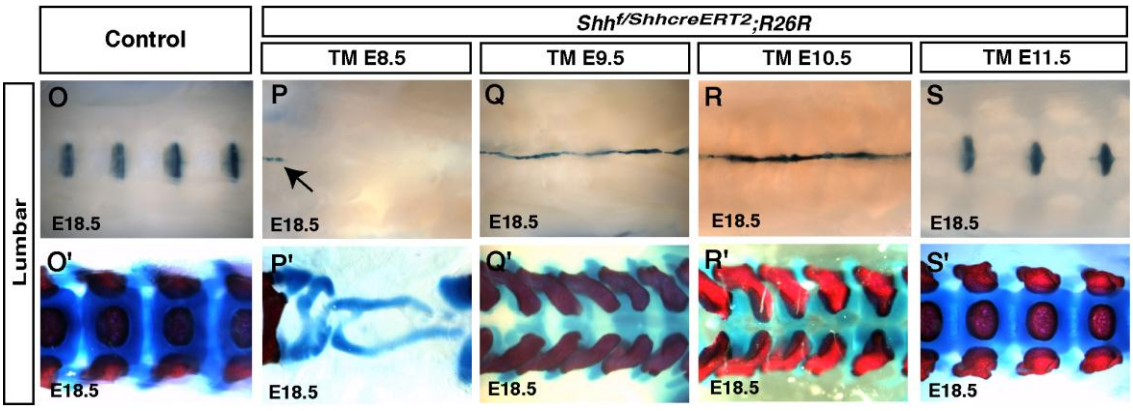

Figure 4-10. Continued

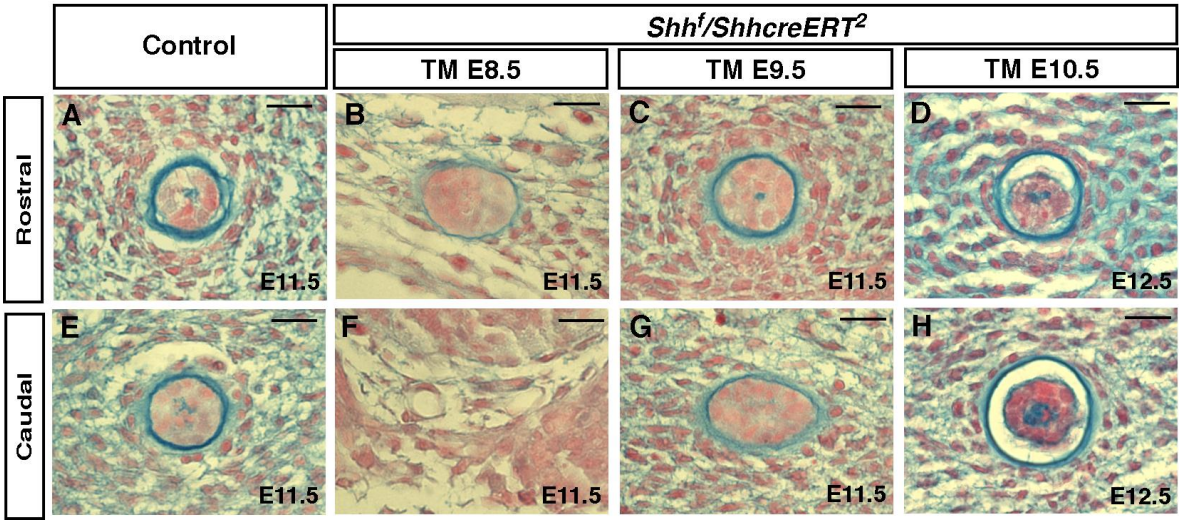

Figure 4-11. *Shh* is required for formation but not maintenance of the notochord sheath. (A-H) Histological analysis of transverse section of E11.5 and E12.5 embryos after a single TM injection at E8.5, E9.5 or E10.5, respectively. (A, E) In controls, the notochord was surrounded by the notochord sheath (blue). (B, F) After removal of *Shh* from E8.5 embryos, a thin notochord sheath was observed surrounding the rostral notochord but was not present surrounding the caudal notochord. (C, D, G, H) Inactivation of *Shh* in either E9.5 or E10.5 embryos did not affect notochord sheath formation. Scale bar= 20μm. Reprinted with permission from *Hedgehog signaling is required for formation of the notochord sheath and patterning of nuclei pulposi within the intervertebral discs*. Choi KS, Harfe BD; Copyright 2011 Proc Natl Acad Sci U S A.

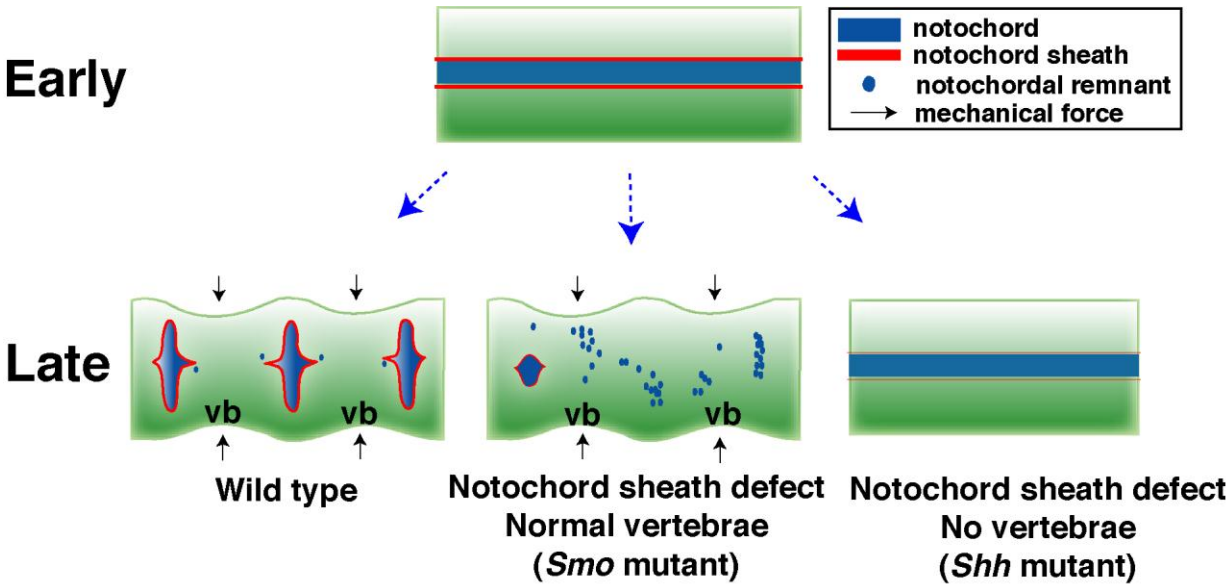

Figure 4-12. Proposed role for the notochord sheath in forming nuclei pulposi of the intervertebral discs. The notochord sheath (red line) begins to form around the notochord (blue line) at E10.0 (early). By E14.5 (late) most notochord cells reside within the intervertebral discs. It has been proposed that swelling pressure (denoted by arrows) exerted by the vertebral bodies serves to push notochord cells into the space between each vertebrae. Loss of a functional sheath, or "wrapper" around the notochord (denoted by a thin red line) results in notochord cells being scattered throughout the vertebral column and formation of small and misshapen nuclei pulposi. Loss of a functional sheath in the absence of swelling pressure results in the continued presence of the rod-like notochord throughout embryonic development. It is important to note that the proposed model does not rule out the possibility that a currently unknown molecular or chemical pathway is responsible for moving notochord cells into the forming discs. Reprinted with permission from *Hedgehog signaling is required for formation of the notochord sheath and patterning of nuclei pulposi within the intervertebral discs*. Choi KS, Harfe BD; Copyright 2011 Proc Natl Acad Sci U S A.

CHAPTER 5
IN THE LIMB AER BMP2 AND BMP4 ARE REQUIRED FOR DORSAL-VENTRAL PATTERING AND INTERDIGITAL CELL DEATH BUT NOT LIMB OUTGROWTH

Introduction

The developmental program that governs formation of the vertebrate limb involves a carefully controlled balance between pathways leading to growth and differentiation, and pathways leading to cell death. Several signaling centers direct differentiation of the early limb bud along each of its three axes; proximal-distal, dorsal-ventral, and anterior-posterior (reviewed in (Tickle, 2003)). The molecules expressed in these centers set off a complex network of signaling cascades that guide the proper organization and patterning of the limb.

Classical experiments identified a region of posterior mesenchyme in the limb bud that controlled the patterning of the vertebrate digits along the anterior-posterior axis named the Zone of Polarizing Activity (ZPA) (Saunders and Gasseling, 1968). Within this signaling center, cells secrete the protein Sonic Hedgehog (SHH), creating a posterior to anterior protein concentration gradient in the early limb bud (Riddle et al., 1993). The consequence of the SHH protein gradient is that cells in the posterior of the limb bud are exposed to higher concentrations of SHH over longer amounts of time than cells located in more anterior positions (Harfe et al., 2004). Cells exposed to high total amounts of SHH have been shown to form posterior digits while cells exposed to lower levels of SHH form more anterior digits (Yang et al., 1997).

Limb outgrowth along the proximal-distal axis is controlled by a separate signaling center, the Apical Ectodermal Ridge (AER; reviewed in (Tickle, 2003)). The AER is a specialized layer of ectodermal cells located along the distal tip of the limb bud. Several members of the fibroblast growth factor (Fgf) family are expressed in the AER. One of

these, *Fgf8* is expressed throughout the AER and has been implicated in controlling limb outgrowth and may also function in patterning the most distal portion of the digits (Lewandoski et al., 2000; Sanz-Ezquerro and Tickle, 2003). *Fgf4* is expressed in the posterior AER and is not required for normal limb development. However, in the absence of *Fgf8*, *Fgf4* is required for proximal-distal outgrowth of the limb (Sun et al., 2000; Sun et al., 2002; Boulet et al., 2004). FGF proteins expressed in the AER have also been demonstrated to regulate the expression of genes in the limb mesenchyme required for proximal-distal patterning and for terminating limb bud outgrowth (Mariani et al., 2008; Verheyden and Sun, 2008).

Genes expressed in the AER and ZPA interact through a complex feedback loop involving FGF proteins in the AER and SHH, Bone Morphogenetic Proteins (BMPs) and the BMP antagonist GREMLIN in the underlying mesenchyme (reviewed in (Panman and Zeller, 2003)). Bmp signaling negatively regulates the feedback loop by inhibiting *Fgf* expression although it is not clear which BMP protein(s) function in this pathway. This signaling loop is important for regulating limb outgrowth and the breakdown of the loop has been proposed to control limb size (Scherz et al., 2004; Verheyden and Sun, 2008).

The Bmp genes *Bmp2*, *Bmp4* and *Bmp7* are expressed in a dynamic pattern during limb development in both the mesenchyme and ectoderm and all three share an overlapping expression pattern in the AER (reviewed in (Robert, 2007)). The diversity of functions that are carried out by BMPs, combined with their distinct expression patterns suggests that BMPs in different locations of the limb may carry out discrete

functions. To test this hypothesis we have investigated the role BMPs expressed within the AER play in patterning the developing limb.

To investigate BMP functions specifically in the AER, several groups have used a transgenic approach to express the BMP antagonist *noggin* in the AER (Plikus et al., 2004; Wang et al., 2004). In these mice, polydactyly, syndactyly, an expanded AER and decreased cell death were observed. A similar result was obtained in the chick system (Pizette et al., 2001). Since all BMPs are affected upon exposure to noggin, the roles of individual BMP ligands cannot be determined in these experiments. In addition, in these experiments it was possible that noggin inhibited additional pathways besides the BMP-signaling pathway and/or that BMP signaling was not completely abolished.

To investigate the role of individual BMPs in the limb ectoderm, we have taken a conditional gene knockout approach. Using conditional floxed alleles of *Bmp2* and *Bmp4* and a transgenic allele that expresses CRE recombinase in the AER we have disrupted expression of these genes, individually and in combination, specifically in the AER. We found that while removal of *Bmp2* or *Bmp4* in the AER had no apparent effect on limb patterning, the combined loss of both *Bmp2* and *Bmp4* resulted in severe polydactyly, syndactyly, retention of interdigital tissue and defects in dorsal-ventral patterning. In addition to examining the morphological consequences of BMP loss, we have uncovered molecular defects in both the limb ectoderm and mesoderm. Surprisingly, no defects in proximal-distal patterning were observed.

Results

Msx2-Cre Inactivation of the Floxed *Bmp2* and *Bmp4* Alleles in the Limb AER

To investigate the roles of individual Bmps in the limb AER, mice were created in which both *Bmp2* and *Bmp4* were removed from the AER using an Msx2-Cre transgene

(see Materials and Methods). This transgene drives expression of CRE recombinase in the AER of the early limb bud and at low levels in the ventral ectoderm (Sun et al., 2002). As documented by several groups, the *Msx2-Cre* transgene is expressed earlier in the hindlimb (24-somite stage) than in the forelimb (28-somite stage) (Sun et al., 2002; Pajni-Underwood et al., 2007).

To determine if the Msx2-Cre allele removed Bmp gene expression we performed section RNA *in situ* hybridizations on E10.5 mice in which both *Bmp2* and *Bmp4* had been removed from the AER (referred to as “double mutant embryos”; see Materials and Methods). In double mutant embryos *Bmp2* and *Bmp4* were absent in the hindlimb AER (Fig. 1A-D). In the forelimbs, occasional patchy *Bmp2* and *Bmp4* expression was observed consistent with the reported delayed expression of the Msx2-Cre transgene in this tissue (data not shown). To determine if both *Bmp2* and *Bmp4* were absent from the same limb bud we performed double RNA *in situs* using probes for both genes on the same double mutant limb bud. In all cases (n=6), expression of both *Bmp2* and *Bmp4* were absent indicating that the Msx2-Cre transgene had successfully recombined both floxed alleles in the same limb bud (data not shown). Expression of a third Bmp, *Bmp7*, in the AER was not altered upon removal of *Bmp2* and *Bmp4* from the limb AER (Fig. 5-1E, F).

Embryos carrying two floxed alleles of *Bmp2* and *Bmp4* (*Bmp2*$^{c/c}$; *Bmp4*$^{c/c}$) and no *Cre* allele were phenotypically normal. These embryos expressed both *Bmp2* and *Bmp4* in the posterior mesenchyme and throughout the AER (Fig. 5-1A, C). Since the floxed alleles of neither *Bmp2* nor *Bmp4* altered gene expression, these mice were used

as normal controls. A “cre control” mouse heterozygous for the floxed *Bmp2* and *Bmp4* alleles and containing the *Msx2-Cre* allele did not result in a visible phenotype.

Removal of *Bmp2* and *Bmp4* in the Limb AER Results in Polydactyly, Syndactyly and Retention of Interdigital Tissue but Does Not Cause Defects in Proximal-Distal Patterning

The AER has been shown to play an important role in limb outgrowth and patterning (Tickle, 2003). To determine the role AER-expressed *Bmp2* and *Bmp4* played in this process we examined the limbs of double mutant animals. In these animals polydactyly, retention of interdigital tissue and syndactyly were observed (Fig. 5-2H). In the forelimbs, double mutants contained an ectopic post-axial digit (n=5/6 limbs; Fig. 5-2C). The ectopic digit contained 2-3 skeletal elements and was not attached to the metacarpal. The forelimbs of double mutant mice also contained bifurcations of at least one of the middle digits, usually at the distal end (n=6/6; Fig. 5-2C).

The hindlimbs of double mutant mice were more severely affected than the forelimbs. Similar to the situation in the forelimbs, polydactyly, retention of interdigital tissue and syndactyly were observed in the double mutant hindlimbs. However, unlike what was found in the forelimbs, the ectopic digit in the hindlimbs was usually a complete digit that attached to the metatarsal (n=5/6; Fig. 5-2F). In addition, double mutant hindlimbs exhibited proximal and distal digit bifurcations (Fig. 5-2F).

In the course of generating double mutants we also created animals in which various combinations of *Bmp2* and *Bmp4* alleles were removed from the limb AER. Mice that lacked either both alleles of *Bmp2* or both alleles of *Bmp4* from the AER were phenotypically wild type (Fig. 5-2B, E and data not shown). In addition, removal of one additional allele of either *Bmp2* or *Bmp4* in these genetic backgrounds did not produce a visible phenotype (data not shown).

Bmp signaling from the mesenchyme has been shown to play an essential role in interdigital tissue regression (Selever et al., 2004; Bandyopadhyay et al., 2006; Ovchinnikov et al., 2006). To determine if ectodermal Bmp expression was important for regression of tissue from between the forming digits we examined the interdigital tissue of double mutant mice. In animals in which both *Bmp2* and *Bmp4* were removed from the AER, interdigital tissue was retained between the digits. In severe cases, digits 1-4 were fused together (Fig. 5-2H). Digit 5 was never found to retain interdigital tissue with digit 4. Removal of *Bmp2* and *Bmp4* from the AER did not result in defects in proximal-distal patterning (Fig 5-2I, J, n=6/6).

Removal of Ectodermal BMPs Resulted in Expansion of the AER and Delayed AER Regression

To determine the morphology of the AER upon removal of *Bmp2* and *Bmp4*, E11.5 limbs were examined using CD44. A section through the AER of an E11.5 limb revealed an elongated and thinner AER (Fig. 5-3A, C and see Fig. 5-1).

Fgf genes are expressed in the AER and are excellent markers for denoting the boundaries of the AER. In double mutant mice the expression domains of both *Fgf4* and *Fgf8* were expanded along both the dorsal-ventral and anterior-posterior axes in E10.5-E12.5 limbs buds (Fig. 5-3D and Fig. 5-4C, G, H). This phenotype is similar to what has been reported upon removal of the Bmp receptor *Bmpr1a* in the AER (Pajni-Underwood et al., 2007). However, unlike the receptor knockout we did not observe gaps in *Fgf4* or *Fgf8* expression in E11.5 limb buds upon AER removal of *Bmp2* and *Bmp4*. Limb buds in which a single allele of either *Bmp2* or *Bmp4* remained did not contain a noticeable dorsal-ventral or anterior-posterior expansion of the AER (Fig. 5-

3G, H). Ectopic Fgf expression was also observed in E11.5-E14.5 limb buds (Fig. 5-4C, D, G, H).

Bmp2 and *Bmp4* Expression in the AER is Required for Regulating Interdigital Apoptosis and Cell Proliferation

Apoptosis between the digits requires BMP signaling. Since multiple BMPs and their receptors are expressed in both the limb ectoderm and mesoderm it has been unclear what role individual BMP ligands play in regulating apoptosis between the forming digits. To determine if the retention of interdigital tissue observed in mutant mice was caused by a decrease in apoptosis we stained animals with Lysotracker Red to mark cells undergoing cell death. Limbs in which *Bmp2* or *Bmp4* were removed contained no reduction in interdigital cell death (data not shown). This is consistent with the lack of any skeletal or visible phenotypes in these genetic backgrounds (see Figs 5-2 and 5-3). Mutant mice in which both *Bmp2* and *Bmp4* were removed from the AER exhibited a drastic decrease in cell death with the anterior portion of the limb being more severely affected (Fig. 5-5A, B).

Msx transcription factors are downstream targets of the BMP signaling pathway and have been shown to play a role in mediating regression of the interdigital mesenchyme via apoptosis (Marazzi et al., 1997; Satokata et al., 2000; Wang et al., 2004). Mutant animals in which *Bmp2* and *Bmp4* were removed from the limb ectoderm lacked detectable *Msx2* expression in the AER (Fig. 5-5C, D). *Msx2* expression in the underlying mesoderm was also decreased (Fig. 5-5C-F).

In addition to a decrease in apoptosis, an increase in cell proliferation can result in webbing between the digits. To determine if there was a proliferation defect in the limbs of mice that lacked AER expression of *Bmp2* and *Bmp4* we used an anti-phospohistone

H3 antibody to detect cells that were actively dividing (Fig. 5-5I-K). Double mutant limbs were found to contain a significant increase in the number of proliferating cells underneath the AER ($p=0.002$) than their corresponding wild type littermates suggesting that AER-expressed Bmps regulate both apoptosis and cell proliferation in the underlying limb mesoderm.

Distal *Gremlin* Expression in the Limb Mesoderm Requires Expression of *Bmp2* and *Bmp4* in the AER

The Bmp-antagonist *Gremlin* is initially expressed in the distal limb bud mesenchyme underneath the AER (Panman et al., 2006). As development progresses, *Gremlin* is excluded from the posterior limb bud due to the expansion of cells that have at one time expressed *Shh* (Scherz et al., 2004). *Gremlin* expression is also excluded from the distal portion of the limb bud underneath the AER during limb development by Fgf signaling (Verheyden and Sun, 2008). Analysis of *Gremlin* expression in double mutant limb buds showed a striking decrease of distal, but not proximal *Gremlin* expression in the early limb bud mesenchyme compared to age-matched littermates suggesting that AER expressed Bmps play a role in regulating *Gremlin* (Fig. 5-5G, H). Hox gene expression but not hedgehog signaling was also expanded in the anterior mesenchyme of the limb buds (Fig. 5-6).

Expression of *Engrailed-1* in the AER and Specification of Ventral Ectodermal Structures Requires *Bmp2* and *Bmp4* Expression in the AER

Engrailed-1 (*En1*) is a homeodomian-containing transcription factor expressed in the ventral AER (Joyner and Martin, 1987). Mice homozygous for a null allele of *En1* contain both dorsal-ventral and proximal-distal defects (Loomis et al., 1996; Loomis et al., 1998). In these mice, the AER is broadened and *Fgf8* expression is expanded. The broadening of the AER in *En1* null embryos is similar to the phenotype we had observed

upon removal of *Bmp2* and *Bmp4* in the AER (see Fig. 5-3D). To investigate if *En1* expression was altered after AER removal of Bmps we performed *En1* RNA *in situ* hybridizations on double mutant embryos. In double mutant embryos lacking both *Bmp2* and *Bmp4* in the AER, *En1* expression was absent from the AER (Fig. 5-7A, B).

In the ventral ectoderm *En1* expression is required to inhibit expression of *Lmx1*, a LIM homeodomain transcription factor expressed in the dorsal mesoderm of wild type embryos (Riddle et al., 1995). To determine if removal of Bmps from the AER affected dorsal-ventral patterning we examined expression of *Lmx1*. In mutant embryos *Lmx1* expression was expanded through the AER but not into the ventral ectoderm (Fig. 5-7.C, D).

In animals in which dorsal-ventral patterning has been reported to be altered, for example in *Wnt7a* null mice, tendons and sesamoid bones are found to be ectopically located on the dorsal side of the limb (Parr and McMahon, 1995). In mice in which both *Bmp2* and *Bmp4* were removed from the AER, we observed tendons and sesamoid bones in their normal location on the ventral side of the limb (Fig. 5-7E, F and data not shown). Interestingly, a distal dorsal ectodermal structure, the nail plate, was duplicated on the ventral side of the limb upon removal of *Bmp2* and *Bmp4* from the AER (Fig. 5-7G, H).

Discussion

In this report we investigated the role Bmp ligands in the AER play in the patterning of the vertebrate limb. Previous work by others has shown that Bmp signaling in the AER is essential for limb patterning. However, these studies were unable to address the role that Bmp ligands expressed in the AER play in limb patterning. Using a conditional knockout approach, we demonstrated that both *Bmp2* and *Bmp4* were

required within the AER for activation of the Bmp signaling pathway in this tissue. Surprisingly, the phenotype observed upon removal of Bmp ligands from the AER did not resemble the previously reported *Bmpr1a* AER knockout.

AER Expression of Bmp2 and Bmp4 is Not Required for Limb Outgrowth

The complete inactivation of Bmp signaling to the AER through the conditional inactivation of *Bmpr1a* results in limb agenesis indicating that Bmp signaling to the AER is required for limb outgrowth (Ahn et al., 2001; Pajni-Underwood et al., 2007). However, in our experiments we did not observe any defects in limb outgrowth. It is often difficult to compare conditional knockout phenotypes due to the use of different Cre alleles. However, we have used an identical Cre allele, Msx2-Cre, that Pajni-Underwod et. al. (2007) used to inactivate *Bmpr1a*. The striking difference in phenotypes observed could be a result of compensation from Bmp ligands present in the limb mesoderm and/or *Bmp7* in the AER.

Removal of different combinations of Bmps has been reported in the limb mesenchyme (Selever et al., 2004; Bandyopadhyay et al., 2006). In these experiments, Bmps were still expressed in the AER but the AER was expanded in the anterior-posterior direction and, at least in the case of *Bmp4* removal from the mesenchyme, the AER was broader (Selever et al., 2004). These data suggest that Bmps expressed in the limb mesenchyme play a role in AER formation. If mesodermally expressed Bmps can activate Bmp signaling in the AER through *Bmpr1a*, as has recently been proposed (Pajni-Underwood et al., 2007), our data indicates that they are incapable of inducing expression of *Msx2* a known target of the Bmp signaling pathway in the AER (at levels that can be detected by RNA *in situ* hybridization). In addition, mesodermally

expressed Bmps apparently cannot rescue expression of *En1* nor defects in AER maturation in mice in which *Bmp2* and *Bmp4* have been removed from the AER.

The source of Bmp ligands that activate the Bmp signaling pathway within the AER is still unclear. Our data supports the hypothesis proposed by Ahn et al. (2001) that Bmp ligand expression in the lateral mesoderm and/or overlying ectoderm may be the source of Bmp activity required for limb outgrowth (Ahn et al., 2001). Removal of additional combinations of Bmp ligands in the AER, limb ectoderm and/or lateral mesoderm could be performed to address this question.

Interdigital Apoptosis Requires Ectodermal Expression of Bmp Ligands

Bmp signaling within the mesenchyme of the interdigital region has long been proposed to regulate programmed cell death between the forming digits (Zou and Niswander, 1996; Marazzi et al., 1997). A recent model has been proposed in which interdigital cell death is controlled by Bmp ligand expression in the interdigital region (Pajni-Underwood et al., 2007). In this model, mesodermal Bmp ligands bind to BMPR1A in the AER and regulate FGF activity in the AER. Our data indicates that AER-expressed Bmps are responsible for interdigital cell death and we propose that Bmps expressed in the AER can activate the Bmp signaling pathway through AER-expressed BMPR1A. In support of this hypothesis, mesenchymal BMP expression was not altered in the double mutants (Fig. 9). Our data does not rule out the possibility that mesodermal Bmps are also required for interdigital cell death, however the lack of *Msx2* expression, a known target of Bmp signaling in the *Bmp2/Bmp4*-null AER indicates that mesodermal Bmps cannot completely compensate for loss of Bmp expression in the AER.

The Role of Bmp Ligands in the AER

In the AER of limbs that lacked *Bmp2* and *Bmp4*, *En1* was lost from the AER. *En1* has been shown to be essential for maturation of the AER and removal of this gene from the entire limb has been reported to result in a wider AER, an expansion of *Fgf8* and the formation of ectopic AERs on the ventral side of the limb bud due to defects in dorsal-ventral patterning (Loomis et al., 1996; Loomis et al., 1998). Interestingly, the only dorsal-ventral defects we observed upon removal of Bmp ligands from the AER were the presence of a nail plate, a dorsal structure, on the ventral side. These data suggest that *En1* expression in different regions of the limb may be required to repress specific dorsal structures.

In the *Bmp2*/*Bmp4* null AER we observed a striking increase in *Fgf4* and *Fgf8* expression in the AER similar to what has been reported in *En1* null embryos (Loomis et al., 1996; Loomis et al., 1998). The loss of *En1* in the AER suggests that AER-expressed BMP ligands function upstream of *En1*. It is not clear how this signaling cascade specifies *En1* expression only in the ventral half of the AER, however, in a wild type limb once *En1* is activated in the ventral AER this structure undergoes maturation and Fgf expression is limited. Removal of *Bmp2* and *Bmp4* or *En1* specifically from the AER results in a failure in AER maturation. Based on our data and previous work from Pajni-Underwood et al. (2007) we support the hypothesis that removal of BMP2/4 from the AER results in expansion of Fgf expression that then leads to an increase in cell proliferation and a decrease in apoptosis in the underlying mesoderm (Fig. 5-8).

A recent report elegantly showed that an Fgf/*Gremlin* inhibitory feedback loop triggers termination of limb bud outgrowth (Verheyden and Sun, 2008). During early stages of limb development FGF levels are low and *Gremlin* is expressed directly

underneath the AER where it inhibits Bmp signaling and maintains the *Shh*-Fgf feedback loop. As limb development proceeds, Fgf signaling increases and distal *Gremlin* expression is repressed. Our data supports a role for AER-mediated Bmp expression in this process. Upon removal of *Bmp2* and *Bmp4* from the AER we observe an increase in Fgf expression and a corresponding decrease in distal *Gremlin* expression in the limb bud mesenchyme.

Our data does not rule out the possibility that BMP ligands expressed in the AER can bind to ectodermal and/or mesodermal Bmp receptors. Since Bmps are secreted molecules it is possible that Bmps produced in the AER bind Bmp receptors in the AER and in the underlying mesoderm. However, we favor a model in which AER-expressed Bmps bind AER-expressed receptors and directly activate Bmp signaling in this tissue. In this model, a decrease in Bmp signaling in the AER results in failure of the AER to undergo maturation resulting in a decrease in *Gremlin* expression underneath the AER. In support of this model, proximal *Gremlin* expression is not altered upon removal of Bmp ligands from the AER suggesting that this domain of expression requires mesodermal and not ectodermal Bmp ligand expression.

We found that *Msx2* expression was downregulated in the AER upon removal of *Bmp2* and *Bmp4* from this tissue. This result is identical to what has been reported upon removal of Bmp signaling from the AER (*Bmpr1a* knockout (Pajni-Underwood et al., 2007)) suggesting that AER-expressed Bmps activate Bmp signaling within the AER. Removal of *Bmpr1a* from the limb mesenchyme has also been reported to cause a decrease in *Gremlin* expression. We propose that ectopic expression of Fgf genes in animals in which *Bmp2* and *Bmp4* have been removed from the AER is a consequence

of the failure of proper AER maturation due to the lack of *En1* and other unknown targets.

Materials and Methods

Mouse Strain Construction and Genotyping

The creation and genotyping of the conditional floxed alleles *Bmp2*$^{c/c}$ and *Bmp4*$^{c/c}$ have been described previously (Kulessa and Hogan, 2002; Bandyopadhyay et al., 2006; Tsuji et al., 2006; Chang et al., 2008). The Msx2-Cre allele has also been described previously (Sun et al., 2002). To create *Bmp2*$^{c/c}$; *Bmp4*$^{c/c}$, Msx2-Cre mice we first recombined the Msx2-Cre allele onto a chromosome containing the floxed *Bmp4*$^{c/c}$ allele. The Msx2-Cre transgene has been reported to have inserted into chromosome 14 very close to the gene *Bmpr1a* (Pajni-Underwood et al., 2007). *Bmp4* is located 12 million base pairs from *Bmpr1a* (Shore et al., 1998). To generate mice containing the *Bmp4* floxed allele in *cis* with the Msx2-Cre allele we mated mice heterozygous for both the *Bmp4* floxed allele and the Msx2-Cre allele to *Bmp4* homozygous females. Offspring obtained from this cross that were homozygous for the *Bmp4* floxed allele and also contained the Msx2-Cre allele must have undergone a meiotic recombination event placing the Msx2-Cre allele in *cis* with the *Bmp4* floxed allele. Screening of ~500 progeny revealed two mice, both males, which had undergone the desired recombination event. Males that were *Bmp4*$^{c/c}$, Msx2-Cre were then mated to *Bmp2*$^{c/c}$ homozygous females. Males from this cross that were heterozygous for both floxed alleles and contained the Msx2-Cre allele were crossed to females homozygous for both the *Bmp2* and *Bmp4* floxed alleles to create *Bmp2*$^{c/c}$; *Bmp4*$^{c/c}$, Msx2-Cre animals (referred to as “double mutants” in this report). Double mutant males were viable and were used to generate double mutant and control (lacking the Msx2-Cre allele) embryos

for analysis. All mouse strains were on a mixed genetic background. Animals were handled in accordance with the University of Florida Institutional Animal Care and Use Committee.

Whole Mount RNA *In Situ* Hybridization, Lysotracker Staining, Skeletal Preparations and Cell Proliferation Analysis

RNA whole mount *in situ* hybridization, Lysotracker Red (Molecular Probes) analysis and skeletal preparations were performed as described previously (Wilkinson, 1992; Murtaugh et al., 1999; Karp et al., 2000; Chi et al., 2003). Probes used to detect *Bmp2* and *Bmp4* deletion were against the floxed regions of these genes and have been described previously (Bandyopadhyay et al., 2006). At least three animals for each genotype were examined in all experiments. To detect cell proliferation levels in E11.5 hindlimbs anti-phospohistone H3 antibody (Cell Signaling Technology) was used at a 1:200 dilution. Three double mutants and three wild type animals were analyzed. The number of anti-phospohistone H3 positive cells from three 10 μm sections of each embryo were counted within a standardized sized box located at the distal edge of the limb (underneath the AER). Unpaired student t-test was used to test significance. All comparisons between mutant and wild type embryos were done with embryos from the same litter.

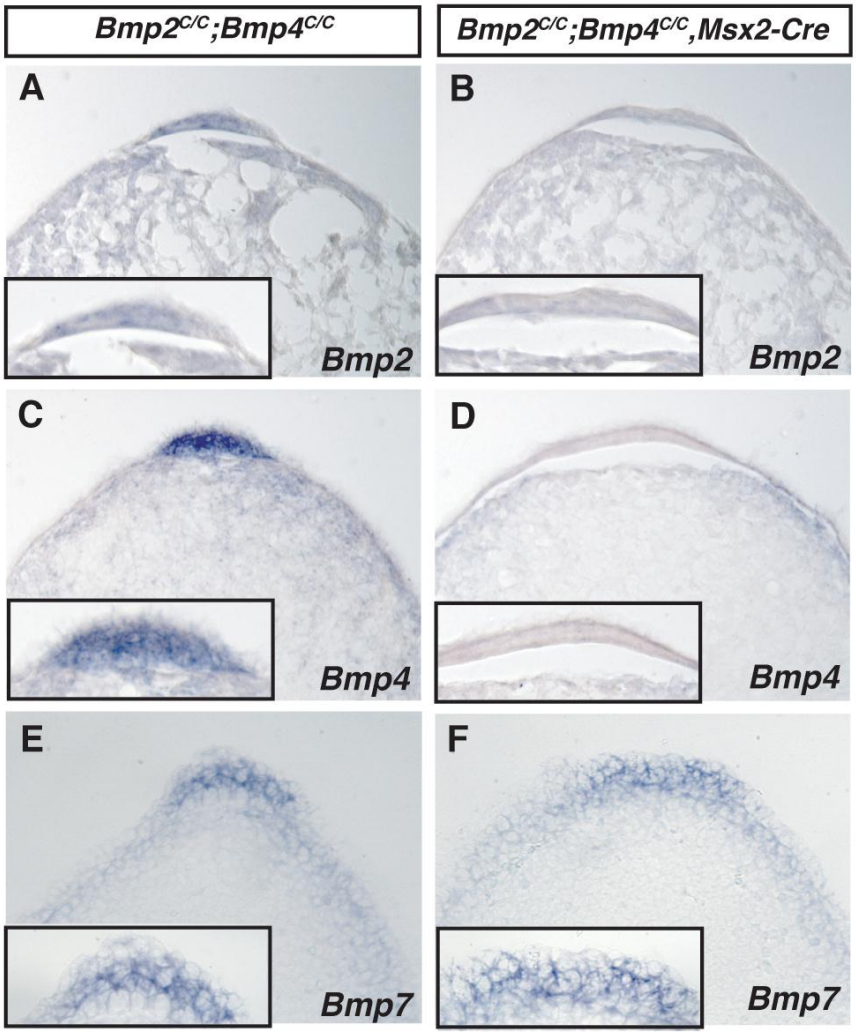

Figure 5-1. Msx2-Cre removes floxed *Bmp2* and *Bmp4* alleles from the AER. Section RNA *in situ* hybridizations for *Bmp2* (A, B), *Bmp4* (C, D) and *Bmp7* (E, F) in wild type ($Bmp2^{c}/^{c}$; $Bmp4^{c}/^{c}$) and double mutant ($Bmp2^{c}/^{c}$; $Bmp4^{c}/^{c}$, Msx2-Cre) hindlimb buds. Animals homozygous for both the floxed *Bmp2* and *Bmp4* alleles were wild type (see text). Double mutant and the corresponding wild type limb buds from littermates are shown. A 20 µm sagittal section containing the AER is shown. Insets are a zoomed view of the AER. Note the expanded AER present in the double mutants (B, D, F). All limbs are from E10.5 embryos. Comparison between mutant and wild type limb buds were done with littermates. Mutant limbs, compared to wild type littermates were usually larger. This was most likely caused by an increase in cell proliferation and a decrease in cell death (see text). Reprinted with permission from *In the limb AER Bmp2 and Bmp4 are required for dorsal-ventral patterning and interdigital cell death but not limb outgrowth*. Maatouk DM, Choi KS, Bouldin CM, Harfe BD; Copyright 2009 Dev Biol.

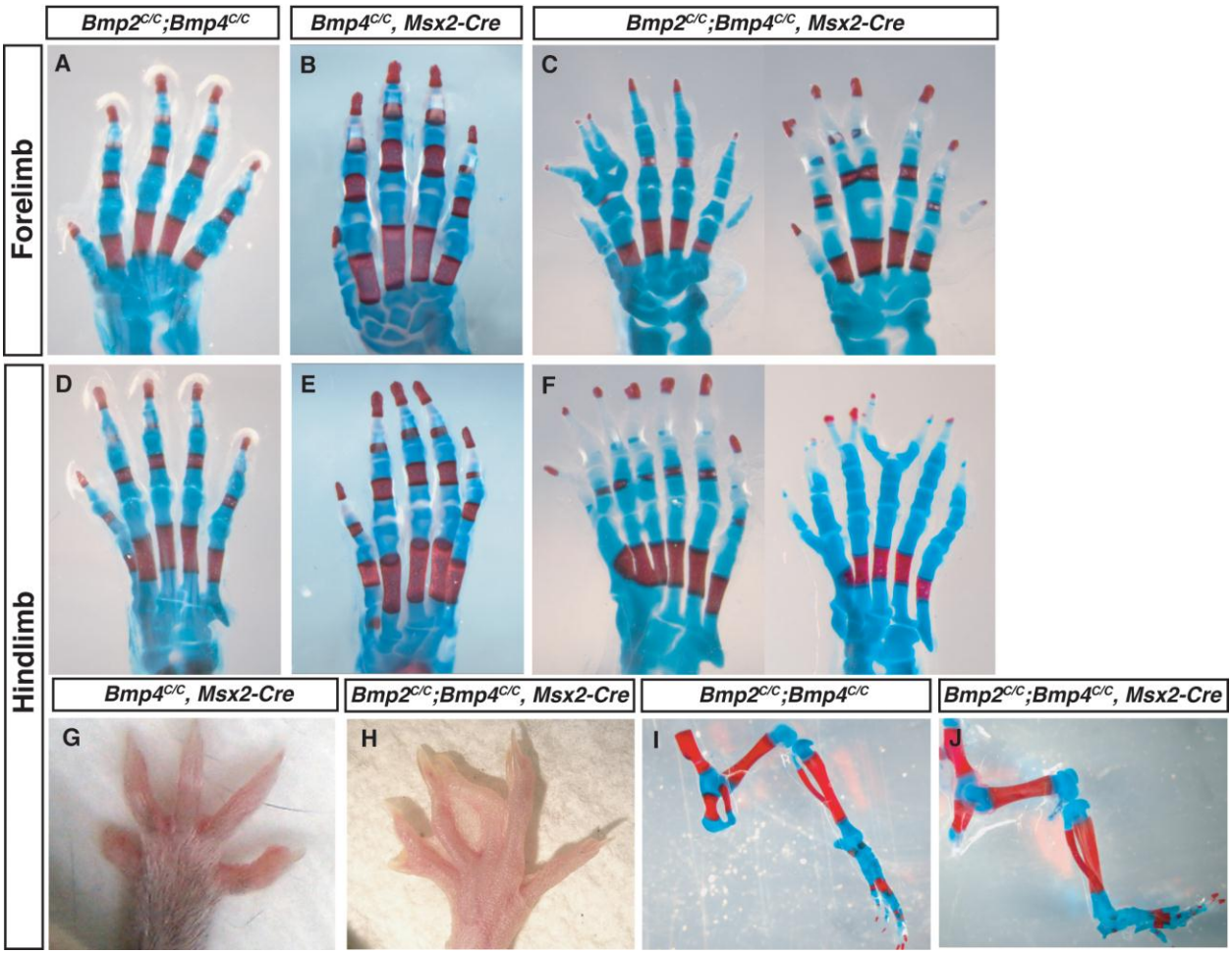

Figure 5-2. Removal of *Bmp2* and *Bmp4* from the AER results in polydactyly, syndactyly and retention of interdigital tissue. Skeletal preparations of wild type (A, D) and mutant (B, C, E, F) fore- and hindlimbs of newborn mice. Removal of both *Bmp4* alleles (B, E) or both *Bmp2* alleles (data not shown) in the AER did not result in the production of a visible phenotype. Two examples of the skeletal defects present in the double mutants are shown (C, F). Bright-field images of adult $Bmp4^{c}/^{c}$, Msx2-Cre (G) and $Bmp2^{c}/^{c}$; $Bmp4^{c}/^{c}$, Msx2-Cre hindlimbs (H). Note the absence of webbing in $Bmp4^{c}/^{c}$, Msx2-Cre limbs. No proximal-distal defects were observed in limbs in which both *Bmp2* and *Bmp4* had been removed from the AER (I, J). Reprinted with permission from *In the limb AER Bmp2 and Bmp4 are required for dorsal-ventral patterning and interdigital cell death but not limb outgrowth*. Maatouk DM, Choi KS, Bouldin CM, Harfe BD; Copyright 2009 Dev Biol.

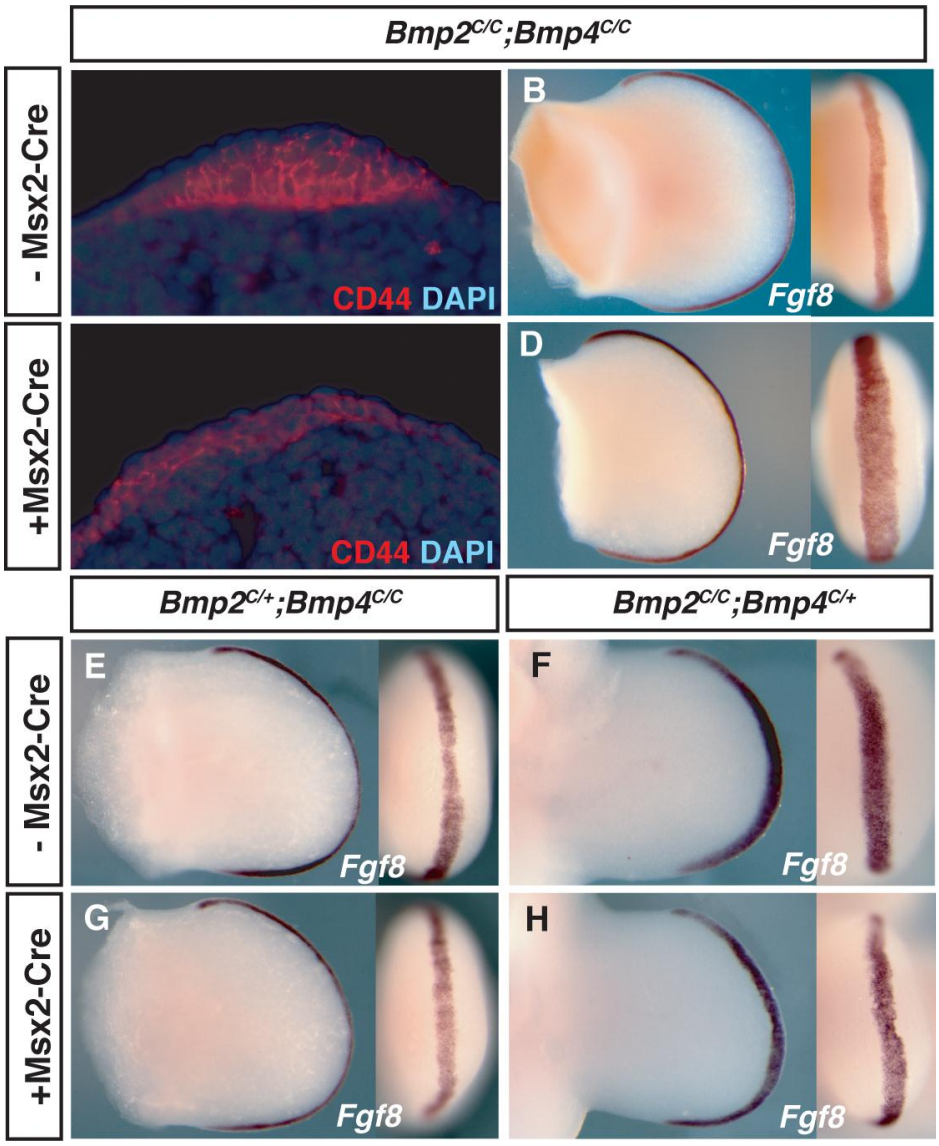

Figure 5-3. AER expansion upon removal of *Bmp2* and *Bmp4*. (A, C) A section stained for CD44 through the AER of an E11.5 limb revealed an elongated and thinner AER in limb buds in which *Bmp2* and *Bmp4* had been removed from the AER. (B, D) In double mutant limb buds *Fgf8* expression was expanded both in the dorsal-ventral and anterior-posterior directions. (E-H) No ectopic expression of *Fgf8* was observed in limbs that contained at least a single wild type allele of either *Bmp2* or *Bmp4*. Reprinted with permission from *In the limb AER Bmp2 and Bmp4 are required for dorsal-ventral patterning and interdigital cell death but not limb outgrowth*. Maatouk DM, Choi KS, Bouldin CM, Harfe BD; Copyright 2009 Dev Biol.

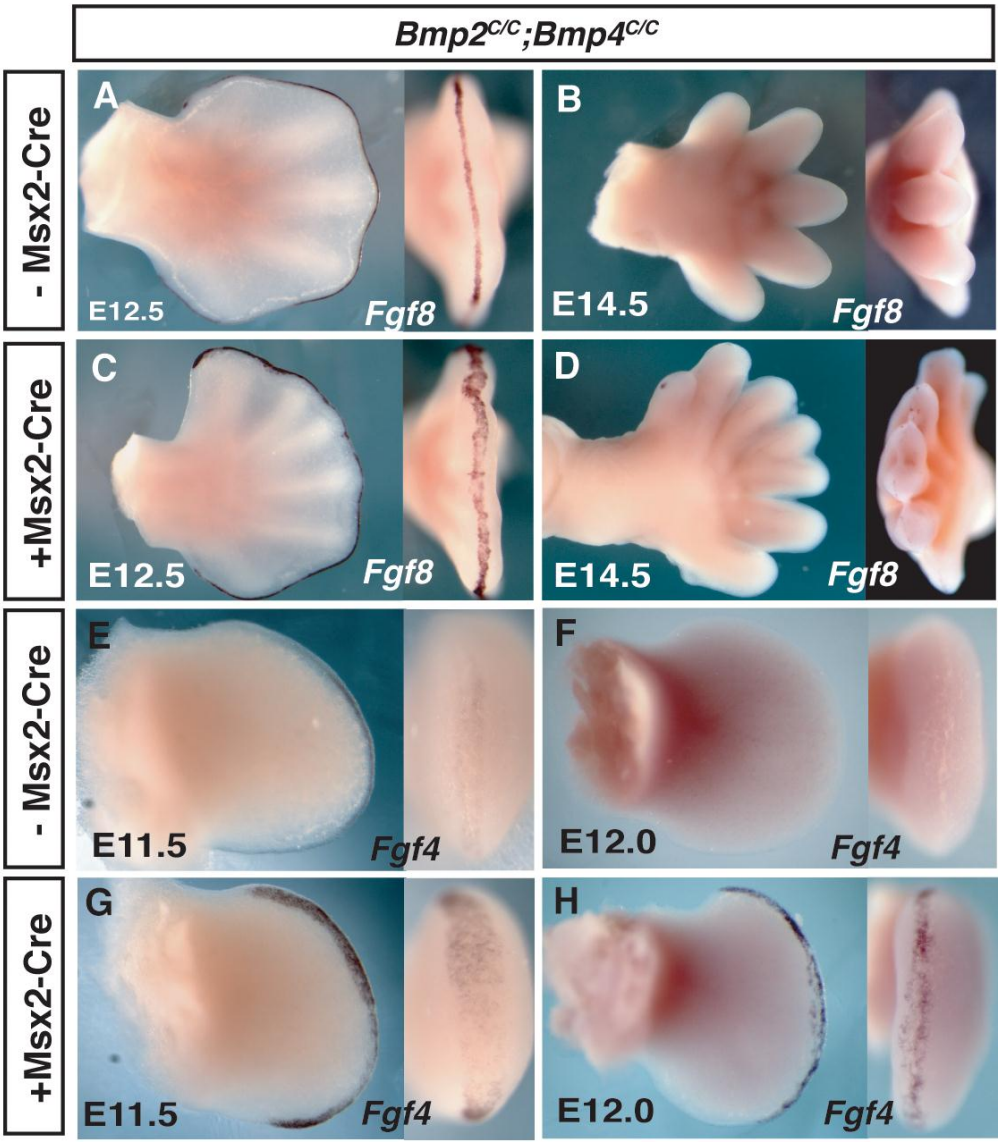

Figure 5-4. Ectopic *Fgf4* and *Fgf8* expression occurs in double knockout limbs. In double mutant E11.5-14.5 limb buds *Fgf8* (A-D) and *Fgf4* (E-H) expression was expanded both in the dorsal-ventral and anterior-posterior directions. In addition, expression of *Fgf4* (F, H) and *Fgf8* (B, D) was observed to persist for an expanded amount of time. Reprinted with permission from *In the limb AER Bmp2 and Bmp4 are required for dorsal-ventral patterning and interdigital cell death but not limb outgrowth*. Maatouk DM, Choi KS, Bouldin CM, Harfe BD; Copyright 2009 Dev Biol.

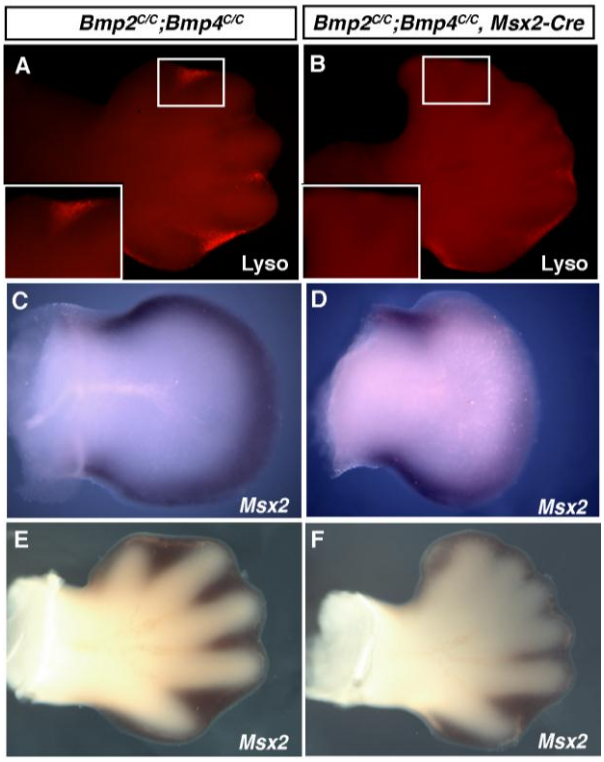

Figure 5-5. AER expression of *Bmp2* and *Bmp4* is required for regulating cell death, cell proliferation and *Gremlin* expression in the limb mesenchyme. (A, B) Lysotracker red assay to measure cell death in E13.5 hindlimb buds. Removal of both alleles of *Bmp2* and *Bmp4* in the AER resulted in a decrease in cell death (compare A and B). The decrease was more pronounced in the anterior of the limb where almost all cell death was abolished (insets in A and B). Box insets are close-up views of the anterior limb. (C, D) In limbs in which both *Bmp2* and *Bmp4* were absent in the AER, *Msx2* was not expressed in this tissue but was still present in the mesenchyme. In both E11.5 (C and D) and E12.5 (E and F), lower amounts of *Msx2* was present in the limb mesenchyme upon removal of *Bmp2* and *Bmp4*. (G, H) Distal Gremlin expression in the limb mesenchyme was reduced in E10.5 hindlimb buds in which Bmp2 and Bmp4 were removed from the AER. Proximal expression was not altered. Lines indicate the distance from the AER to distal limit of Gremlin expression. (I-K) Bmp ligands expressed in the AER are required for regulating cell proliferation in the underlying mesenchyme. Removal of both alleles of Bmp2 and Bmp4 resulted in an increase in cell proliferation (compare I and J). Images shown are transverse sections of the distal end of an E10.5 limb bud. (K) Quantification of the number of anti-phosphohistone H3 positive cells demonstrated that there was a significant increase in the number of proliferating cells in double mutant limbs (*p-value = 0.002). Reprinted with permission from *In the limb AER Bmp2 and Bmp4 are required for dorsal-ventral patterning and interdigital cell death but not limb outgrowth*. Maatouk DM, Choi KS, Bouldin CM, Harfe BD; Copyright 2009 Dev Biol.

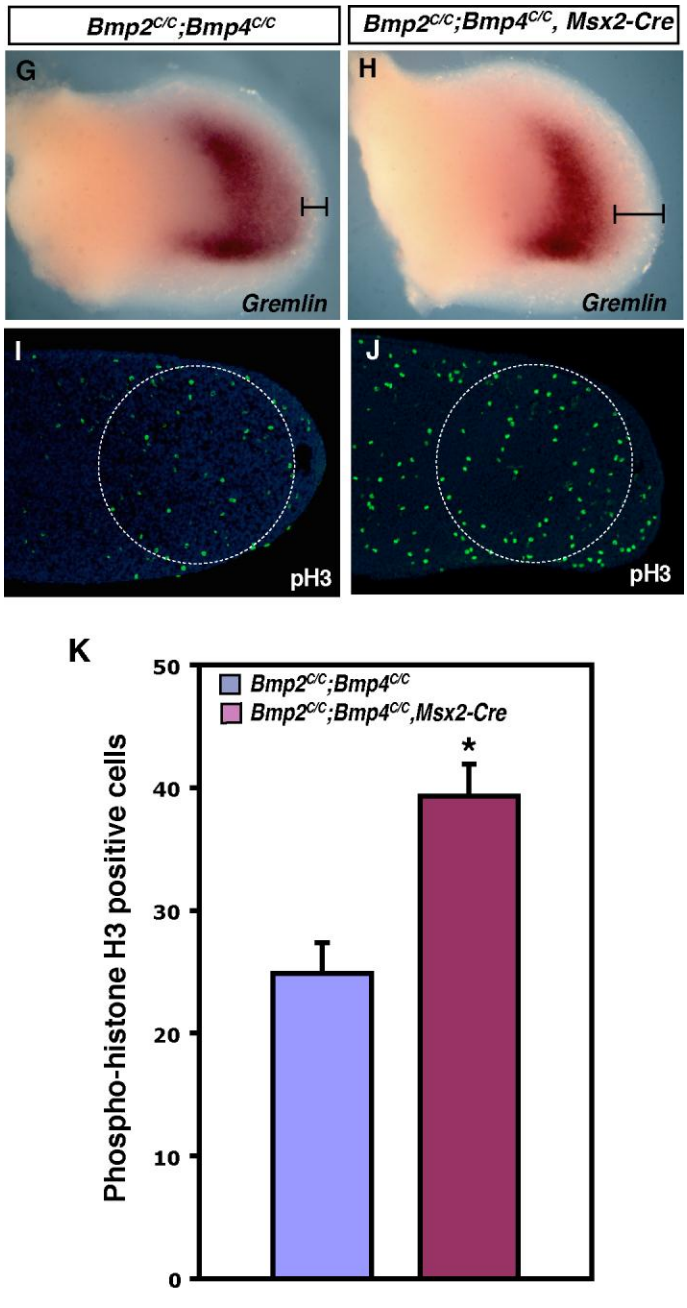

Figure 5-5. Continued

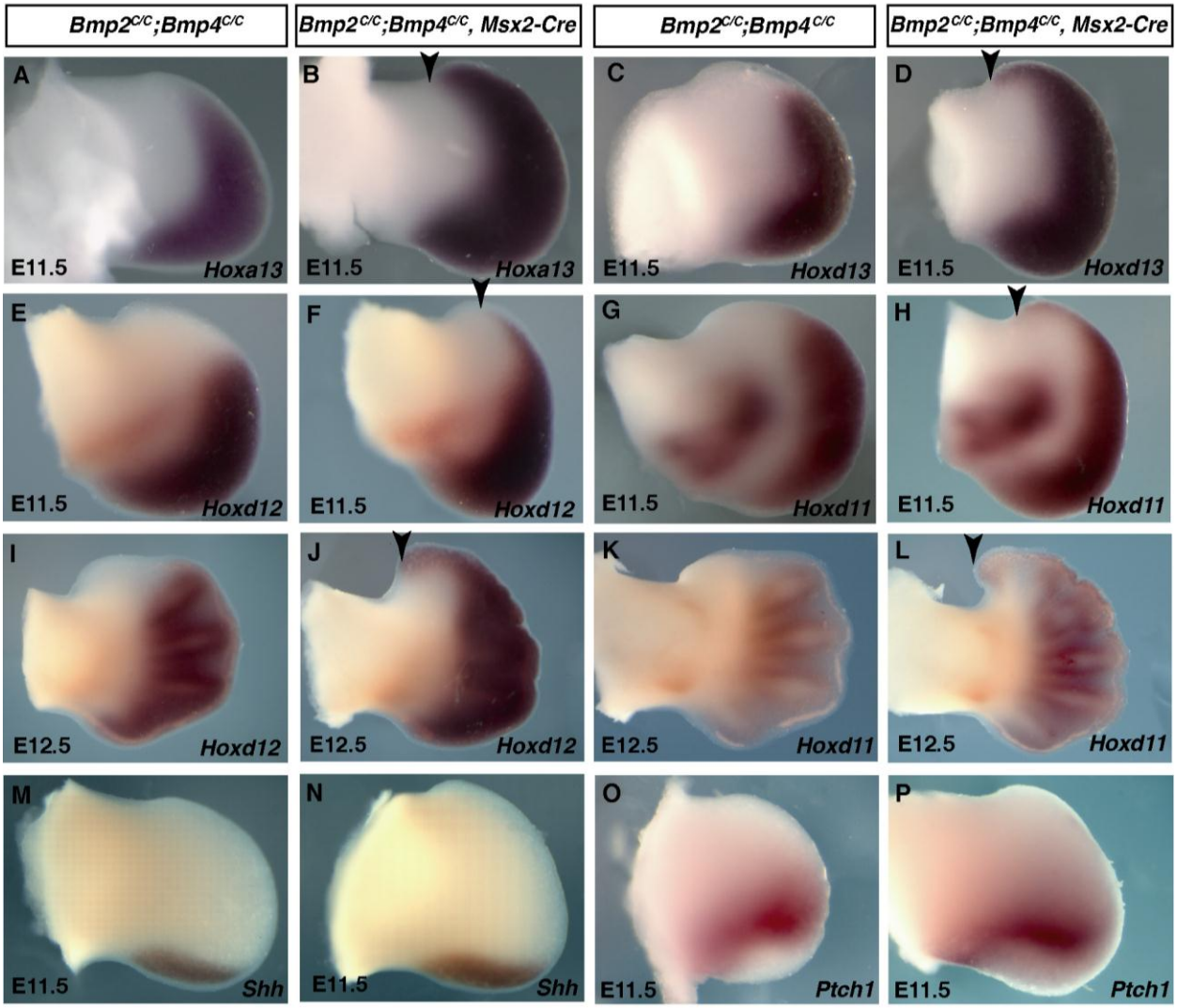

Figure 5-6. Anterior expansion of Hox genes but not Hedeghog signaling occurs in the limb mesenchyme upon removal of *Bmp2* and *Bmp4* from the AER. *Hoxa13* and *Hoxd11-13* expression was expanded in the anterior of E11.5 (A-H) and E12.5 (I-L) limb buds in which both *Bmp2* and *Bmp4* were removed from the AER (arrowheads). Expression of *Shh* (M, N) and the Hedgehog target gene *Ptch1* (O, P) were not altered upon removal of *Bmp2* and *Bmp4* from the AER. All images shown are of hindlimbs. Reprinted with permission from *In the limb AER Bmp2 and Bmp4 are required for dorsal-ventral patterning and interdigital cell death but not limb outgrowth*. Maatouk DM, Choi KS, Bouldin CM, Harfe BD; Copyright 2009 Dev Biol.

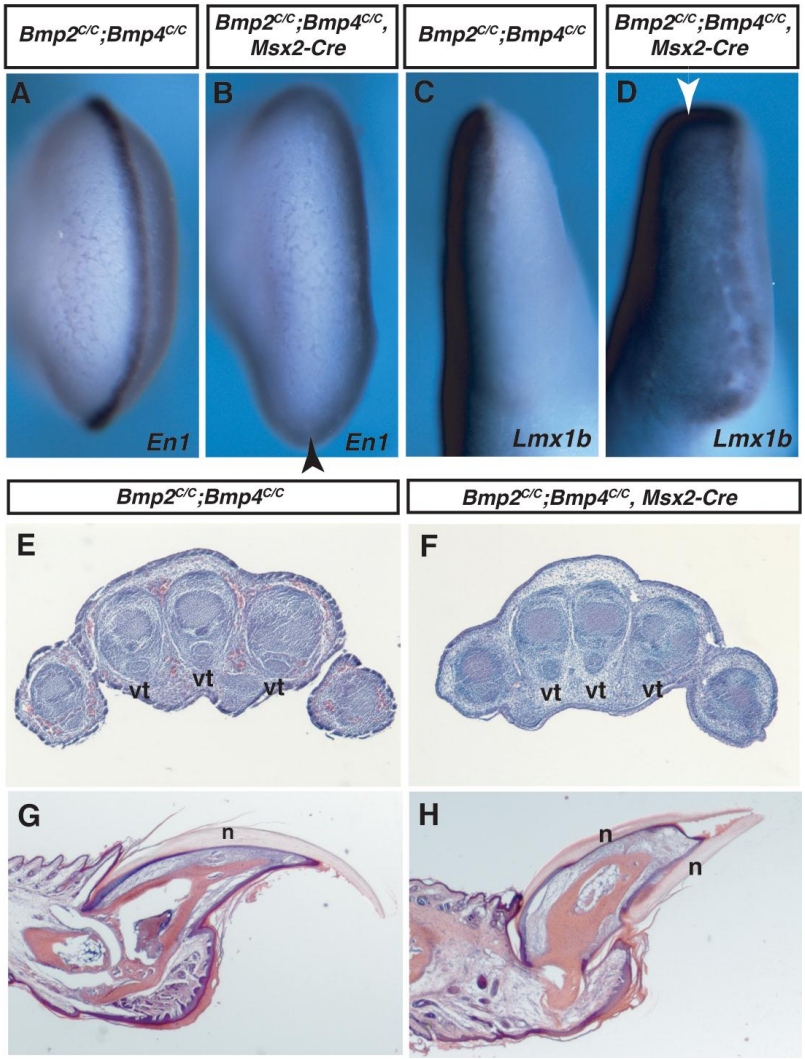

Figure 5-7. Bmp ligands expressed in the AER are required for dorsal-ventral patterning. (A) *En1* is expressed in the AER of E11.5 hindlimb buds. (B) Upon removal of both *Bmp2* and *Bmp4* from the AER, *En1* expression was abolished in the AER of E11.5 limb buds. (C, D) *Lmx1b*, a gene expressed only in the dorsal mesoderm, was ectopically expressed in the ventral mesoderm in double mutant limbs (arrows in D). A-D are "edge" views of E11.5 hindlimb buds. (E, F) The positions of tendons, a ventral mesodermal structure, were not altered in double mutants. Transverse section through an E15.5 limb is shown. vt=ventral tendon. (G, H) Sagittal section of a one month-old limb showing the presence of an ectopic nail plate (n) on the ventral side of a limb in which *Bmp2* and *Bmp4* were removed from the AER. Reprinted with permission from *In the limb AER Bmp2 and Bmp4 are required for dorsal-ventral patterning and interdigital cell death but not limb outgrowth*. Maatouk DM, Choi KS, Bouldin CM, Harfe BD; Copyright 2009 Dev Biol.

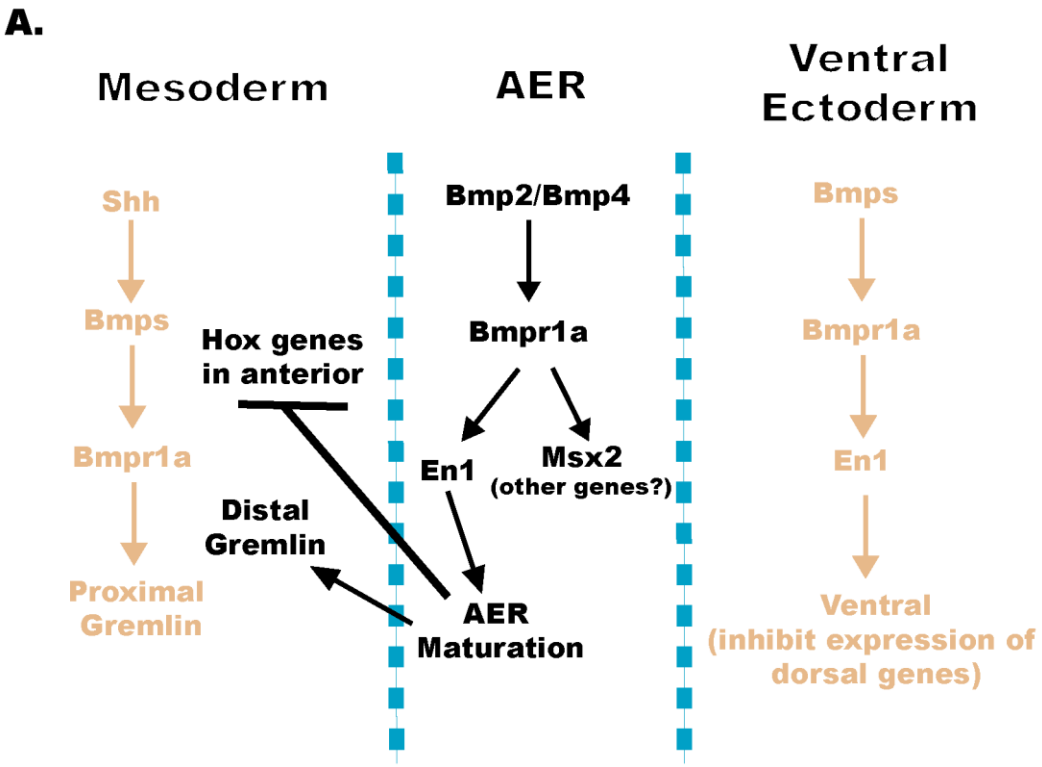

Figure 5-8. Model: Proposed role of BMP ligands in the AER. (A) In wild type limb buds *Bmp2* and *Bmp4* expressed in the AER activate the Bmp signaling pathway through the Bmp receptor *Bmpr1a* cell autonomously. Activation of this pathway results in *En1* and *Msx2* expression in the AER. (B) Removal of *Bmp2* and *Bmp4* from the AER results in loss of *En1* and *Msx2* expression which causes a failure in AER maturation leading to an expanded AER in both the dorsal-ventral and anterior/posterior directions. The expanded AER expresses Fgf genes at elevated levels leading to a decrease in distal *Gremlin* expression, a decrease in cell death and an increase in cell proliferation in the underlying mesenchyme. In the mesenchyme the BMP signaling pathway is still functional upon AER Bmp removal. Model shows limb development during the "promoting" phase, prior to regression of distal *Gremlin* expression (Verheyden and Sun, 2008). See text for additional details. Reprinted with permission from *In the limb AER Bmp2 and Bmp4 are required for dorsal-ventral patterning and interdigital cell death but not limb outgrowth*. Maatouk DM, Choi KS, Bouldin CM, Harfe BD; Copyright 2009 Dev Biol.

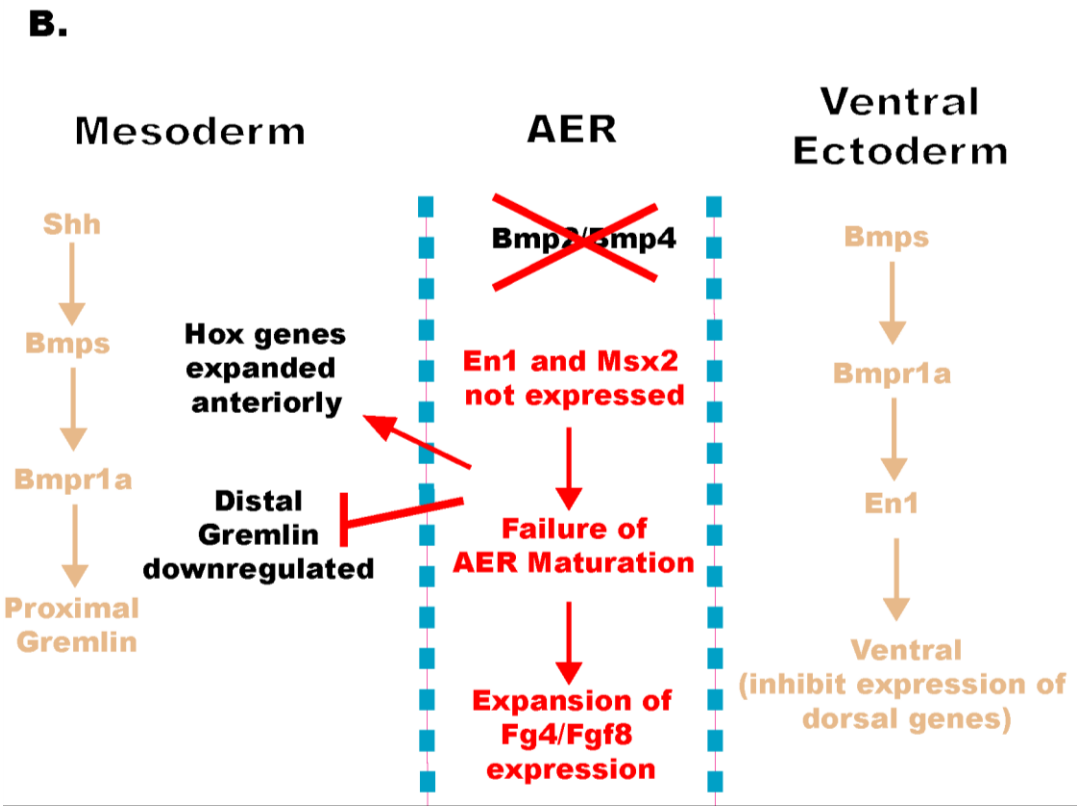

Figure 5-8. Continued

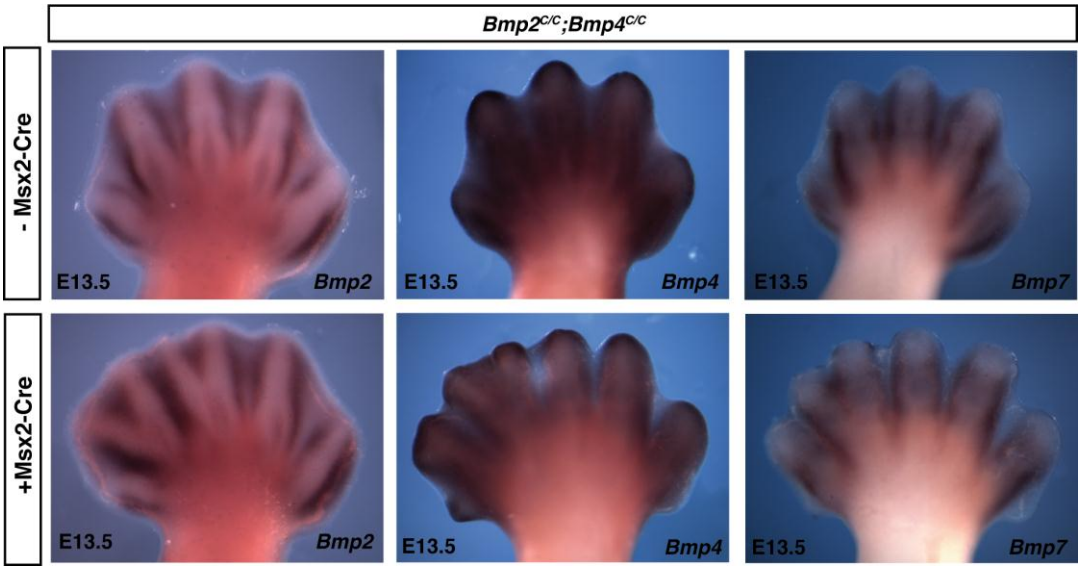

Figure 5-9. Bmp expression in the mesenchyme is not altered in the double mutants. In E13.5 limbs, Bmp2 (A, B), Bmp4 (C, D) and Bmp7 (E, F) were expressed in the interdigital tissue in double mutants. Reprinted with permission from *In the limb AER Bmp2 and Bmp4 are required for dorsal-ventral patterning and interdigital cell death but not limb outgrowth*. Maatouk DM, Choi KS, Bouldin CM, Harfe BD; Copyright 2009 Dev Biol.

CHAPTER 6
CONCLUDING REMARKS

This study investigated how signaling molecules control morphogenesis of the intervertebral disc and vertebrate limb. In Chapter 3 we demonstrated that the entire nucleus pulposus originates from the embryonic notochord. Prior to this study, the origin of nucleus pulposus was under debate. To address this question, we generated a fate map of notochord cells using the *Shhcre* allele and *R26R* reporter allele and confirmed that the notochord gives rise to the entire nucleus pulposus during embryogenesis. To determine if the adult nucleus pulposus was derived from the embryonic notochord we fate-mapped notochord cells using the *Shhcre* allele and examined reporter expression in skeletally mature animals. From our data we concluded that mature nucleus pulposus cells (chondrocyte-like cells) are derived from the embryonic notochord but not from the surrounding mesenchyme.

It is important to note that not all notochord cells give rise to the nucleus pulposus. Some notochord cells that did not end up in the nucleus pulposus formed notochordal remnants (Chapter 3). These notochordal remnants were first found during the transition from the notochord to nucleus pulposus. Most notochordal remnants underwent apoptosis but some of them persisted throughout life. A rare tumor called chordoma is thought to arise from these notochordal remnants. Although it is unclear how notochordal remnants cause chordoma, the identification of notochordal remnants in mice might make it possible to generate a mouse model for the study of chordoma *in vivo*.

In addition to fate mapping notochord cells, we determined the role hedgehog signaling plays in the formation of intervertebral discs (Chapter 4). We genetically

abolished *Smoothened (Smo)* from all *Shh*-expressing cells and demonstrated that hedgehog signaling from the notochord was required for the formation of the notochord sheath. The notochord sheath is essential for maintaining the rod-like structure of the notochord. Moreover, the notochord sheath might support the structure of notochord from swelling pressure from vertebral bodies and internal swelling pressure from vacuolization of cells located within the notochord.

We also investigated the temporal role of Shh signaling during the formation of the intervertebral disc (Chapter 4) by removing Shh signaling at different time points. We propose that Shh signaling regulates the formation of the intervertebral disc in two phases. Initially, Shh signaling regulates the formation of the notochord sheath in order to maintain the structure of the notochord. The later, second phase of Shh signaling indirectly regulates the migration of notochord cells into the forming. Our data cannot exclude the possibility that hedgehog signaling regulates unknown pathways that are responsible for the migration of notochord cells into intervertebral regions.

In addition to intervertebral disc development, we investigated the role of BMP signaling in the AER (Chapter 5). *Bmp2, Bmp4* and *Bmp7* are expressed in the AER. Removal of individual Bmps did not show defects in limb patterning suggesting that individual BMP proteins may play partially redundant roles during limb development. To investigate the role of *Bmp2* and *Bmp4*, we genetically removed *Bmp2* and *Bmp4* in the AER. Our result showed that *Bmp2* and *Bmp4* are required for normal cell proliferation in the mesenchyme, normal cell death in the interdigital region and dorsoventral boundary formation in the limb. However, *Bmp2* and *Bmp4* signaling are not required

for limb outgrowth. It is possible that *Bmp7* might compensate for loss of *Bmp2* and *Bmp4* expression in the AER.

In this work we have identified and characterized signaling molecules that play essential roles during patterning of the intervertebral discs and limbs. Knowledge of the role hedgehog signaling and Bmp signaling play in the formation of these structures will allow us to begin to understand that signaling pathways required for formation of these structures during normal development. This is a required first step for developing therapies to heal damaged discs and limbs.

APPENDIX A
OLIGONUCLEOTIDES USED AS GENOTYPING PRIMERS

Table A-1. Oligonucleotides used as genotyping primers.

Allele	Name		Sequence
Bmp2	Bmp2	F	5'-GTGTGGTCCACCGCATCAC
Bmp2	Bmp2	R	5'-TGCCATCATCACTTCCTGAC
Bmp4	Bmp4	F	5'-AGACTCTTTAGTGAGCATTTTCAAC
Bmp4	Bmp4	R	5'-AGCCCAATTTCCACAACTTC
Bmp7	Bmp7 wt	F	5'-ACCCCAGGTCAAGACACCAAA
Bmp7	Bmp7 wt	R	5'-AGGCGCTGAATTGTCAGCTA
Bmp7	Bmp7 floxed	R	5'-CAGCAGCCTCTGTTCCACATACAC
Cre	Cre	F	5'-TGACGGTGGGAGAATGTTAAT
Cre	Cre	R	5'-GCCGTAAATCAATCGATGAGT
Eyfp	Eyfp wt	F	5'-GGAGCGGGAGAAATGGATATG
Eyfp	Eyfp wt	R	5'-AAAGTCGCTCTGAGTTGTTAT
Eyfp	Eyfp mutant	R	5'-AAGACCGCGAAGAGTTTGTC
Msx2cre	Msx2cre	F	5'-AACATCTTCAGGTTCTGCGG
Msx2cre	Msx2cre	R	5'-GACTTTTCAGTTTGGGCG
R26R	R26R wt	F	5'-AAAGTCGCTCTGAGTTGTTAT
R26R	R26R wt	R	5'-GGAGCGGGAGAAATGGATATG
R26R	R26R mutant	R	5'-GCGAAGAGTTTGTCCTCAACC
Shh	Shh floxed	F	5'-ATGCTGGCTCGCCTGGCTGTGGAA
Shh	Shh floxed	R	5'-GAAGAGATCAAGGCAAGCTCTGGC
Smo	Smo wt	F	5'-CCACTGCGAGCCTTTGCGCTAC
Smo	Smo wt	R	5'-CCCATCACCTCCGCGTCGCA
Smo	Smo floxed	F	5′-ATGGCCGCTGGCCGCCCCGTG
Smo	Smo floxed	R	5'-GGCGCTACCGGTGGATGTGG

APPENDIX B
PROBES USED FOR RNA *IN SITU* HYBRIDIZATION

Table B-1. Probes used for RNA in situ hybridization.

Gene name	Plasmid number	Restriction enzyme	Antisense polymerase
Bmp2	BH63	SacII	SP6
Bmp2	BH219	XbaI	T3
Bmp4	BH62	HindIII	T7
Bmp7	BH33	HindIII	T7
En1	BH213	ClaI	T7
Fgf4	BH144	BamHI	T3
Fgf8	BH61	PstI	T7
Gremlin	BH75	PstI	T3
Hoxa13	Bh84	NcoI	SP6
Hoxd11	Bh143	SalI	T7
Hoxd12	BH172	BamHI	T7
Hoxd13	BH216	XhoI	Sp6
Lmx1b	BH71	HindIII	T3
Msx2	Bh215	BamHI	T7
Pax1	BH264	T7-T3 PCR	T3
Pax3	BH259	T7-T3 PCR	T7
Ptch1	BH141	BamHI	T3
Shh	BH39	HindIII	T3
Shh exon2	BH271	HindIII	T7

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BIOGRAPHICAL SKETCH

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