The Function of Smad4 in Skeletal Development

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For the degree of Masters of Science in Biology

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ABSTRACT

The Function of Smad4 in Skeletal Development

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Smad4 is the central intracellular mediator of transforming growth factor-β (TGF-β) and BMP signaling pathways. To study the role of Smad4 in skeletal development, we introduced a conditional mutation of the gene in chondrocytes using Cre-loxP system. A high level of Smad4 gene deletion was verified in Cre expressing mice. In vivo, deletion of Smad4 resulted in normal early limb development. That is, the mesenchyme condensed in the correct pattern. However, later in development, depletion of Smad4 resulted in defects in the development of the skeletal elements. The skull was smaller and the limbs were shorter than controls, the deltoid tuberosity was missing, and the joints of the phalanges were fused. The rib cage was smaller and flattened which could lead to respiratory distress and subsequent post-natal death. The abrogation of Smad4 in chondrocytes resulted in dwarfism with a severely disorganized growth plate characterized by expanded resting zone of chondrocytes, reduced chondrocyte proliferation. My data also indicated that loss of Smad4 has a huge impact on the activation of Smad2 and p38 and that phosphorylation of Smad2 and p38 are Smad4 dependent. However, Smad4 is not required for the activation of Smad1/5. Therefore, since loss of Smad4 caused a decreased pSmad2 and p-p38 level, the cells might respond to TGF-β or BMP signaling with a compensatory upregulation of pSmad1/5 level. All
these data provided evidence demonstrating that Smad4-mediated TGF-β and BMP signals are required for maintaining the normal organization of chondrocytes in the growth plate, and that Smad4 is required for normal development of the skeleton.
INTRODUCCIÓN

Bones are rigid organs that form a major part of the endoskeleton of vertebrates. Their function is to move, support, and protect the various organs of the body, produce red and white blood cells and store minerals. Bone formation during the fetal stage of development occurs by two processes: Intramembranous ossification and endochondral ossification. Intramembranous ossification mainly occurs during formation of the flat bones of the skull, the mandible, maxilla, and clavicles, formation of bone tissue occurs by differentiating the cells of these condensations directly into bone forming osteoblast without a preliminary cartilage stage. Endochondral ossification, on the other hand, occurs in long bones and most of the rest of the bones in the body; and it involves the conversion of cartilage into bone.

Most of skeletal system forms through endochondral ossification in which a series of consecutive and strictly regulated differentiation steps are involved. Chondrogenesis is the earliest step for endochondral bone formation and it begins with the condensation of mesenchymal cells, which differentiate into chondrocytes forming cartilaginous templates. Chondrocytes have a characteristic shape and secrete a matrix rich in type II collagen and the proteoglycan aggregan. The surrounding mesenchymal cells differentiate into fibroblastic cells to form the perichondrium. The cartilage tissues known as the growth plate locating at the both extremities of long bones continuously proceed through programmed proliferation, maturation, hypertrophic and finally terminal hypertrophic differentiation. Hypertrophic chondrocytes direct adjacent perichondrial cells to
differentiate into osteoblasts that form a bone collar. The region of terminal hypertrophic chondrocytes is further invaded by blood vessels followed by osteoblasts and osteoclasts, which start to replace the cartilaginous extracellular matrix (ECM) with the bone ECM. In humans, growth plates disappear at the time of adolescence after a burst of pubertal activity. The cartilage will eventually be replaced by the bone tissue through the endochondral ossification. Most importantly, this differentiation process is driven along the defined orientation and with the synchronous rate. During this process, the proliferation and differentiation of chondrocytes should be tightly regulated to maintain the normal skeletal development.
Figure 1. Endochondral Bone Formation. a) Mesenchymal cells condense. b) Cells of condensations become chondrocytes (c). c) Chondrocytes at the centre of condensation stop proliferating and become hypertrophic (h). d) Perichondrial cells adjacent to hypertrophic chondrocytes become osteoblasts, forming bone collar (bc). Hypertrophic chondrocytes direct the formation of mineralized matrix, attract blood vessels, and undergo apoptosis. e) Osteoblasts of primary spongiosa accompany vascular invasion, forming the primary spongiosa (ps). f) Chondrocytes continue to proliferate, lengthening the bone. Osteoblasts of primary spongiosa are precursors of eventual trabecular bone; osteoblasts of bone collar become cortical bone. g) At the end of the bone, the secondary ossification centre (soc) forms through cycles of chondrocyte hypertrophy, vascular invasion and osteoblast activity. The growth plate below the secondary centre of ossification forms orderly columns of proliferating chondrocytes (col).
Members of the TGF-β superfamily play important roles in the regulation of bone development. The bone morphogenetic proteins BMPs form a subgroup of molecules within the TGF-β superfamily and were identified by their ability to induce early cartilage formation. Misexpression of the BMP antagonist, Noggin, prior to the onset of chondrogenesis results in the absence of formation of condensations. Overexpression of Noggin in the cartilage of the transgenic mice causes the absence of nearly all cartilages. During the latter stages of endochondral ossification, BMP genes and their receptors are expressed in specific regions of the developing cartilage elements (Yi et al., 2000; Daluiski et al., 2001). Several in vitro and in vivo studies have shown that BMP signaling promotes the chondrocyte proliferation and expansion of all zones of differentiation in the skeletal elements (Lyons et al. 2009). Notably, all these functions of BMPs have been established using in vitro culture systems or overexpression systems. Targeted disruption of BMPs either leads to mice with no significant phenotypes or embryonic lethality due to the functional redundancy of large number of ligands and receptors, as well as basic roles of BMPs during early embryonic development (Kronenberg, 2003). Studies that delete several BMP genes in cartilage tissues are done to fully understand the roles of BMPs during endochondral ossification.

TGF-β genes are also expressed abundantly during endochondral ossification. Many studies have shown that TGF-β genes play important roles in regulating chondrocyte proliferation and differentiation. The transgenic mice expressing a dominant-negative mutation of the TGF-β gene type II receptor developed degenerative joint disease resembling human osteoarthritis, suggesting that TGF-β signaling can
inhibit the hypertrophic differentiation of articular chondrocytes (Serra et al., 1997). However, the underlying molecular mechanism is still largely unclear.

Smad transcription factors lie at the core of TGF-β and BMP signaling pathways. The striking ability of Smad proteins to accumulate in the nucleus in response to TGF-β and BMP was one of the key observations placing Smads downstream of the TGF-β and BMP receptors (Massague et al. 2005). There are eight Smad proteins that are encoded in the human and mouse genomes, and they are divided into three functional classes: the receptor-regulated Smads or RSmads (Smad1, 2, 3, 5 and 8). Smads 1, 5, and 8 serve as substrates for BMP receptors and Smads 2 and 3 serve as substrates for the TGF-β receptors and activin. The co-activator Smad (Smad4) also known as Co-Smad serves as a common partner for all the RSmads. The inhibitory Smads (Smad6 and Smad7) that serve as decoys interfering with Smad-receptor or Smad-Smad interactions.

At least two distinct pathways mediate BMP and TGF-β signaling: the canonical Smad pathway and a mitogen-activated protein kinase (MAPK) pathway, also known as noncanonical pathway. In Smad-canonical pathway, receptor-regulated Smads form heterodimers with Smad4, and then translocate into the nucleus to induce or repress the expression of BMP and TGF-β target genes. TGF-β and BMP transduce signals through formation of heteromeric complexes of type I and II serine/threonine kinase receptors. Upon TGF-β or BMP binding, type II receptors phosphorylate serine/threonine residues in type I receptors. Activated type I receptors phosphorylate and thereby activate RSmads. Subsequently, RSmads recruit and bind Co-Smads to form heteromeric complexes. These Smad complexes enter the nucleus and bind DNA directly, or interact with DNA binding proteins to regulate the transcription of target genes. In noncanonical
pathway, BMPs can signal by activating TGF-β activated kinase 1 (TAK1). TAK1 leads to the activation of p38 MAPKs. The aspects of BMP signaling in the chondrocytes mediated by p38 MAPKs are unclear (Lyons et al., 2009).

**Figure 2. Smad4 Canonical Pathway.** The ligand binds to pairs of membrane receptor serine/threonine kinases (receptor types I and II), promoting the formation of a heterotetrameric receptor complex. The cytoplasmic region of the type I receptor contains a canonical protein kinase domain (purple) preceded by a regulatory region (green) to which the inhibitor (red) binds to enforce the inactive basal state. Phosphorylation of the regulatory region by the type II receptor creates a site for receptor-regulated Smad proteins (RSmad). Anchor proteins capture RSmads for presentation to the activated type I receptor. Receptor-mediated phosphorylation of RSmads allows the accumulation of RSmad in the nucleus and the recognition of Smad4. The resulting Smad complex incorporates different DNA-binding cofactors that confer target gene selectivity and influence the recruitment of either transcriptional coactivators or corepressors. Several
hundred genes are regulated by TGF-β and BMP in this fashion. Rsmad dephosphorylation terminates the signaling cycle.

The Smad4 gene is located on the long arm of chromosome 18 at position 21.1 in humans. Smad4 is also known as DPC4, JIP, MADH4, and MAD. The name Smad was coined with the identification of human Smad1 in reference to its sequence similarity to the Sma and Mad proteins (Massague et al. 2005). People with mutations in the Smad4 gene appear to have an increased risk of developing various cancers. Some of these gene mutations are inherited, while others are acquired during a person's lifetime. Cells with mutations in the Smad4 gene, whether inherited or somatic, may proliferate out of control and result in a tumor, often in the colon or pancreas (Massague et al. 2005).

Mutation analyses in mice using gene targeting have revealed multiple important functions of Smad genes in various developmental processes (Weinstein et al., 2000), including endochondral ossification. Previous studies have shown that targeted disruption of Smad3 resulted in a degenerative joint disease resembling human osteoarthritis. This suggests that Smad3 is required for maintaining articular cartilage by repressing chondrocyte hypertrophic differentiation (Yang et al. 2001). A missense mutation of the Smad3 gene is found in an individual with osteoarthritis, indicating that the TGF-β/Smad3 signaling pathway is involved in the onset of osteoarthritis in human (Yao et al., 2003). As a common mediator Smad of TGF-β signaling, Smad4 is expressed ubiquitously in all zones of the epiphyseal plate (Sakou et al., 1999), suggesting that the Smad4 may have important functions during endochondral ossification. However, the Smad4 deficient mice die at the early stages of embryogenesis (Sirard et al., 1998; Yang et al., 1998), which makes it difficult to access the function of Smad4 in organogenesis. Other studies also showed that all endogenous Smad complexes described to date have
been shown to contain Smad4. All Smad target genes characterized by ChIP assay showed Smad4 binding along with RSmads and Phospho-RSmads have been shown to interact with factors distinct from, and in competition with, Smad4. Nevertheless, Smad4-deficient tumor cells and fibroblasts from Smad4-deficient mice still display some gene responses to TGF-β (Subramanian et al. 2004). Certain pancreatic carcinoma cells that lack Smad4 contain high levels of phosphorylated RSmads and respond to TGF-β receptor signaling with increased motility (Subramanian et al. 2004). Whether Smad4 is always required in Smad transcriptional complexes remains an important yet elusive question.

The conditional knockout of Smad4 in chondrocytes resulted in dwarfism with a severely disorganized growth plate characterized by an expanded resting zone, deceased proliferation, increased apoptosis and premature hypertrophic differentiation of chondrocytes. Overall, studies show that Smad4 is required for maintaining the regular arrangement and sequential differentiation of chondrocytes in the growth plate (Zhang et al. 2005).

In order to comprehensively understand the requirement of Smad4 in TGF-β and BMP canonical and noncanonical signaling pathways in endochondral ossification, Smad4 gene was specifically deleted in the chondrocytes. A mouse strain that carries a Smad4 conditional allele (Smad4Co) (Yang et al., 2002) was crossed with the chondrocyte specific Cre (Prx1-Cre) transgenic mice (Hao et al., 2002). A high level of Smad4 gene deletion was verified in limb mesenchyme in Cre expressing mice.

Based on previous studies we assume that loss of Smad4 would have an impact on the non-cononical pathway, or the TAK1-p38 pathway. Since Smad4 is required for the
canonical pathway, in its absence, the non-canonical pathway would compensate for Smad4. Therefore, we expect to observe an upregulation of the phospho-p38 level. Together we assume that Smad4 is required for normal skeletal development.
MATERIALS AND METHODS

Generation and Genotyping of Smad4<sup>fx/fx</sup>; Prx1-Cre mice. To get the conditional Smad4 gene knockout mice (Prx1-Cre+/Smad4<sup>fx/fx</sup>), mice that were homozygous for the ‘‘floxed’’ Smad4 allele (Smad4<sup>fx/fx</sup>) (Yang et al., 2002) were crossed with Prx1-Cre transgenic mice (Hao et al., 2002). One centimeter of each tail was cut for genotyping. The tail samples were digested using 500 µl of lysis buffer and 10 µl proteinase kinase in 55°C water bath overnight. After centrifuging digested tubes for 5 min, 300 µl of supernatant was aliquoted into new tubes. 150 µl of phenol: chloroform was added to each tube. After vortexing and centrifugation, 200 µl of top aqueous layer was collected and aliquoted into new tubes. An equal amount of 100% Isopropanol was added to wash the pellet. After mixing and centrifugation the supernatant was discarded and pellet was washed one last time, repeating the previous step using 70% EtOH. The pellet was air-dried overnight and the next day 50µl of water was added to each pellet. Using PCR the DNA was amplified. The wild-type Smad4 allele was detected using primer 1 (5'GGGCAG CGT AGC ATA TAA GA3') and primer 2 (5'GACCCA AAC GTC ACC TTC AC3'). These amplify a fragment of 385 bp from the DNA of wild-type and heterozygous (Smad4<sup>fx/+</sup>) mice. A fragment of 438 bp was amplified from the floxed allele of heterozygous (Smad4<sup>fx/+</sup>) and homozygous (Smad4<sup>fx/fx</sup>) mice using the same pair of primers. Primer 3 (5'GCC TGC ATT ACC GGT CGA TGC3') and primer 4 (5'CAG GGT GTT ATA AGC AAT CCC3') that amplified a fragment of 481 bp were used to detect the existence of the Cre gene. In addition, primer 5 (5'CCT TAG TTG AAT CCC3') and primer 2 were used to amplify a fragment of 234 bp from the
Smad4 allele after the Cre-mediated recombination. Each DNA sample was electrophoresed on a 1.5% agarose gel.

**Skeletal Preparation and Histology.** Skeletal preparations were generated by placing the embryos in tap water for 24 hours. The skin was peeled off and the embryos were eviscerated. The embryos were fixed in 95% EtOH for 3 days, in acetone for at least 2 days to remove fat and to keep specimen firm, and in staining solution for 3 days. The embryos were washed in distilled water and placed in 1% aqueous KOH until it is almost clear. The embryos were cleared completely through 20%, 50%, and 80% glycerol/1% aqueous KOH solution. Finally, they were placed in 100% glycerol for storage. For immunofluorescence, the knee joints were fixed in 4% paraformaldehyde at 4°C overnight and then decalcified in 5% EDTA/PBS. Decalcified tissues were dehydrated and embedded in wax (Leica) using standard procedures. Sections were boiled for 15 minutes in citrate buffer (Ivkovic et al., 2003). Sections were blocked with 5% goat or donkey serum for 1 hour and incubated with primary antibody overnight at 4°C, this was followed by incubation with secondary antibody for 1 hour at room temperature, then with fluorophore for 30 minutes at room temperature. The primary antibodies were as follows: phospho-Smad1/5, phospho-Smad2, phospho-Smad4 and phospho-p38 (Cell Signaling Technology); type II and type X collagen (Abcam). The secondary antibodies were conjugated with AlexaFluor-555 and AlexaFluor-488. Tissue sections were counterstained with DAPI (Vectashield).

**Cell Culture.** Mouse primary sternal chondrocytes were isolated by excision from the rib cage of E19.5 embryos. The excised rib cages were cultured in CM (DMEM + 10% FBS + 1% Pen/Strep (P/S)). The rib cage was digested in a 12-well dish using 2 mg/ml
pronase in PBS + 1% P/S. After 15 minutes the ribs were rinsed using 1X PBS and were
digested further using 3 mg/ml collagenase in DMEM + 1% P/S. Next, the ribs were
rinsed as previously described and 0.3 mg/ml collagenase II in DMEM + 1% P/S + 1X
anti-microbial solution was added to digest the ribs further. The collagenase digest mix
was incubated overnight at 37°C/5% CO₂. Then the cells were filtered through a 70 µm
cell strainer and were washed using DMEM + 10% FBS + 1% P/S. Cells were seeded at
1×10⁶ cells/well in 6-well plates, and cultured in DMEM supplemented with 10% FBS
and P/S. For western blot cells were serum-starved in DMEM containing 1% FBS
overnight, then stimulated the next day with 50-100 ng/ml BMP2, 10 ng/ml TGF-β2
(R&D Systems) for 60 minutes.

**Western Analysis of Sternal Cartilage.** Sternal cartilage was isolated, cultured as
described and then trypsinized using trypsin/EDTA. The pellet was lysed with 100 µl
lysis buffer on ice for 10 min. After centrifugation the supernatant was transferred to a
new tube and the pellet was discarded. Protein concentration was determined using
nanodrop spectrophotometer. Protein samples were taken and mixed with 4X sample
buffer and then boiled for 5 min at 85°C. Whole-tissue lysates were electrophoresed on
10% SDS-polyacrylamide gels at 200 V for 30 minutes. A piece of PVDF membrane was
cut and soaked in methanol for 30 minutes on a rocker at room temperature. Once
methanol removed, 1X Blotting buffer was added. After membrane transfer and blocking
overnight, the membrane was incubated with primary antibody diluted in Blocking buffer
for 60 minutes at room temperature. After 3 washes, 10 minutes each with 0.05% Tween
in PBS, the membrane was incubated with secondary antibody diluted in Blocking buffer
for 45 minutes at room temperature. After three more washes we detect the signals using Amersham ECL kit.
RESULTS

**Targeted deletion of Smad4 gene in mouse cartilage.** To investigate the role of Smad4 in endochondral ossification, we generated the chondrocyte specific Smad4 knockout mice using the Cre–loxP system. To disrupt the Smad4 gene in chondrocytes, we crossed a mouse strain containing the Smad4 conditional alleles (Smad4fx/fx) (Zhang et al., 2005) with the Prx1-Cre transgenic mice (Hao et al., 2002). The achieved Smad4fx/+; Prx1-Cre mice were further crossed with Smad4fx/fx mice to generate Smad4fx/fx; Prx1-Cre mice. The Smad4fx/+; Prx1-Cre mice were used as controls because there were no apparent difference in the structure and arrangement of growth plate cartilage observed between Smad4fx/+; Prx1-Cre and Smad4fx/fx or Smad4fx/+ or wild-type mice. The offspring was genotyped by PCR analysis. According to Table.1, no mutant offspring was found. This indicated that deletion of Smad4 in chondrocytes would lead to pre or post-natal death, since many mutant embryos were found. Deletion of the Smad4 gene in chondrocytes isolated from Smad4fx/fx; Prx1-Cre embryo was evaluated by PCR and Southern blot analysis. Southern blot analysis confirmed that the Smad4 was deleted effectively in cartilage tissues by Cre-mediated recombination (Fig. 3). These results indicated that the Smad4 gene has been disrupted efficiently in chondrocytes by Prx1-Cre-mediated recombination. And Smad4 gene deletion leads to pre or post-natal death.
<table>
<thead>
<tr>
<th>Pups Genotype</th>
<th>Number of Pups</th>
<th>Parents Genotype</th>
<th>DOB</th>
</tr>
</thead>
<tbody>
<tr>
<td>fx/fx</td>
<td>3</td>
<td>fx/fx; fx/+, Cre+</td>
<td>3/27/10</td>
</tr>
<tr>
<td>fx/+</td>
<td>3</td>
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<td>fx/+, Cre+</td>
<td>1</td>
<td>fx/fx; fx/+, Cre+</td>
<td></td>
</tr>
<tr>
<td>fx/fx</td>
<td>2</td>
<td>fx/fx; fx/+, Cre+</td>
<td>1/10/10</td>
</tr>
<tr>
<td>fx/+</td>
<td>7</td>
<td>fx/fx; fx/+, Cre+</td>
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</tr>
<tr>
<td>fx/+, Cre+</td>
<td>2</td>
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<td>1</td>
<td>fx/+; fx/+, Cre+</td>
<td>12/27/09</td>
</tr>
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<td>fx/+; fx/+, Cre+</td>
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</tr>
<tr>
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</tr>
<tr>
<td>wt, Cre+</td>
<td>2</td>
<td>fx/+; fx/+, Cre+</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.** Pups Mendelian ratios

**Figure 3.** Western blot analysis of Smad4 gene expression in Smad4<sup>fx/fx</sup>; Prx1-Cre mice. Chondrocytes were treated with rBMP2 30, 60, 120 minutes prior to protein isolation.
Prx1-Cre induced deletion of the Smad4 gene resulted in alteration and growth retardation of long bones. The mutant embryos were smaller than Smad4\textsuperscript{fx/+} or Smad4\textsuperscript{fx/fx} or Smad4\textsuperscript{fx/+}; Prx1-Cre embryos (Figs. 4). Whole skeletons from the Smad4\textsuperscript{fx/fx}; Prx1-Cre and control embryos were examined by alcian blue and alizarin red S staining. The E19.5 Smad4\textsuperscript{fx/fx}; Prx1-Cre mice exhibited shortening in the length of all bones, as shown on the hind limbs (Figs. 5). The ossification was delayed in the mutant mice compared with that in the control mice.

My results suggested that the conditional knockout of Smad4 in chondrocytes resulted in normal early limb development. That is, the mesenchyme condensed in the correct pattern. However, by E15.5 days, defects in the development of the limbs were apparent. The skull was smaller and the limbs were shorter than controls, the deltoid tuberosity was missing, and the joints of the phalanges were fused. The rib cage was smaller and flattened which could lead to respiratory distress and subsequent post-natal death.
Fig 4. Analysis of the skeletal phenotype in Smad4\textsuperscript{fx/fx}; Prx1-Cre embryos. Gross appearance of P0 mice. Mutant embryos had smaller skull and the limbs were shorter than controls, and the joints of the phalanges were fused.
Figure 5. Analysis of the skeletal phenotype in Prx1-Cre+/Smad4<sup>fl/fl</sup> and WT embryos. The skull was smaller and the limbs were shorter than controls, the deltoid tuberosity was missing, and the joints of the phalanges were fused. The rib cage was smaller and flattened.
Targeted disruption of Smad4 in chondrocytes caused disorganization of the growth plate. To characterize the skeletal abnormalities in the Smad4<sup>fx/fx</sup>; Prx1-Cre mice in more details, the proximal tibias at E19.5 were sectioned for histological analysis. The growth plate of the mutant mice showed the apparent disorganization. The periarticular regions were broader than that of the control mice (Figs. 6), and columns of proliferating chondrocytes were sparser and shorter (Figs. 6). In addition, the number of hypertrophic chondrocytes was decreased, and the interface between the proliferating and hypertrophic zone was irregular in the Smad4 mutant mice (Fig. 6). The results showed that the chondrocyte proliferation in the mutant mice was slightly decreased compared with that in the control mice. Collectively, these results indicated that the loss of Smad4 caused impaired proliferation, ectopic hypertrophic differentiation of chondrocytes, which resulted in the disorganization of growth plate cartilage.
Figure 6. Histological analysis of growth plate in Smad4 fx/fx; Prx1-Cre and control mice. Sections of control and mutant proximal tibias from E19.5 mice are used. Shortened proliferative region and disorganized hypertrophic zone of chondrocytes were present in Smad4 mutant mouse.

**Targeted disruption of Smad4 in chondrocytes and its impact on the activation of Smad2, p38, and Smad1/5.** My data also indicated that loss of Smad4 has a profound impact on the activation of Smad2 and p38. The phosphorylation level of both Smad2 and p38 are decreased in the absence of Smad4 (Fig. 7). This means, the phosphorylation (or activation) of Smad2 and p38 are Smad4 dependent. However, Smad4 is not required for the activation of Smad1/5 (Fig. 7). Therefore, since loss of Smad4 caused a decreased pSmad2 and p-p38 level, the cells might respond to TGF-β or BMP signaling with a compensatory upregulation of pSmad1/5 level.
Figure 3. Cartilage-specific excision of mouse Smad4. Immunofluorescence analysis of Smad phosphorylation (pSmad1/5, pSmad2) and p38 in wild-type (wt) and mutant cartilage, counterstained with DAPI.
DISCUSSION

Chondrogenesis is a series of consecutive and strictly regulated steps that start with condensation of mesenchyme followed by differentiation of cells into chondrocytes. The factors that regulate the early stages of chondrogenesis are just beginning to be elucidated. Several previous reports suggested that TGF-β and BMP play a critical role in early chondrogenesis (Carrington and Reddi, 1990; Chimal-Monroy and Diaz de Leon, 1997; Kulyk et al., 1989; Leonard et al., 1991; Macias et al., 1999; Roark and Greer, 1994; Verrecchia and Mauviel, 2002). It is thought that Smad4 is required for the majority of canonical BMP signaling (Feng and Derynck, 2005; Ross and Hill, 2008). In vitro (Sirard et al., 2000; Subramaniam et al., 2004) and in vivo (Wisotzkey et al., 1998; Chu et al., 2004) studies have shown that some TGF-β/Smad-dependent processes occur in the absence of Smad4. R-Smads can form homotrimers that do not contain Smad4 (Wu et al., 1997; Chacko et al., 2001), but the signaling capacity of these complexes is unknown. Even less is known about the requirement for Smad4 in BMP pathways.

Genetic studies reveal that some BMP receptor Smad-dependent processes occur in the absence of Smad4 (Chu et al., 2004; Wisotzkey et al., 1998). BMPs induce R-Smad nuclear translocation in Smad4-null colon cancer cells (Liu et al., 1997). In this study, mice lacking Smad4 have smaller skull and the limbs were shorter than controls, the deltod tuberosity was missing, and the joints of the phalanges were fused. In the developing joint, cartilage replaced the interzone cells leading to joint fusion. The rib cage was smaller and flattened which could lead to respiratory distress and subsequent post-natal death. Therefore, the phenotype of mice lacking Smad4 in cartilage demonstrates that Smad4 is required for endochondral bone formation.
I observed that Smad4\textsuperscript{fx/fx}; Prx1-Cre mice exhibited a growth plate abnormality due to the altered proliferation, differentiation and survival of chondrocytes revealed the critical functions of Smad4 in endochondral ossification. Previous studies showed that mice overexpressing the Noggin under the control of Col11a2 promoter/enhancer sequences lacked nearly all cartilages (Tsumaki et al., 2002), BmprIB; Bmp7 double mutants develop the forelimb autopod with severely reduced or absent elements (Yi et al., 2000). Unexpectedly, the cartilage formation and the bone morphogenesis were not apparently affected in the chondrocyte specific Smad4 knockout mice. This implicated the complexity of cytoplasmic transduction of BMP and TGF-\(\beta\) signals. Based on my observations on the Smad4 mutant mice, I thought that Smad4 could have redundant functions during chondrogenesis. And it is possible that there is co-Smad other than Smad4 in chondrocytes.

Smad4-independent pathways such as various MAPKs in the chondrocytes acting as the compensatory mechanism to transduce the extracellular BMP and TGF-\(\beta\) signals (Iwasaki et al., 1999; Yu et al., 2002; Ijichi et al., 2004; Chu et al., 2004). Previous studies have shown that both Smad2/4 as well as the p38 MAPK pathways are rapidly activated following the treatment with TGF-\(\beta\) in the ATDC5 chondrogenic cell line (Watanabe et al., 2001). There is also evidence showing that BMPs function in the developing limb is mediated not only through Smad proteins but also through p38 MAPK (Zuzarte-Luis et al., 2004). Additional experiments need to be done to elucidate the mechanisms underlying the Smad4-independent TGF-\(\beta\) signals in regulation of the growth and differentiation of chondrocytes. My data indicates that loss of Smad4 has a profound impact on the activation of Smad2/3 and MAPKs. The phosphorylation level of
both Smad2 and p38 are decreased in the absence of Smad4. This means, the phosphorylation (or activation) of Smad2 and p38 are Smad4 dependent. However, Smad4 is not required for the activation of Smad1/5. Therefore, since loss of Smad4 caused a decreased pSmad2 and p-p38 level, the cells might respond to TGF-β or BMP signaling with a compensatory upregulation of pSmad1/5 level. But what causes this upregulation remains unknown.

The most striking phenotype in Smad4\textsuperscript{fx/fx}; Prx1-Cre embryos was the dramatic disorganization of the growth plate characterized by expanded resting zone of chondrocytes (Figs. 5), indicating that Smad4-mediated TGF-β and BMP signals were required for regulating the normal organization of the growth plate. The mammalian growth plate is composed of three basic layers that play crucial roles in endochondral ossification. The resting zone of chondrocytes lies uppermost in the growth plate, followed by proliferating zone that is composed of flattened, coin-like chondrocytes with a clear orientation that directs the lengthening of the bone. The hypertrophic chondrocytes stop dividing and then enlarge, serving as the principal engine of bone growth. Many previous studies have shown that TGF-βs, BMPs and their antagonists, receptors and intracellular Smads were expressed differentially in all different types of chondrocytes (Pathi et al., 1999; Sakou et al., 1999; Kronenberg, 2003). My analysis demonstrates the requirement for Smad4 in the early chondrogenesis.

Further experiments are required to validate how this network controls the normal organization of the growth plate. And further studies need to be done to demonstrate what other transcriptional regulators RSmads bind to in the absence of Smad4. Unraveling these pathways and understanding how they intersect with other regulators will be
considerable challenge, but it provides insights that will enable new therapeutic
approaches to cartilage repair and replacement.
REFERENCES


