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Roles of Inhibitory Smads in Endochondral Bone Formation

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by

Kristine David Estrada

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ABSTRACT OF THE DISSERTATION

Roles of Inhibitory Smads in Endochondral Bone Formation

by

Kristine David Estrada

Doctor of Philosophy in Molecular, Cell, and Developmental Biology

University of California, Los Angeles, 2012

Professor Karen M. Lyons, Chair

Endochondral ossification involves a highly coordinated program of chondrocyte differentiation, proliferation, maturation, and hypertrophy. The bone morphogenetic protein (BMP) and transforming growth factor beta (TGFβ)/activin pathways are important regulators of these processes. The importance of extracellular antagonists as regulators of the duration, intensity and extent of BMP and TGFβ/activin signaling has been defined. For example, mice lacking the BMP receptor antagonist Noggin exhibit cartilage overgrowth concurrent with excess BMP activity. However, very little is known about the roles of intracellular inhibitors of BMP and TGFβ/activin pathways, such as the inhibitory Smad (I-Smad) proteins, Smad6 and Smad7. *In vitro* studies reveal that I-Smads 6 and 7 can regulate BMP- and TGFβ-mediated effects on chondrocytes. Although *in vivo* studies in which I-Smads were overexpressed in cartilage have shown that I-Smads have the potential to limit BMP signaling *in vivo*, the physiological relevance of I-Smad activity in skeletal tissues remains unknown. Furthermore, whether I-Smads impact TGFβ signaling in cartilage during development is unclear.

This thesis includes two reviews (both have been published) and two original articles (one of which has been published). First, the known mechanisms by which BMP signaling regulate chondrogenesis, osteogenesis, and adipogenesis will be highlighted Chapter One, "BMP signaling in Skeletogenesis." Then, the roles of Smad proteins, which are the mediators of the canonical BMP/TGFβ pathways, in regulating skeletal development will be highlighted in Chapter Two, "Smad signaling in skeletal development and regeneration." Finally, the roles of Smad6 and Smad7 in endochondral bone formation will be described in Chapters Three ("Smad6 is essential to limit BMP signaling in cartilage development") and Four ("Smad7 regulates terminal maturation of chondrocytes during cartilage development in mice"). The last chapter will discuss the overall conclusions and future directions of my thesis project.

The dissertation of Kristine David Estrada is approved.

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CHAPTER ONE:

BMP Signaling in Skeletogenesis

Kristine D. Estrada and Karen M. Lyons

8.1 Introduction

Bone is a dynamic tissue that provides skeletal support to the body and is essential in the maintenance of hematopoiesis and calcium homeostasis. Bone development and remodeling are tightly regulated by local paracrine factors and systemic hormones. Bone morphogenic proteins (BMPs) were first identified in the 1960s as proteins with the ability to induce ectopic cartilage and bone formation in vivo [87]. The proteins, however, were not identified until the late 1980s, when several polypeptides with BMP activity were cloned and purified [94]. To date, more than 20 BMP-related proteins have been identified and characterized. These molecules constitute the BMP family of secreted factors and form a subgroup of the transforming growth factor β (TGFβ) superfamily. Extensive studies have shown that BMPs are also essential for nonosteogenic developmental processes. For example, BMPs play roles in dorsal-ventral patterning, specification of the epidermis, development of neuronal phenotypes, tooth development, and regulation of apoptosis [10, 16, 21, 26, 61, 105]. The mechanisms by which BMPs regulate these processes will not be discussed here. This chapter will focus on how BMP signaling and crosstalk between other signaling pathways controls chondrogenic, osteogenic, and adipogenic processes.

8.2 The BMP Signaling pathway

The regulation of cellular responses by BMPs is mediated by at least two distinct pathways: the canonical Smad pathway and the noncanonical mitogen-activated protein kinase (MAPK) pathways [11, 53, 89]. BMP signaling is transduced through type I and type II transmembrane serine/threonine kinase receptors. So far, three type II receptors that bind to the BMP ligands have been identified: type II BMP receptor (BMPRII), and type IIA and IIB activin receptors (ActRIIA and ActRIIB) [38, 64, 74, 96]. Three type I receptors have also been identified: activin receptor-like kinase (ALK) 2, ALK3/BMPRIA, and ALK6/BMPRIB [42, 51, 85]. The pattern of oligomerization of the type I and type II BMP receptors is flexible and susceptible to modulation by ligand binding. Specifically, prior to ligand binding, a low level of preformed type I and II BMP heteromeric complexes is present on the cell surface. However, the major fraction of BMP receptors is recruited into the heteromeric complexes only after ligand binding [19]. Binding of BMP-2 to preformed heteromeric BMP receptor complexes triggers the canonical Smad pathway, whereas binding of BMP-2 with the consequent formation of heteromeric receptor complexes triggers the MAPK pathway [63].

In the canonical Smad pathway, the type II receptors phosphorylate serine and threonine residues of the type I receptors upon ligand binding. In turn, activated type I receptors phosphorylate and thereby activate a subgroup of the Smad family of proteins, called receptor-regulated Smads (R-Smads: Smad1, 5, and 8). Activation is at serines present in their conserved C-terminal SSXS motif. Subsequently, the activated R-Smads form a trimeric complex with a common-partner Smad, Smad4, a component of both the TGFβ and BMP signaling pathways. The R-Smad/Smad4 complex translocates into the nucleus and regulates the transcription of genes by interacting with the DNA-binding proteins or by binding directly to the DNA containing Smad-binding elements. In the noncanonical Smad signaling pathway, BMPs signal via the MAPK pathway by activating TGFβ activated kinase 1 (TAK1). TAK1 leads to the activation of several MAPKs, including JNK, p38, and extracellular signal-regulated kinases (ERKs) [57, 89].

Several mechanisms regulate the duration and intensity of BMP signaling. BMP-mediated responses are regulated extracellularly by antagonists, such as decorin, noggin, and chordin, while BMP responses are regulated intracellularly through attenuation of R-Smad activity by scaffolding proteins that can sequester R-Smad proteins, by inhibitory phosphorylation of R-Smads by MAPK that blocks their nuclear entry, and by the actions of the inhibitory Smads (I-Smads), Smads6 and 7.

I-Smads are structurally related to R-Smads, but lack the C-terminal phosphorylation site present on R-Smads. I-Smads can act as intracellular antagonists of BMP signaling by forming stable associations with activated type I receptors, thereby preventing the phosphorylation of R-Smads [28, 59]. I-Smads can also compete with activated R-Smads for interaction with Smad4 [24]. I-Smads inhibit BMP signaling by recruiting the Smad ubiquitin regulatory factor (Smurf) family of E3 ubiquitin ligases to their respective type I receptors, causing the ubiquitination and degradation of activated type I receptors [37, 58, 83, 84].

8.3 BMP Signaling in Mesenchymal Cell Condensation and Commitment to Chondrogenic Lineage

The process of bone formation begins with aggregation and condensation of mesenchymal cells. This process is associated with an increase in cell–cell and cell–matrix contacts and interactions, which are the result of increased expression and activity of cell adhesion molecules (CAMs), such as neural cadherin (N-cadherin) and neural cell adhesion molecule (N-CAM). N-cadherin interacts with the actin cytoskeleton through the formation of a functional complex with cytoplasmic catenins, such as α- and β-catenin [25, 60]. The spatiotemporal expression pattern of N-cadherin in the developing limb bud suggests that it is required in chondrogenesis. In turn, when N-cadherin activity is disrupted in vitro by NCD-2, an antibody directed against the functional region of N-cadherin, cell condensation and chondrogenesis are inhibited in the micromass cultures of the limb-bud mesenchymal cells. Inhibiting N-cadherin activity in vivo interferes with the development of the embryonic limb bud [65].

BMP signaling is essential in prechondrogenic condensations, with inhibition of BMP signaling by overexpression of either Smad7 [30] or noggin [6, 71] blocking condensation. BMPs promote mesenchymal cell condensation partly by upregulating N-cadherin expression and function [22]. This may involve crosstalk with the Wnt signaling pathway, because BMP-2 modulates the expression of β-catenin and Wnt family members, including Wnt-3a and Wnt-7a, in high-density micromass cultures of the C3H10T1/2 mesenchymal progenitor cell line [15]. Specifically, BMP-2 upregulates Wnt-3a expression and overexpression of Wnt-3a enhances BMP-2-mediated chondrogenesis of C3H10T1/2 cells through the stabilization of β-catenin and regulation of N-cadherin-mediated adhesion [14]. In contrast, BMP-2 downregulates Wnt-7a expression [15] and retroviral expression of Wnt-7a

blocks the progression of condensation of limb-bud micromass cultures through alterations in the expression of CAMs [82]. It can be inferred from these studies that crosstalk between BMP and Wnt signaling pathways regulates the initial events in mesenchymal condensation and promotes the commitment of these cells to the chondrogenic lineage.

8.4 BMP Signaling in Chondrogenesis

Bone is formed by intramembranous or endochondral ossification [44, 72, 102]. Intramembranous ossification leads to the formation of flat bones, especially those found in the skull, where mesenchymal cells condense and directly differentiate into osteoblasts. Endochondral ossification is the process by which most other bones are formed. Subsequently, mesenchymal cells condense and then differentiate into chondrocytes that form a matrix template, the growth plate, which is invaded by blood vessels and osteoblasts to initiate ossification.

The chondrocytes in the growth plate undergo a complicated differentiation program of proliferation, maturation, and apoptosis [44, 72, 104]. At the center of condensation, mesenchymal cells differentiate into round, slowly proliferating chondrocytes that express various extracellular matrix and CAMs, such as type II collagen, aggrecan, N-cadherin, and N-CAM, as well as the transcription factor, Sox9. The cells at the border of condensation form the perichondrium. The chondrocytes then align to form a columnar layer of flattened cells that proliferate rapidly and express low levels of the transcription factors, Runx2 and Osterix. Chondrocytes then exit the cell cycle to undergo hypertrophic differentiation and to express the signaling factor, Indian hedgehog (Ihh), as well as type-X collagen. The enlarged, hypertrophic chondrocytes terminally differentiate, mineralize, and undergo apoptosis. Expression of type-X collagen, Runx2, and growth factors that control chondrocyte proliferation and differentiation is enhanced in these cells. Following chondrocyte apoptosis, the residual cartilage matrix serves as a scaffold for trabecular bone.

During embryonic development, BMP signaling is essential for chondrogenesis. Mutations of individual BMPs, as well as compound deletions of either type-I BMP receptors or R-Smads, result in skeletal defects [40, 49, 73, 81, 98]. Moreover, individual BMPs and type-I BMP receptors are expressed in distinct and overlapping regions of the growth plate; this suggests that BMP molecules act in synergy to mediate chondrogenic events [13, 27, 50, 52, 62, 103].

8.4.1 Effect of BMP Signaling on Sox9 Expression

One of the earliest events in chondrogenesis is the commitment of mesenchymal cells to the chondrogenic lineage. Sox9, a member of the Sry-related high-mobility superfamily of transcription factors, is essential in this process. Sox9 is expressed in all cartilage primordia [3], specifically in prechondrogenic condensations during embryogenesis [95]. In mouse chimeras, *Sox9* null cells do not participate in mesenchymal condensations and fail to express chondrocyte-specific markers, such as type-II collagen and aggrecan [3]. Sox9 also plays a role in chondrocyte differentiation and maturation. *Sox9* haploinsufficiency in vivo results in defective cartilage primordia and premature mineralization [4]. In vitro, both $Sox9^{+/-}$ and $Sox9^{-/-}$ mouse embryonic stem cells show reduced type-II collagen expression and Alcian blue staining, exhibiting defects in maturation [23]. Several studies indicate that BMP signaling directly regulates *Sox9* expression. In particular, *Sox9* expression is upregulated in vitro in BMP-2-induced chondrogenesis of C3H10T1/2 cells and in mouse embryonic fibroblasts. In both cell types, *Sox9* expression is required for BMP-2-mediated chondrogenesis. When antisense *Sox9* nucleotides downregulate *Sox9* expression and when Sox9 expression is downregulated by *Sox9*-targeted siRNA, type-II collagen expression and Alcian blue staining are reduced [69, 101]. The regulation of *Sox9* expression by BMP is direct; a

CCAAT box on the *Sox9* promoter is the regulatory sequence responsible for BMP-2-induced *Sox9* expression [69]. The mechanism by which BMP-2 activates *Sox9* expression is attributed to BMP-2-induced association of NF-Y transcription factors with p300, which may contribute to chromatin remodeling at the *Sox9* proximal promoter region [68, 69].

8.4.2 Crosstalk Between BMP Signaling and Other Signaling Pathways: Ihh/PTHrP and FGF

One of the other signal pathways besides BMP that regulates chondrocyte proliferation and differentiation involves the secreted signaling factors, Ihh and parathyroid hormone-related protein (PTHrP). Loss of *Ihh* leads to reduced proliferation and premature maturation of chondrocytes [80]. Similarly, loss of PTHrP results in increased chondrocyte differentiation and accelerated bone growth [35]. *Ihh* is initially expressed in early mesenchymal condensations and, with the initiation of hypertrophic differentiation, it becomes restricted to prehypertrophic chondrocytes. Ihh stimulates the production of PTHrP in the periarticular region of the developing bone. Ihh expression is suppressed when PTHrP signals its receptor, PP-R, which is expressed at high levels in the transitional region between the proliferating and hypertrophic chondrocytes [45, 90]. The interaction between Ihh and PTHrP constitutes a negative feedback loop that regulates hypertrophic differentiation by keeping the chondrocytes in the proliferative state and thereby controlling bone growth (Fig. 8.1d, f).

BMP signaling interacts with the Ihh/PTHrP pathway (see Chap. 4) by increasing the Ihh expression in prehypertrophic chondrocytes (Fig. 8.1h) [54, 55]. Consequently, Ihh upregulates the BMP expression in the adjacent perichondrium and proliferating chondrocytes (Fig. 8.1g). This creates in a positive feedback loop between the two pathways to maintain the rate of chondrocyte proliferation [55, 70]. Evidence that the *Ihh* promoter contains BMP-responsive elements and is activated by treatment with BMP indicates direct regulation of *Ihh* expression by BMPs [73, 77]. Additional evidence of direct

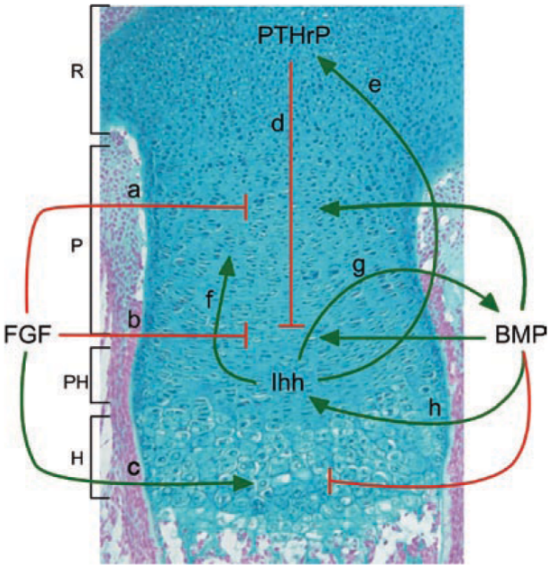

Figure 8.1. Crosstalk between BMP signaling and the Ihh/PTHrP and FGF signaling pathways. FGF signaling has the opposite effect of BMP signaling on chondrogenesis. FGFs inhibit proliferation (*a*), hypertrophic differentiation (*b*), and promote terminal differentiation (*c*). BMP signaling interacts with the Ihh/PTHrP feedback loop to maintain the rate of chondrocyte proliferation and to regulate hypertrophic differentiation. PTHrP inhibits hypertrophic differentiation by maintaining chondrocytes in the proliferative state (*d*). Ihh stimulates the expression of PTHrP in the periarticular region (*e*). Ihh is expressed in the prehypertrophic region and promotes chondrocyte proliferation (*f*). Ihh and BMPs promote the expression of each other (*g, h*). *R* resting zone; *P* proliferative zone; *PH* prehypertrophic zone; *H* hypertrophic zone.

regulation comes from Gli transcription factors that upregulate the promoter activity of *Bmp*-4 and -7, as key effectors of hedgehog signaling [39]. Even though both the BMP and Ihh/PTHrP pathways regulate chondrocyte proliferation, neither pathway acts downstream of the other. In particular, double treatment with BMP-2 and cyclopamine, which blocks Ihh signaling, also blocks chondrocyte proliferation. Similarly, overexpression of Ihh does not overcome the block to chondrocyte proliferation, induced by treatment with the BMP antagonist, noggin [55]. Regulation of PTHrP expression by Ihh is also independent of BMP signaling [55, 73].

BMP signaling also interacts with the fibroblast growth factor (FGF) signaling pathways, which plays an important role in chondrogenesis (see Chap. 6 of this volume). This is suggested by the expression patterns of FGFs and their receptors in distinct regions of the growth plate and at various

stages of endochondral bone formation [67, 97]. The role of FGF receptors as negative regulators of chondrocyte proliferation has been demonstrated in humans with missense activating mutations in *Fgfr3* [75,79]. In addition, *Fgf18* deficiency in mice leads to increased zones of proliferative and hypertrophic chondrocytes [48, 66]. These studies indicate that the effect of FGF signaling on chondrocyte proliferation is the opposite of BMP signaling (Fig. 8.1a–c): FGF signaling inhibits chondrocyte proliferation, whereas BMP signaling promotes it. Similarly, FGF signaling inhibits *Ihh* expression, while BMP signaling induces it [48]. Antagonism between FGF and BMP signaling in regulating chondrocyte proliferation and *Ihh* expression has been confirmed in limb explant cultures treated with FGF-2. The molecular mechanism by which these two signaling pathways antagonize each other is unclear. Inactivation by the suppression of ligand expression is not involved, because FGF-2 upregulates the expression of *Bmp*-4 *and Bmp*-7, whereas BMP-2 upregulates the expression of *Fgf18* [54]. Defects in BMP signaling resulting from targeted deletion of type-I BMP receptors [99] or R-Smads [73] in mouse cartilage suggest that the inhibition of FGF signaling by BMP signaling is due to the inactivation of ERK1/2 and STAT1, partly, as the result of inhibiting *Fgfr1* expression in the growth plate.

The mechanism by which the effectors of FGF signaling, ERK1/2 and STAT1, are involved in BMP/FGF antagonism has been examined in vitro. In particular, FGF signaling inhibits BMP signaling through ERK2-mediated phosphorylation of the linker region of Smad1, thereby inactivating Smad1 via Smurf1-mediated ubiquitination and subsequent degradation. In addition, binding of Smurf1 to linker-phosphorylated Smad1 limits the nuclear accumulation of Smad1 by inhibiting its association with nucleoporin [76]. As these studies were conducted with immortalized cell lines and neuroectodermal explants from *Xenopus* embryos, it is uncertain whether linker phosphorylation of R-Smads by ERKs or ERK/STAT inactivation is the general model for BMP/FGF antagonism. Inhibition of BMP signaling by FGFs in the growth plate may involve inactivation of R-Smads by inhibition of C-terminal Smad phosphorylation, rather than via Smad linker phosphorylation. In fact, stimulation or antagonism of FGF pathways leads to respective decreases or increases in C-terminal phosphorylated (activated) Smad1/5, but causes no change in the levels of linker-phosphorylated Smad1/5. Interestingly, Smad1/5 linker phosphorylation was detected primarily in proliferating, but not in resting and hypertrophic chondrocytes [73]. Conceivably, the regulation of linker and C-terminal phosphorylation of R-Smads by BMP and FGF signaling is required to tightly control the duration and intensity of BMP signaling in distinct zones at the growth plate.

8.5 BMP Signaling in Osteogenesis

Progression from chondrocyte proliferation to endochondral ossification requires the upregulation of genes for matrix proteins, transcription factors, and growth factors that coordinate the initiation of mineralization and induction of vascular invasion. BMPs can stimulate ectopic bone formation through increased expression of genes associated with osteoblast differentiation, such as alkaline phosphatase, osteocalcin, osteopontin, and the bone-specific transcription factor, Runx2 [7, 46]. The function of BMPs in osteoblasts has been extensively examined in vitro [9]. More recently, the Cre-*lox*P system, in conjunction with a promoter of an osteoblast-specific *Cre* transgene, such as *Col1a1-Cre* or *Osteocalcin2-Cre*, has been used to investigate the role of BMP signaling in vivo. This system has made the essential role of BMP signaling apparent not only in osteoblast, but also in osteoclast differentiation. Targeted deletion of BMPR1A in osteoblasts, obtained by mating conditional BMPR1A knockout mice with transgenic mice expressing Cre under the control of the *Osteocalcin2* promoter, led to reduced osteoblast activity and bone mass in 3-month-old mice. In 10-month-old mutant mice, on the other hand, bone mass was increased because of a decrease in osteoclast activity [56]. This indicates that BMP signaling plays an essential role in bone cell metabolism. The age-dependent function of BMP signaling in bone formation was further investigated with the aid of a tamoxifen-inducible

Cre-*lox*P system, where the administration of tamoxifen was observed to disrupt BMP signaling in osteoblasts. Targeted deletion of BMPR1A in osteoblasts of mice, either 2 days or 2 months old, caused the bone mass to increase after 3 weeks of tamoxifen administration because bone resorption had decreased [33]. The difference in the bone phenotypes of the conditional BMPR1A knockout mice, in which Cre expression was controlled by either the *Osteocalcin2* or the *Col1a1* promoter, may involve the differences in recombination efficiencies or the timing of recombination. These findings indicate that loss of BMP signaling leads to reduced osteoclast activity, because osteoblast-mediated osteoclast differentiation has been diminished.

8.5.1 Mechanistic Role of BMP Signaling in Osteoclastogenesis

The decrease in osteoclast function that leads to decreased bone resorption may involve interactions between the receptor activator of NF-κB ligand (RANKL) and its receptor, RANK. Osteoblasts express RANKL, while osteoclast precursors express RANK. Thus, cell–cell interactions between RANKL-expressing osteoblasts and RANK-expressing cells promote osteoclast differentiation [36]. Osteoblasts also produce osteoprotegerin (OPG), a decoy receptor for RANKL that can prevent RANKL–RANK interactions [41]. BMP signaling modulates the RANKL–OPG pathway in vitro, as BMP-2 treatment stimulates *Rankl* expression [29,88]. In vivo studies have also shown that BMP signaling regulates the RANKL–OPG pathway by BMP-induced *Opg* expression via Hoxc-8-binding sites located on the *Opg* promoter [33, 34]. Smad1 competes with Hoxc-8, which inhibits *Opg* promoter activity, by binding to the *Opg* Hox sites [91]. BMP signaling may also mediate the RANKL–OPG pathway via secondary mediators such as Wnts, which regulate *Rankl* and *Opg* expression [20]. *Bmpr1a*-deficient calvaria showed upregulation of canonical Wnt signaling via downregulation of the expression of sclerostin, the Wnt pathway inhibitor. In these calvaria, reduction of *Rankl* expression is accompanied by an increase in *Opg*. Treatment of *Bmpr1a*-deficient calvaria with sclerostin reverses the expression patterns of *Rankl* and *Opg* [34]. Similarly, secreted Wnt inhibitors from the Dickkopf (Dkk) family facilitate osteoclastogenesis by enhancing *Rankl* expression and reducing *Opg* expression [17]. It is thus apparent that BMP signaling controls the extent of bone formation by regulating osteoblast-induced osteoclastogenesis by its mediation of the RANKL–OPG pathway, and/or indirectly by its downregulation of Wnt signaling.

8.5.2 Effects of BMP Signaling on Runx2 Activity

Matrix protein expression by osteoblasts during intramembranous and endochondral bone formation is regulated by the transcription factor, Runx2/Cbfa1/Osf2 (hereafter referred to as Runx2), which binds to responsive elements on osteoblast-specific genes to regulate their transcription. The essential role of Runx2 in osteoblast differentiation has been brought out by studies with mice that have a homozygous mutation in *Runx2*. These mice die perinatally and completely lack bone owing to defects in osteoblast maturation [43]. Interestingly, overexpression of either Runx2 or dominant-negative Runx2 causes osteopenia attributed to diminished matrix production and mineralization. In either case, overexpression of Runx2 resulted in diminished function of fully differentiated osteoblasts, as indicated by reduced expression of *osteocalcin*, a marker for terminally differentiated osteoblasts [12,47]. Thus, transcriptional regulation of *Runx2* is crucial in controlling its function during both early and late stages of osteoblast differentiation.

The canonical BMP signaling pathway has been implicated in the regulation of Runx2 transcription and activity [46]. The nuclear matrix targeting signal (NMTS) on the C-terminal region of *Runx2* directs Runx2 to specific sites within the nuclear matrix, thus promoting the expression of osteoblast-specific genes [100]. Deletion mutant studies have identified a R-Smad/Runx2 interaction domain on *Runx2* that overlaps the NMTS [1], where HTY residues on the R-Smad/Runx2 interaction domain initiate BMP-induced osteoblast differentiation [31]. Therefore, R-Smads may facilitate subnuclear targeting of Runx2 to promote the expression of osteoblast-specific genes.

Degradation of Runx2 modulates its transcriptional activity. BMP signaling regulates Runx2

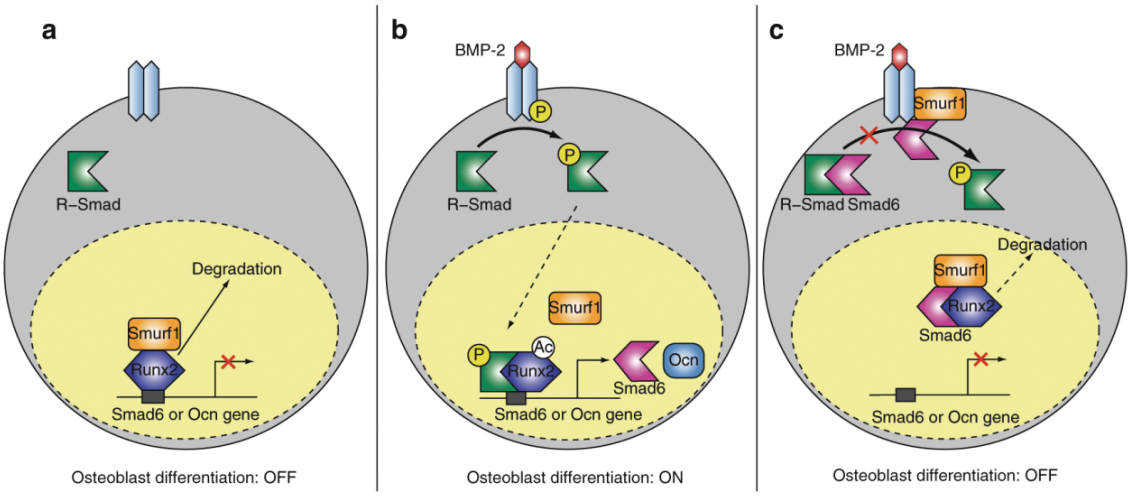

Figure 8.2. Proposed model for BMP-mediated Smad6/Runx2 feedback loop to control osteogenesis. (**a**) Osteoblast differentiation is inhibited in the absence of BMP signaling. In the absence of BMP-2, Smurf1 binds to Runx2 on the Smad6 promoter and induces Runx2 degradation, thus inhibiting *Smad6* transcription. Degradation of Runx2 can also inhibit transcription of genes associated with osteoblast differentiation, such as *osteocalcin* (*Ocn*). (**b**) BMP signaling promotes osteoblast differentiation. In the presence of BMP-2, Runx2 is acetylated, which inhibits Smurf1-mediated degradation. Also, phosphorylated R-Smads replace Smurf1 to promote Runx2-mediated *Smad6* and/or *Ocn* transcription. (**c**) Expression of *Smad6* results in the attenuation of BMP signaling. Smad6 of Smad interacts with Smurf1 to induce Runx2 degradation. Smad6 can also interact with R-Smads and/or BMP receptors to prevent activation (phosphorylation) of R-Smads.

activity by acting on Runx2 stability (Fig. 8.2). Treatment of the pluripotent mesenchymal cell line, C2C12, with BMP-2 causes R-Smads to interact with Runx2 and precludes Smurf1 binding on the *Smad6* promoter, thus promoting *Smad6* gene transcription (Fig. 8.2b) [93]. In turn, Smad6 interacts with Smurf1 to induce Runx2 degradation (Fig. 8.2c) [78]. Treatment of C2C12 cells with BMP-2 has shown that BMP signaling also protects Runx2 from degradation. BMP-2 stimulates Runx2 acetylation, which inhibits Smurf1-mediated degradation and promotes BMP-induced osteoblast differentiation and bone formation (Fig. 8.2b) [32]. It is unclear, however, whether the tight regulation of Runx2 stability by BMP signaling occurs in vivo.

8.6 BMP Signaling in Adipogenesis and Energy Metabolism

Adipocytes, crucial for the maintenance of proper energy balance, store energy in the form of lipids and expend energy in response to hormonal stimulation. Obesity develops when energy intake exceeds energy expenditure. Understanding the development and regulation of adipogenesis is important in managing the health implications of obesity. Like cartilage and bone, adipose tissue arises from a multipotent stem cell population of mesodermal origin. BMP signaling commits mesenchymal stem cells to the adipocyte lineage, and thus represents the initial stage of adipocyte differentiation [5, 92]. Interestingly, adipocyte and osteoblast commitment can be altered by selective blockage or activation of type-I BMP receptors [8]. Also, BMP-induced commitment of the mesenchymal to the adipocyte lineage is dose-dependent [92].

Once committed, preadipocytes differentiate into adipocytes. Adipose tissue exists as either white (WAT) or brown adipose tissue (BAT). WAT is the primary site of energy storage and is dispersed throughout the body of mammals and birds. Most WAT is subcutaneous and intra-abdominal. BAT provides basal and inducible energy expenditure in the form of thermogenesis. This in turn involves increased expression of uncoupling protein 1 (UCP-1). In humans and rodents, BAT, localized in the intrascapular and paraspinal regions, is abundant during the prenatal and neonatal periods. After birth, only small amounts of BAT remain and its function

in adults has been considered negligible [18]. However, recent reports have shown that high energy expenditure in obesity-resistant mice correlates with high expression of UCP-1 in BAT, interspersed between muscle bundles, and that UCP-1 expression is inducible [2]. The regulation of UCP-1 in BAT, as well as brown adipogenesis, may protect against obesity. Moreover, treatment with BMP-7, but not with BMP-2, -4, or -6, induces differentiation of brown preadipocytes, as indicated by increased *UCP-1* expression. Overexpression of BMP-7 in mice has also led to increased brown fat mass, increased energy expenditure, and reduced weight gain [86]. Thus, BMP-7 may prove to be a therapeutic option for treating human obesity.

8.7 Perspectives

Studies using cell-specific Cre-mediated recombination in mice, coupled with in vitro molecular and biochemical assays, have revealed that BMP signaling plays an essential role in all aspects of skeletogenesis. BMP signaling induces the commitment of mesenchymal progenitor cells into the chondrogenic, osteogenic, and adipogenic lineages, and regulates the progression of these cell types through their complex differentiation program. A major challenge is whether the mechanisms by which BMP signaling regulates the expression of target genes in vitro are the general mode for regulation of skeletogenesis in vivo. How BMP signaling interacts with other signaling pathways, such as the Ihh/PTHrP, FGF, and Wnt, is essential to understand how imbalance in signaling affects bone development and homeostasis. Such understanding may lead to the development of new therapies for the treatment of skeletal diseases.

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CHAPTER TWO:

Smad signaling in skeletal development and regeneration

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ABSTRACT

Smad proteins are intracellular molecules that mediate the canonical signaling cascade of TGFβ superfamily growth factors. The TGFβ superfamily comprises two groups of growth factors, BMPs and TGFβs. Both groups can be further divided into several sub-groups based on sequence homologies and functional similarities. Ligands of the TGFβ superfamily bind to cell surface receptors to activate Smad proteins in the cytoplasm; then the activated Smad proteins translocate into the nucleus to activate or repress specific target gene transcription. Both groups of growth factors play important roles in skeletal development and regeneration. However, whether these effects reflect signaling through canonical Smad pathways, or other non-canonical signaling pathways *in vivo* remains a mystery. Moreover, the mechanisms utilized by Smad proteins to initiate nuclear events and their interactions with cytoplasmic proteins are still under intensive investigation. This review will discuss the most recent progress understanding Smad signaling in the context of skeletal development and regeneration.

1. Introduction

1.1. TGFβ superfamily

BMPs were initially discovered by the fact that demineralized bone matrix can initiate bone formation when transplanted to ectopic sites in rodents [1]. Later, TGFβs were discovered in studies of platelet derived growth factor (PDGF) and epidermal growth factors (EGF/TGFα) [2,3]. Eventually, other related ligands were identified, leading to the definition of the TGFβ superfamily, consisting of BMPs, TGFβs, and other groups of proteins such as growth and differentiation factors (GDFs), activins, inhibins and Mullerian inhibitory factor (MIF) [4]. Interestingly, although BMPs reserve a bone forming capability in different species, mammalian TGFβs have been found to only induce bone formation with site and tissue specificity in non-human primates [5,6].

1.2. Smad proteins

The first description of Smad proteins was the finding of Mothers Against Dpp (MAD) in Drosophila, which modified the phenotype of *decapentaplegic* (dpp; a BMP ligand) mutants [7]. Later studies identified Sma proteins in *C. elegans* as closely related to MAD, and both mediated signaling downstream of serine/threonine kinase receptors of TGFβ superfamily proteins [8]. Therefore, homologs of Mad and Sma have been named Smad. So far, 8 mammalian Smad proteins have been isolated, designated Smad1 through Smad8. The Smad proteins are divided into three groups according to their functions. The first group is the receptor-regulated Smads (R-Smads), which include Smads1, 2, 3, 5 and 8. These Smad proteins bind to membrane bound serine/threonine receptors, and are activated by the kinase activity of the receptors. The second group includes only one member, Smad4. Smad4 acts as a co-factor that binds to the activated R-Smads to form a complex that translocates into the nucleus. Therefore Smad4 has been named Co-Smad. The third group comprises the inhibitory Smads (I-Smads), which includes Smads 6 and 7. These two Smads exert an inhibitory effect on the signaling cascade by various mechanisms (Fig. 1).

Smad proteins also share similar structures. A typical Smad structure includes N-terminal MH1 domain, linker region and a C-terminal MH2 domain. The MH1 domain is highly conserved in all R-Smads and Smad4, but not in I-Smads. The major function of the MH1 domain is to mediate DNA binding of Smad proteins. The linker region is highly variable in different Smads. It is the target of regulation by other intracellular proteins through phosphorylation, ubiquitination, or sumoylation. The MH2 domain is present in all Smads. Activation of R-Smads is through the phosphorylation of a Ser-X-Ser motif in the MH2 domain by activated receptors. The MH2 domain is also responsible for Smad protein interactions with

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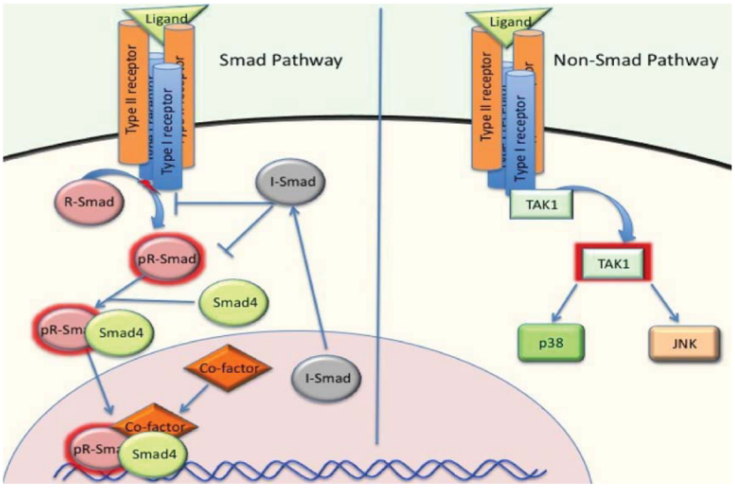

Fig. 1. The Smad and non-Smad pathway of BMP/TGFβ signaling. BMP/TGFβ signaling *in vivo* is mediated by either the Smad pathway (canonical) or the non-Smad (non-canonical) pathways. Both pathways could mediate important functions of BMP/TGFβ in skeletogenesis. The non-Smad pathways also regulate Smad proteins by modulating linker region and C-terminal phosphorylation. Moreover, Smad proteins also modulate activities of molecules in non-Smad pathways. The preference of intracellular pathways by different receptor complexes, the cross talk between these two pathways, and the exact signaling mechanisms of the non-Smad pathways in skeletal system remain to be investigated.

other intracellular proteins and transcriptional activation of target genes [9]. Different receptors in the TGFβ superfamily have different preferences for binding to R-Smad proteins. For example, Smads1, 5 and 8 mediate BMP signaling by interacting with the BMP receptors ALKs 1, 2, 3, and 6, whereas Smads2 and 3 mediate TGFβ and activin signaling through the TGFβ/activin receptors ALKs 4 and 5. Smad6 is more specific for the inhibition of BMP signaling, whereas Smad7 has inhibitory effects on both BMP and TGFβ signaling (for more detailed reviews, see [9,10]). The consensus understanding so far is that R-Smads require Smad4 binding before they can translocate into the nucleus, Recent evidence has challenged this dogma, as *Smad4* conditional deletion in mice did not cause significant skeletal defects [11]; while, conditional deletion of *Smad1/5/8* led to lethality at birth due to severe chondrodysplasia [12].

1.3. Skeletal development and regeneration

Skeletal development in mammals is accomplished via two different mechanisms. In intramembranous bone formation, mesenchymal cells from neural crest and cephalic mesoderm differentiate into osteoblast cells to form the major cranial vault and clavicles. Endochondral bone formation accounts for the development of the majority of the skeleton. Initially, mesenchymal cells from the mesoderm condense at the sites where the bones will form. The condensed mesenchymal cells then proliferate and differentiate into chondrocytes, forming cartilaginous anlagen of the future bones. Later, the chondrocytes undergo terminal differentiation and become replaced by invading osteoblasts to form the mineralized bone tissue (for a more detailed review of endochondral bone formation, see [13]). BMPs are important for condensation, and they are required for the initiation of chondrocyte differentiation by inducing Sox9 expression. BMPs also regulate chondrocyte differentiation at later stages by interacting with other signaling pathways such as Indian Hedgehog (Ihh), Parathyroid Hormone Related Peptide (PTHrP) and Fibroblast Growth Factor (FGF) (see later in this review). In the osteoblasts, BMP signaling is required for the commitment of mesenchymal cells toward the osteoblast lineage. Similarly, TGFβ/activin signaling is important in chondrogenesis and osteogenesis, working synergistically or antagonistically with BMP signaling, depending on the stage of differentiation.

Skeletal regeneration is a process of new bone formation after trauma or injury. The new bones form at the site of injury, and could involve both intramembranous and endochondral bone formation. Therefore, skeletal regeneration is considered a process recapitulating development. However, there are particular differences between these two processes, and these will be discussed more in this review later.

In the past few decades, studies with gene deletions, targeted gene modification, and overexpression of BMP/TGFβ signaling pathway components have revealed essential roles of BMPs and TGFβs in skeletal development *in vivo*. Several excellent reviews have addressed this topic [14–16]). Therefore, in this review, we focus on a less well-understood aspect of BMP/TGFβ biology: the roles of Smad family members. Until recently, it was widely assumed that BMP and TGFβ signaling are mediated predominantly by R-Smad proteins acting in concert with Smad4. However, recent studies have increased awareness of the potential importance of non-Smad mediated BMP and TGFβ signaling [17,18]. The existence of the non-Smad pathways raises the question of the relative importance of canonical Smads *vs.* non-canonical (non-Smad) pathways. Moreover, intensive studies have described a sophisticated Smad-dependent network of signaling events that are important in many processes, including normal development, regulation of immune system, tumor initiation, tumor metastasis and others. Conclusions from these studies indicated that the mechanisms of Smad signaling are quite species, tissue, and cell type specific. Therefore, understanding the mechanisms of Smad signaling in the context of skeletal development and regeneration requires additional focused studies that are carried out *in vivo* under physiological conditions.

2. Role of Smads in chondrogenesis

Smad proteins are ubiquitously expressed in chondrocytes during the entire process of chondrogenesis [41]. However, due to the different functions of BMP and TGFβ pathways, Smads have overlapping but distinct patterns of activity in different stages of chondrogenesis. Elucidating the roles of Smad proteins *in vivo*

Table 1
Consequences of global deletion, conditional deletion, and over-expression of Smad proteins in mice.

Gene	Mutation	Promoter	Phenotype	References
Smad1	−/−	–	Lethal at E 10.5, defect in extra-embryonic tissues and germ cell formation	[19]
Smad2	−/−	–	Lethal at E 7.5–12.5, defects in primitive streak formation, A–P axis formation in epiblast, and gastrulation	[20–23]
Smad2	ES chimera	Lac-z marked	Absence of Smad2-deficient cells in definitive endoderm	[24]
Smad3	−/−	Smad2-deficient cells	Colorectal carcinoma, immuno-function defect, and osteoarthritis later in life	[25–29]
Smad4	−/−	–	Lethal at E 6.5–8.5, defects in gastrulation (mesoderm), anterior truncation of embryos	[30,31]
Smad4	CKO	*Col2a1-Cre*	Dwarfism	[11]
Smad4	CKO	*Osteocalcin-Cre*	Reduced osteoblast proliferation and function	[32]
Smad5	−/−	–	Lethal at E 10.5–11.5, defects in angiogenesis	[33]
Smad6	−/−	–	Partially lethal, defects in endocardial cushion formation, and aortic ossification and high blood pressure in viable mutants	[34]
Smad6	Overexpression	*Col11a2*	Delayed chondrocyte hypertrophy, dwarfism with osteopenia	[35]
Smad7	MH2 domain deletion	–	Partially lethal, defects in endocardial cushion formation	[36]
Smad7	Exon1 deletion	–	Mutant is smaller, altered B cell response to TGFβ signaling, increased fibrogenesis	[37–39]
Smad7	Overexpression	*Prx1-Cre*; *11Enh-Cre*; *11Prom-Cre*	Decreased chondrocyte proliferation; differentiation	[40]
Smad1/5/8	Smad1cko; Smad5cko; Smad8−/−	*Col2a1-Cre*	Severe chondrodysplasia with embryonic lethality	[12]

involves several important questions. First, within the two different groups of R-Smads, Smad1/5/8 and Smad2/3, does each of the Smads play distinct roles in signal transduction, or do they have essentially overlapping functions? Second, to what extent is BMP/TGFβ signaling mediated by R-Smads at distinct stages of skeletal development? Third, what is the evidence that Smad4 is required for Smad1/5/8 and/or Smad2/3 signaling? Finally, what role, if any, do the I-Smads play in skeletal development and regeneration? Several mouse models with knockout, conditional knockout, and overexpression of Smad proteins have been generated to study functions of Smads (see Table 1). The phenotypes of these mice, if not lethal before skeletogenesis, normally include skeletal defects, confirming the important functions of Smads in this process.

Since BMP/TGFβ signaling is important for nearly every aspect of development, it is not surprising that global knockout of an R-Smad generally leads to early embryonic lethality. *Smad1* knockout mice die in mid-gestation due to extra-embryonic defects [19]. *Smad2* knockout mice die at embryonic day 7.5–12.5 due to defects in primitive streak formation and failure to establish an anterior–posterior axis within the epiblast or defects during gastrulation [20–23]. Moreover, studies of *Smad2*-deficient chimeric mice revealed that *Smad2*, but not *Smad3*, mRNAs were expressed in visceral endoderm, and definitive endoderm formation is Smad2-dependent, indicating a unique function of Smad2 [24]. Global knockout of *Smad4* causes early embryonic lethality at day E6.5–E8.5, due to defects in gastrulation [30,31], *Smad5* knockout mice die between embryonic day 10.5 and 11.5, because of defects in angiogenesis [33]. Interestingly, *Smad8* knockout mice do not have apparent defects, suggesting Smads1 and 5 could compensate for most of the functions of Smad8 [12,42,43]. *Smad3* knockout mice survive birth, but develop colorectal cancer, impaired immunonological functions, and osteoarthritis later in life [25–29]. These studies suggested that Smads1 and 5 are both indispensable in BMP signaling, especially in early development, although they might share some overlapping functions. Similarly, Smad2 signaling is indispensable in embryonic development. Smad2 or other mechanisms could compensate for the loss of Smad3 in early development, suggesting the functional overlap of Smads2 and 3. However, in later stage of life, Smad3 has several indispensable functions that are different from Smad2. In particularly, Smad3 is required for maintenance of normal immuno-suppressive responses, articular chondrocyte homeostasis, and the tumor suppressive functions of TGFβ.

With the advancement in the genetic technologies, use of Cre-LoxP system allowed conditional deletion of *Smad* genes in skeletal tissue. Since direct evidence of the functional role of Smad1/5/8 in early chondrogenesis and growth plate chondrocytes is still missing, we have recently generated conditional deletions of *Smad1/5/8* in cartilage using *Col2a1-Cre* [12]. *Smad1/5/8* triple deletions yielded early embryonic lethality and closely phenocopied mice lacking the BMP receptors ALK3 (BMPR1A) and ALK6 (BMPR1B) [44]. These mice do not form any endochondral skeleton; condensations form, but with the onset of *Col2a1-Cre* expression, any further development is blocked. *Smad1/5* double mutant mice have very similar phenotypic presentations to *Smad1/5/8* triple mutants, suggesting that Smad8 plays a very minor role in chondrogenesis. In contrast, individual loss of Smads1, 5 or 8, and mice carrying only a single allele of *Smad5* (*Smad1*$^{-/-}$; *Smad5*$^{+/-}$; *Smad8*$^{-/-}$) in cartilage are viable and form a nearly normal skeleton. This observation demonstrates that Smads1 and 5 exhibit extensive functional overlap. Additional studies demonstrated that the BMP signaling mediated by Smad1/5 is required for the regulation of the Ihh/PTHrP feedback loop and the antagonism between BMP and FGF signaling in the growth plate [12].

An important finding from these studies is that the majority of BMP signaling in endochondral bone formation appears to be mediated by canonical Smad1/5 pathways as opposed to non-canonical pathways. Moreover, it is surprising that Smad4 is not required for skeletogenesis. As discussed above, the current dogma is that Smad4 is a required co-Smad for canonical BMP and TGFβ signaling, and Smad4 is expressed ubiquitously in all zones of growth plate [41]. However, conditional deletion of Smad4 in cartilage leads to fairly minor defects; *Smad4*^{cko} mice develop dwarfism post-natally, mainly as a result of a disorganized growth plate. The *Smad4*-deficient growth plate showed an expanded resting zone, reduced proliferation, accelerated differentiation and increased apoptosis of chondrocytes, as well as ectopic bone collar formation in the perichondrium and loss of responsiveness to TGFβ1 [11]. Given that loss of Smads1 and 5 leads to a total arrest in chondrogenesis at the condensation stage, these data indicate that BMP signaling in skeletogenesis is largely independent of Smad4.

Potential mechanisms by which BMP R-Smads may mediate their effects in skeletal cells have been the topic of numerous *in vitro* studies. As aforementioned, BMPs induce chondrogenesis by regulating Sox9 expression in mesenchymal cells. However, the molecular mechanism is still unclear. It has been suggested that BMP alone is not sufficient to induce Sox9 expression [45], although later studies indicated that BMP/Smad pathways regulate Sox9 expression through a CCAAT-box in the *Sox9* promoter, as well as by chromatin remodeling at the proximal promoter [46,47]. A number of transcriptional targets of Smads1 and 5 in the growth plate have been described, including Ihh, Col2, Col10, and Runx2 [48–51]. In addition to a role as a transcriptional activator, BMP R-Smads also act as transcriptional repressors through specific recruitment of transcriptional repressor complexes. For example, the Smad1/4 complex is required to recruit a HDAC/Sin3A complex for modulating a transcription repressor, Nkx3.2, which promotes chondrocyte differentiation [52]. At the same time, other nuclear proteins regulate Smad protein functions by regulating Smad stability, DNA binding, and transcriptional activities. For example, most Smads can be degraded by the proteasome through ubiquitination; on the other hand, sumoylation, seems to protect Smads from being ubiquitinated. The capacity of R-Smads to bind DNA is enhanced by the presence of stabilizing co-factors. Some transcriptional co-activators have been shown to interact with the MH2 domain of R-Smads to fully activate target gene transcription [9]. Other protein–protein interactions influence Smad signaling without participating in Smad–DNA complexes. For example, calponin 3, an actin binding protein, has been found to interact directly with Smads 1 and 5 to negatively regulate the BMP-dependent cellular response of human chondrocytes, possibly by sequestering Smads to the cytoskeleton [53]. Jab1, a subunit of the COP9 signalosome [54] interacts directly with Smad5 to attenuate the BMP-signaling response in chondrocytes, possibly by inducing Smad5 degradation [55].

With respect to the TGFβ/activin R-Smads, even less is known. The extent by which TGFβ/activin signaling *in vivo* is mediated by Smad2/3 remains an important and unanswered question. It is possible that Smad3 plays an essential role, since the *Smad3* null mice phenotype is similar to that of the mice expressing a transgenic dominant-negative TGFβ type II receptor (TgfbrII) [26,56]. However, conditional deletion of the *TgfbrII* with *Col2a1-Cre* and *Prx1-Cre* causes axial skeleton defects, alteration in hypertrophic differentiation in growth plates, and joint fusions in phalanges [57,58]. These defects are not present in the *Smad3* null mice, indicating that either Smad2 is more dominant in mediating TGFβ signaling in skeletal tissue or the non-Smad pathways are major players (Fig. 1). Moreover, TGFβ/activin signaling may be more dependent on Smad4 than in BMP signaling, as the cartilage-specific loss of *Smad4* resembles in many aspects the phenotype of mice lacking *Smad3* [11,26]. Nevertheless, the full repertoire of effects mediated by TGFβ signaling in cartilage has not yet been defined *in vivo*, nor have any studies yet addressed potential overlapping functions for Smads2 and 3.

Previous studies have shown that TGFβ may play important functions at early stages of chondrogenesis. An *in vitro* study showed that Smad3, but not Smad2, forms a complex with Sox9 and CEBP/p300 to activate genes for chondrogenesis [59]. A recent study showed that Smad3 works cooperatively with Sox9 to initiate target gene transcription through chromatin remodeling [60]. However, the fact that *Smad3* knockout mice survive birth and only have limited defects in the skeleton argues that the role of Smad3 in early chondrogenesis is not critical, or Smad2 could largely compensate for the loss of Smad3 in early chondrogenesis. Organ culture studies demonstrated that Smad3 is required for TGFβ1-induced chondrocyte proliferation in mice, but share redundant functions with Smad2 in terms of inhibiting hypertrophic differentiation [61]. In the post natal stage of life, Smad3 has been shown to play an essential role in maintaining articular cartilage by preventing articular chondrocytes from undergoing terminal hypertrophic differentiation [28]. In accordance, chondrocytes in *Smad3* deficient mice show accelerated differentiation in the growth plate shortly after weaning, resulting in dwarfism. Accelerated differentiation was also observed in articular chondrocytes, such that they escape from quiescence and continue the process of maturation, resulting in the loss of articular cartilage. Later studies carried out with primary chondrocytes isolated from these mice demonstrated increased BMP responsiveness, decreased responsiveness to TGFβ1, and increased apoptosis [29]. Altogether, these observations suggested that for TGFβ signaling, Smad2 could compensate for most of Smad3's functions in early development. However, Smad3 is required to maintain cartilage homeostasis by mediating the TGFβ signaling that inhibits terminal differentiation of chondrocytes. Whether this is due to elevated levels of expression of Smad3 relative to Smad2 in the chondrocytes, or to a distinctly different activity of Smad3 compared to Smad2, remains to be investigated.

Data from *in vitro* experiments indicated extensive differences between Smads2 and 3 in terms of DNA binding capacity, interactions with other nuclear proteins, and target gene selection. For example, an additional 30 amino acids encoded by exon3 in the MH1 domain of Smad2 prevents its direct binding to DNA, such that a complex of Smad2/4 and other transcription factors is required for DNA binding. However, Smad3 homomers can form DNA binding complexes even without Smad4. A whole list of transcription factors and nuclear proteins that interact with Smad3 has been generated; most of them also interact with Smad2 [62]. Studies with modulating Smad2 and 3 levels by siRNA have revealed that Smads2 and 3 not only share redundant functions, but also have unique roles. Smad3 appears more important than Smad2 in TGFβ's function in cell growth arrest (for a detailed review, please see [62]).

It is becoming increasingly clear that R-Smads also have essential functions in processes other than initiating or repressing transcription directly on DNA. A recent finding linked R-Smads to the post-transcriptional processing of microRNAs. Smads1, 3, and 5 interact with primary transcripts of miR-21, in a complex with the RNA helicase p68. This complex is a component of the DROSHA microprocessor complex, which processes primary microRNAs to mature forms. BMP and TGFβ signaling increase the expression of mature miR21 by stimulating the activity of microprocessor in an R-Smad-dependent manner [63]. A noteworthy point is that this process does not need Smad4. Although miR-21 has no known function in skeletal cells, it is reasonable to speculate that Smad signaling has an effect in skeletal tissue by modulating the processing of different miRNAs. For example, miR-141 and miR-200 modulate BMP2-induced pre-osteoblast differentiation through translational repression of the transcription factor Dlx5 [64]. Moreover, a BMP responsive miRNA199a has been shown to target Smad1 and down-regulate its level to negatively regulate BMP2-induced target gene transcription in C3H10T1/2 cells [65].

Taken together, R-Smads 1/5/8 appear to mediate the majority of BMP effects in chondrogenesis, and they may do so through both transcriptional regulation and non-transcriptional regulatory mechanisms. Smads 1 and 5 share a high level of functional redundancy, whereas Smad8 is less important in chondrogenesis. On the other hand, Smads2 and 3 have been shown to have quite unique functions from each other. The non-Smad pathways may significantly contribute to TGFβ signaling in chondrogenesis suggested by the difference between the $TgfrII^{cko}$ and $Smad3^{-/-}$ phenotypes. However, conditional deletion of either or both of Smads 2 and 3 will reveal additional valuable information.

3. Roles of Smads in osteogenesis

BMP's function in osteogenesis is tightly related to runt related factor Runx2 (Cbfa1/AML3). Runx2 is a platform for the assembly of a multi-component regulatory complex that controls activation and repression of genes during cell fate determination and differentiation. In the skeletal system, Runx2 is critical for osteogenic lineage commitment and formation of the skeleton [66,67]. The interaction of Runx2 with BMP signaling is bidirectional. Runx2 is induced by BMP2 in osteoblast and chondrocyte cultures. On the other hand, Runx2 also induces BMP2 and 4 expressions by binding to a region in their promoters. In the process of osteogenic induction, Runx2 works together with Smads through direct binding in a transcriptional activator complex. Runx2 recruits R-Smads to the complex to initiate BMP responsive gene transcription [68,69]. The carboxyl terminus of Runx2 interacts with R-Smads via a Smad interacting domain (SMID), which overlaps with the nuclear matrix targeting signal (NMTS) [69]. Furthermore, the specific residues responsible for the interaction of Runx2 with R-Smads have been identified. A triple mutation of amino acids 426–428 (HTY-AAA) in the Runx2 C-terminal domain abolished both interaction with Smads and osteogenic differentiation [70].

Other mechanisms by which Smad proteins promote osteogenesis have been described. Smad1 interacts with Hoxc8, a transcription inhibitor, and dislodges it from its binding sites to stimulate *osteoprotegrin* transcription [71]. Osterix, an Sp1 transcription family member, is essential for osteogenesis. It is up-regulated by BMP2 during osteoblast differentiation and is considered to work downstream of Runx2 [73]. Recent studies have suggested that in addition to Runx2-mediated induction of Osterix, BMP signaling can also induce Osterix expression through Msx2 [74], which is one of the three members of Msh family of homeobox genes and induced directly by BMP-specific R-Smads [75]. Other possible mechanisms of BMP induction of osterix expression could be through Dlx5 (reviewed in [76]). Taken together, BMP R-Smads mediate BMP function in osteogenesis by interacting with Runx2 to activate target gene transcription, in parallel with direct induction of important osteogenic genes like MSX2 and Dlx5, leading to the induction of osterix (Fig. 2).

On the other hand, TGFβ R-Smads have been shown to work both synergistically and antagonistically with BMP in osteogenesis. For example, Smad3 has been shown to bind to the *osteopontin* promoter as a sequence specific activator [72]. However, TGFβ activated Smad3 inhibits osteocalcin expression by forming a repressive complex with Runx2 and histone deacetylase (HDAC) at osteocalcin promoter, and this mechanism is cell type and promoter specific [77].

Other transcriptional regulators working upstream, downstream or in parallel with BMP signaling in osteogenesis include Bapx1, Msx1, Dlx6, and inhibitor of differentiations (Ids) (for a detailed review, see [78]). Although these molecules participate in BMP signaling, direct evidence of interactions with Smad proteins in the context of osteogenesis is still missing. How TGFβ work synergistically or antagonistically with BMPs in this process through different Smad activities requires further investigation.

4. Roles of Smads in skeletal tissue regeneration

Tissue regeneration in bone and cartilage more or less recapitulates the process of development. However, there are noteworthy differences in terms of the cytokines and growth factors involvement. Therefore, it is reasonable to speculate that the process of bone/cartilage tissue regeneration has its own requirements for specific BMP/TGFβ ligands, receptors, and Smads

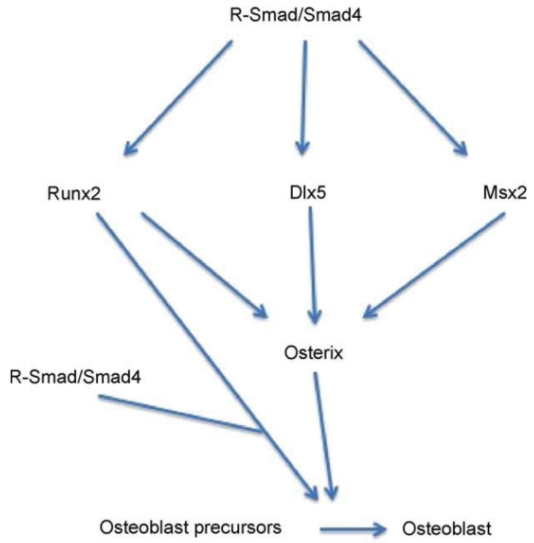

Fig. 2. BMP signaling regulates important osteogenic genes through direct induction of Runx2, Msx2 and possibly Dlx5 in a Smad-dependent manner. Then these molecules induce other important osteogenic gene expression, such as osterix. Runx2 work with R-Smad in an activator complex to activate BMP responsive genes. Osterix also activate osteogenic gene transcription, promoting the osteoblastic differentiation.

[79]. The bone healing process has been artificially divided to four different stages including (1) inflammation, (2) cartilage formation and periosteum response, (3) cartilage resorption and primary bone formation, and (4) secondary bone formation and remodeling. Multiple cytokines and growth hormones are involved in the four temporally overlapping stages. These include Interleukins, TNFα, PDGF, VEGF, and BMP/TGFβ. The major challenge in understanding the roles of BMPs and TGFβ in tissue regeneration at the molecular level is to characterize the spatial/temporal activity profile of different BMP/TGFβ ligands, receptors, and Smads, so that appropriate therapeutic strategies can be developed [80]. Fracture healing is the most widely studied process in bone tissue regeneration. Most bones heal by a combination of intramembranous and endochondral ossification. Endochondral bone formation occurs closer to the fracture site, which is mechanically unstable. It occurs external to the periosteum. Intramembranous bone formation occurs at both ends of the callus and internal to the periosteum [81]. During this process, BMP/TGFβ signaling is responsible for recruiting bone-forming cells, initiation of chondrogenesis and osteogenesis, and regulation of bone remodeling. Many studies have been done in animals and humans to elucidate the expression patterns of different ligands, leading to speculation on their functions [79]. A recent study demonstrated an essential role for BMP2 in fracture healing. Mice lacking BMP2 in limb cartilage and bone suffer from spontaneous fractures and an impaired fracture response in which cells are recruited to the site of injury but are unable to commit to chondrogenic or osteogenic fates [82]. For clinical applications, human recombinant BMP2 and BMP7 have been approved for promoting fracture healing. Direct evidence for the specific roles of Smad proteins in this process is very limited. A study in a rat fracture model indicated that in the fracture healing process, Smad1 and 5 expression patterns are similar to those of BMP2 and 7; whereas Smad2 and 3 expression patterns are similar to those of TGFβ [83]. This suggested that, at least partially, BMP/TGFβ signaling in fracture healing is mediated by canonical Smad

pathways. However, in addition to mediating the BMP/TGFβ signaling, it is conceivable that Smad proteins have multiple interactions with other signaling molecules to promote chondrogenesis and osteogenesis.

Studies of BMP/TGFβ signaling in cartilage repair or regeneration are mostly within the context of osteoarthritis (OA). TGFβ signaling promotes chondrocyte production of extracellular matrix (ECM), and maintains articular cartilage homeostasis. A number of ECM proteins have been shown to be TGFβ target genes [84]. Its protective role has been confirmed by the fact that *Smad3*$^{-/-}$ mice develop OA [28]. This also indicated that TGFβ exerts its protective role in articular cartilage at least partially through Smad3, rather than Smad2. Moreover, TGFβ has been shown to promote cartilage repair and to alleviate OA in animals. However, a major complication of applying TGFβ for OA treatment is that TGFβ application also induces the unwanted effects of tissue fibrosis and osteophyte formation. Recent studies have explored the possibility of applying TGFβ I-Smads locally in soft tissue to avoid this problem, but whether this is practicable is still under evaluation [85,86].

5. Role of I-Smads

I-Smads (Smads6 and 7) are key factors in intracellular regulation of BMP and TGFβ signaling. The protein-binding MH2 domain of I-Smads is structurally similar to that of R-Smads, but lacks the C-terminal Ser-X-Ser motif that is phosphorylated by the activated type I receptor. I-Smads act as competitive inhibitors of R-Smad phosphorylation by forming stable associations with activated type I receptors [87–89]. Smad6 can also inhibit R-Smad signaling in a phosphorylation-independent manner by interacting with receptor-activated Smad1, thus forming an inactive Smad1–6 complex [90]. In addition to their role as competitive inhibitors, I-Smads can inhibit BMP and TGFβ signaling by recruiting E3 ubiquitin ligases to type I receptors, R-Smads or Smad4, leading to their ubiquitination and degradation [91–94]. I-Smads, however, are not immune to E3 ligases. Smad7 can be targeted by the RING-domain E3 ligase, Arkadia [95], resulting in amplified TGFβ signaling. Interestingly, the expression of I-Smads is directly induced by BMP and TGFβ signaling, thus forming a negative feedback loop that limits the intensity and duration of BMP and TGFβ signaling. In addition, the complex interaction between I-Smads and E3 ligases may fine-tune BMP and TGFβ signaling.

I-Smads are strongly expressed in the prehypertrophic and hypertrophic zones of the growth plate [41], suggesting a role for I-Smads in regulating chondrocyte maturation. Indeed, gain-and loss-of-function studies have shown that I-Smads regulate chondrocyte maturation *in vitro* [96,97]. *In vivo* analyses revealed that I-Smads play multiple roles in development. Mice with a global deletion of *Smad6* or *Smad7* exhibit defects in the cardiovascular system [34,36]. Moreover, mice with a hypomorphic allele of *Smad7* have altered immune responses [37]. Gain of function studies has recently been conducted to determine role of I-Smads in endochondral bone formation. Cartilage-specific overexpression of *Smad6* results in delayed chondrocyte hypertrophy leading to dwarfism [35]. Overexpression of *Smad7* at various stages of endochondral bone formation in mice results in inhibition of mesenchymal cell condensation and chondrocyte proliferation, as well as delayed chondrocyte maturation [40]. Because of the high levels of I-Smad expression in these transgenic mice, the results may only highlight the pathological role of I-Smads in endochondral bone formation. Furthermore, it is not known whether Smad7 is normally expressed in condensing mesenchymal cells or proliferating chondrocytes. Hence, the physiological role of I-Smads is still unknown.

6. Smads and other signaling pathways

BMP/TGFβ signaling interacts with other signaling pathways to form a complicated network regulating cellular growth, differentiation, migration and apoptosis. These interactions and their end effects are usually species, tissue, and temporal–spatial specific. They also occur at different regulatory levels by engaging ligands, Smads, target genes, and intracellular proteins. In the context of skeletal development, BMP/TGFβ signaling predominantly interacts with Wnt, Indian Hedgehog (Ihh), PTHrP, FGF, PI3K/Akt, and MAPK signaling pathways through Smad-dependent interactions.

6.1. Smad interaction with Hedgehog and PTHrP pathway

Ihh signaling is important in growth plate chondrocytes. By interacting with PTHrP, Ihh promotes proliferation and inhibits differentiation of growth plate chondrocytes, thus regulating bone growth (reviewed in [98]). As mentioned above, Ihh is a direct target gene of BMP [48], and Ihh also promotes BMP expression levels. *In vitro* studies demonstrated direct association of Smad1 with truncated Gli3 protein [99], indicating direct roles of Smad proteins in the interaction of these two signaling pathways. *In vivo* data from *Smad1/5*^{cko} mice showed that Ihh and PTHrP receptor (PPR) mRNA levels were significantly reduced, indicating that BMP regulation of *Ihh* and *PPR* is direct and Smad1/5 dependent [12]. Little is known about interaction between TGFβ and Hedgehog signaling. A mouse metatarsal culture study suggested that the signaling relay from Ihh to PTHrP in the growth plate is mediated by TGFβ2 in the perichondrium [100]. Another study has shown that Smad3-dependent TGFβ signaling up-regulates *Gli2* expression in fibroblasts, keratinocytes, and several cancer cell lines. Mice with TGFβ1 overexpression showed Smad3-dependent increased expression of *Gli1* and *Gli2* in the skin [101]. However, whether these are true in the skeletal system remains to be investigated.

6.2. Smad interaction with FGF pathway

FGF signaling is essential in both endochondral and intramembranous bone formation. Disruption of FGF signaling is the cause of several human craniosynostosis and chondrodysplasia syndromes. FGF controls chondrocyte and osteoblast proliferation and differentiation through Jak/Stat and MEK1 pathways (reviewed in [102]. Previous studies have shown the antagonistic functions of FGF and BMP signaling in chondrocytes, however, the particular mechanisms underlying this antagonism remain largely unknown. Mouse model studies involving deletion of BMPR1A (ALK3) and BMPR1B (ALK6) in chondrocytes have confirmed this antagonism by showing the up-regulation of Stat1, Stat5, ERK1/2 in mutant growth plates, as well as increased levels of FGF receptor I [103]. In terms of Smad involvement, studies in other systems have shown that the linker region of Smad1 can be phosphorylated by MEK1 pathways, thereby inhibiting BMP signaling [104,105]. However, our recent study in a limb culture system argues this is not true for growth plate chondrocytes *in vivo*. FGF signaling did not affect linker phosphorylation of Smad. Instead, linker phosphorylation was induced by BMP treatment. Interestingly, the C-terminal phosphorylation of Smads is reduced in cartilage after FGF treatment, suggesting that FGF signaling antagonizes BMP by an indirect mechanisms, possibly by inducing de-phosphorylation of Smads or regulating BMP ligand and receptor expression [12].

6.3. Smad interaction with Wnt signaling

Wnt is another key signaling molecule in skeletal cells, regulating proliferation, differentiation, migration and apoptosis.

The interactions between BMP/TGFβ and Wnt pathways are profound and bidirectional. They occur at the level of ligands, cytoplasmic signaling intermediates, and transcriptional targets. First of all, Wnt and BMP/TGFβ pathways regulate ligand expression reciprocally. For example, in chicken embryos, Wnt-8c induces Nodal (a member of TGFβ superfamily) expression in a β-catenin-dependent manner [106], and BMP2 down-regulates Wnt7a and β-catenin in a p38 dependent manner, leading to enhanced chondrogenesis in mesenchymal cells [107]. An interaction also happens through connective tissue growth factor (CTGF). Wnt and BMP co-regulate CTGF expression in mesenchymal stem cells, and its induction inhibits osteoblastic differentiation [108]. CTGF is also co-regulated by Wnt and TGFβ in *Xenopus* embryos. In this scenario, CTGF interacts directly with BMP4 and TGFβ through its cystine rich (CR) domain. This direct binding prevents BMP4 from binding to its receptor, but enhances TGFβ1-receptor binding [109]. (For a more detailed review, see [110].)

Smad proteins play an important role in the cross talk between BMP/TGFβ and Wnt signaling. The first evidence of direct Smad interaction with Wnt signaling components was in *Xenopus* embryos. It was shown that Smad4, β catenin and Lef1 form a complex to activate expression of the Wnt target gene *twin* (*Xtwn*) [111]. Interestingly, this process does not necessarily require active TGFβ/activin signaling, implying an independent function of Smad4 [112]. Another study showed a direct interaction of Smads2, 3 and 4 with Lef1/TCF in mammalian cells. But these mechanisms of transcriptional regulation cannot be generalized since not all target genes of Wnt are regulated this way, and BMP signaling does not affect expression of these genes [111–113]. However, there are other genes that are regulated by a similar mechanism, including Msx1, Msx2 and Id2, which are relevant to skeletal system development, although the evidences comes from studies of neural development and human carcinoma [114–116]. In reciprocity, Wnt signaling can regulate BMP/TGFβ signaling by regulating GSK3-β activity. Wnt signaling deactivates GSK3-β and stabilizes Smad1, by preventing the ability of GSK3-β to phosphorylate the Smad linker region [117]. Linker phosphorylation of Smad1/5 by GSK3-β facilitates degradation, and prevents R-Smad interaction with nuclear pores [118]. Axin interacts with Smad3 to facilitate TGFβ signaling [119]. However, a recent study reported that Axin promotes Smad3 degradation to inhibit TGFβ signaling [120]. In a skeletal context, TGFβ1 has been shown to increase β-catenin nuclear translocation and to exert effects similar to those of Wnts on human bone marrow derived mesenchymal stem cells (MSC) (stimulating proliferation while inhibiting differentiation of these cells toward osteoblasts) or adipocytes. This process is Smad3-dependent and involves direct interaction between Smad3 and β-catenin. Using siRNA to knock down Smad3 abolished this effect, suggesting that Smad2 is unable to compensate for Smad3 for this specific function [121]. BMP signaling has been shown to have an opposite effect on Wnt signaling. Smad1 interacts with Dvl-1 at the linker region and this interaction accounts for the inhibitory effect of BMP2 on Wnt signaling in mouse mesenchymal stem cells (MSCs) [122].

6.4. Smad interaction with PI3K/Akt pathway

Phosphatidylinositol 3 kinase converts phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-triphosphate (PIP_3). PIP_3 then regulates downstream effectors such as Akt, a serine/threonine kinase. This pathway usually promotes cell survival, growth, and migration. The PI3K/Akt pathway is negatively regulated by phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which is a phosphatase that dephosphorylate PIP_3 to PIP_2 thus deactivating PIP_3-dependent pathways. Just like TGFβ-Wnt interactions, interactions between PI3K/Akt and BMP/TGFβ have been discovered in different cell types. PI3K/Akt pathways antagonize the pro-apoptotic effects and cell cycle arrest induced by TGFβ signaling. The mechanism of the pro-apoptotic effect of TGFβ has been shown in different cell types, and is mostly Smad3-dependent [123]. Consistently, the mechanism of PI3K/Akt-mediated restriction of TGFβ signaling appears to be through Smad3. Different studies have suggested mechanisms involving direct or indirect interaction of Akt with Smad3, and enhanced or attenuated TGFβ signaling regulated by PI3K/Akt (for a more detailed review, see [110]). On the other hand, PI3K/Akt signaling is also regulated by BMP/TGFβ in the context of cell migration, epithelial–mesenchymal transition, cell survival, and cell growth; but again, these interactions are all likely cell type dependent [110]. In a skeletal development context, studies carried out in mesenchymal precursor 2T3 cells showed dominant-negative PI3K and dominant-negative Akt have been shown to inhibit Smad5-dependent target gene transcription, as well as nuclear translocation of BMP-specific R-Smads after ligand stimulation [124].

6.5. Smad interaction with MAPK pathway

MAPK pathways are evolutionarily conserved and regulate a variety of cellular events. There are three distinct MAPK pathways, Erk1/2, JNK1/2/3, and p38/MAPKs. The interaction of MAPK pathways with BMP/TGFβ and Smads generates a complicated network, involving transcriptional regulation of Smads, as well as phosphorylation of Smad linker regions. Some of these interactions have been discussed in the context of Smad/FGF interactions. Again, although the interactions have been intensively studied, most of the evidence is derived from *in vitro* studies. For example, Smad1/5 and Smad2/3 could all be phosphorylated by MAPKs at the linker region, and this phosphorylation had different effects on each individual Smad (reviewed in [110]). So far, *in vivo* data from the skeletal system are limited.

However, both BMP and TGFβ/activins have been shown to activate certain MAPK pathways, such as TAK1/p38 pathway. These pathways mediate the effects of non-Smad signaling, or non-canonical signaling of TGFβ superfamily proteins [17,18], although the mechanisms of how BMP/TGFβ activate the MAPK signaling pathways are still under intensive investigation. The most recent study showed that, in glomerular mesangial cells, TGFβ1 activate TAK1 through TAB1-mediated auto-phosphorylation of TAK1, without the requirement of the kinase activity of type I receptor [125]. As mentioned in the beginning of this review, such non-canonical signaling may be more important than suggested by our current understanding. The fact that MAPK pathways also regulate Smad protein activities raised the possibility of cross talk between these two branches of BMP/TGFβ signaling in skeletal genesis. In deed, in addition to linker region phosphorylation, studies on TAK1 conditional knock out mice in chondrocytes suggested TAK1 also regulate C-terminal phosphorylation of Smad1. Moreover, TAK1 deficient chondrocytes showed reduced activity of Smad1/5/8, and decreased expression level of multiple BMP target genes, suggesting extensive regulation of BMP signaling by TAK1 through acting on both Smad and non-Smad proteins [126]. Further investigation is needed to reveal this emerging complex picture of BMP/TGFβ signaling crosstalk.

7. Perspectives

Smad proteins have been long considered the major intracellular signaling transduction molecules for TGFβ superfamily members. Since TGFβ superfamily signaling has a key role in skeletal development and regeneration, it is then very important to understand the role of Smad proteins in this context. However, fewer studies have been focused on Smad family members than on

the corresponding ligands and receptors. With the emerging picture of a complicated network of intracellular molecules involved in TGFβ superfamily signaling, it is more important to unveil the functions of Smad proteins. A major challenge here is that the functions of Smads and their interactions with other signaling molecules seem to be cell type, tissue and species specific. This emphasizes the importance of *in vivo* studies in animal models. Currently, mouse models are the most accessible and convenient for studying Smad functions in skeletal development. Recent studies have already pointed out the importance of Smad1/5 in skeletal formation; however, roles of TGFβ specific Smads, co-Smad, and inhibitory Smads have not been fully addressed. For the investigation of these topics, conditional knockouts of Smad proteins in different compartments or stages of skeletal development will yield valuable information. At the same time, interactions with other signaling pathways or intracellular molecules could be studied in these animal models. Such studies will reveal more information about the major regulators in skeletal development and regeneration, identify new participants/targets in these processes and lead to new strategies for disease treatment and tissue engineering.

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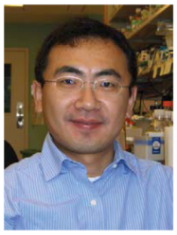

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Karen M. Lyons Professor Karen M. Lyons is a world-renowned expert in the research of developmental biology. Her research interest has been focused on BMP/TGFβ signaling in bone and cartilage. She received a PhD degree in Medical Genetics from the University of Wisconsin, Madison in 1988. She then completed two postdoctoral fellowships in Developmental Biology. The first fellowship was with Dr. Brigid Hogan at Vanderbilt University in Nashville, Tennessee, where she cloned one of the first BMP genes to be discovered, BMP6. The second fellowship was with Dr. Liz Robertson at Harvard University in Cambridge, Massachusetts, where she generated mice lacking BMPs 6 and 7. In 1995, she accepted a faculty position at the University of California in Los Angeles, where she is currently a full professor in the Department of Orthopaedic Surgery and has a joint appointment in Molecular, Cell and Developmental Biology. At UCLA, Karen generated mice lacking the BMP receptor type IB, and demonstrated that this receptor is essential for chondrogenesis. More recently, she has shown that the majority of BMP signaling in skeletal cells occurs through canonical pathways.

CHAPTER THREE:

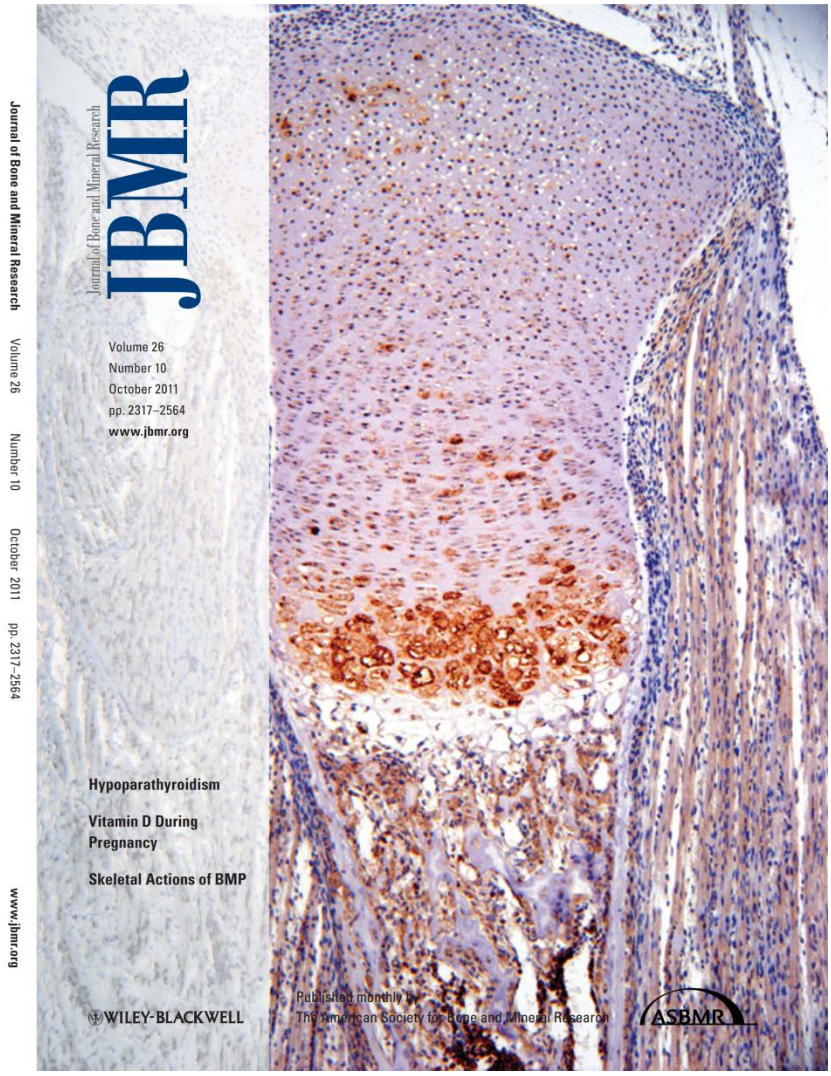
Smad6 is essential to limit BMP Signaling during cartilage development

Preface

In vitro studies have shown that Smad6 is a key intracellular inhibitor of BMP signaling in many different cell types, including chondrocytes. Prior to our work, *in vivo* studies in which Smad6 was overexpressed in chondrocytes have shown that Smad6 inhibits BMP signaling during cartilage development, as Smad6 transgenic mice exhibit postnatal dwarfism and osteopenia associated with an inhibition of BMP signaling in chondrocytes (Horiki et al., 2004). In these overexpression studies, however, Smad6 is expressed in all chondrocyte subpopulations, and thus it does not recapitulate the physiological expression pattern and consequently the physiological function of Smad6 in developing cartilage. Thus, although Horiki and colleagues have shown that Smad6 has the potential to inhibit BMP signaling during cartilage development, it is unknown whether it is actually required. Therefore, our work provides the first evidence that Smad6 is required for normal cartilage development, and that Smad6 regulates endochondral bone formation by limiting BMP signaling in chondrocytes.

Preliminary phenotypic analyses via histological staining were performed by Kelsey Retting, a previous graduate student in the lab. I continued the phenotypic analyses and performed all of the *in vitro* experiments, with the help of two wonderful undergraduate researchers, Alana Chin and Christina Hong. I prepared all of the figures and wrote the initial draft of the manuscript. Karen Lyons and Alana Chin provided critical comments, after which I revised and then prepared the manuscript for submission to the Journal of Bone and Mineral

Research. This paper was accepted with minor revisions and a figure from the paper was chosen as cover art (see below) for the October 2011 issue (Volume 26, Issue 10).



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ORIGINAL ARTICLE

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Smad6 Is Essential to Limit BMP Signaling During Cartilage Development

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ABSTRACT

Bone morphogenetic protein (BMP) signaling pathways regulate multiple aspects of endochondral bone formation. The importance of extracellular antagonists as regulators of BMP signaling has been defined. In vitro studies reveal that the intracellular regulators, inhibitory Smads 6 and 7, can regulate BMP-mediated effects on chondrocytes. Although in vivo studies in which inhibitory Smads were overexpressed in cartilage have shown that inhibitory Smads have the potential to limit BMP signaling in vivo, the physiological relevance of inhibitory Smad activity in skeletal tissues is unknown. In this study, we have determined the role of Smad6 in endochondral bone formation. Loss of *Smad6* in mice leads to defects in both axial and appendicular skeletal development. Specifically, *Smad6*−/− mice exhibit a posterior transformation of the seventh cervical vertebra, bilateral ossification centers in lumbar vertebrae, and bifid sternebrae due to incomplete sternal band fusion. Histological analysis of appendicular bones revealed delayed onset of hypertrophic differentiation and mineralization at midgestation in *Smad6*−/− mice. By late gestation, however, an expanded hypertrophic zone, associated with an increased pool of proliferating cells undergoing hypertrophy, was evident in *Smad6* mutant growth plates. The mutant phenotype is attributed, at least in part, to increased BMP responsiveness in *Smad6*-deficient chondrocytes. Overall, our results show that Smad6 is required to limit BMP signaling during endochondral bone formation.

KEY WORDS: BMP/SMAD; GROWTH PLATE; CHONDROCYTE; RODENT; GROWTH; DEVELOPMENT

Introduction

Endochondral bone formation is the multistep process that is responsible for the development of the majority of the skeleton.[1] First, condensed mesenchymal cells in the developing limb bud differentiate into chondrocytes, which then contribute to the formation of the cartilage growth plate that is gradually replaced by bone. Growth plate chondrocytes undergo a complex program of proliferation, hypertrophic differentiation, and apoptosis. The zone of hypertrophic chondrocytes is invaded by blood vessels, osteoblasts, and osteoclasts to initiate ossification of the cartilage matrix.

Bone morphogenetic protein (BMP) signaling pathways play important roles at multiple stages of endochondral bone formation. In particular, BMP signaling promotes chondrocyte proliferation, in part through upregulation of *Sox9* and type II collagen expression.[2,3] BMP signaling also has been shown to induce chondrocyte hypertrophy,[4] but to delay terminal differentiation of chondrocytes.[5] Thus, BMP signaling regulates chondrocyte proliferation and differentiation. The BMP signaling pathway is required for endochondral bone formation, as evidenced by severe chondrodysplasia in mice lacking BMP signaling components.[6–8]

BMP signaling is initiated by the binding of ligands to heterotetrameric complexes of types I and II serine/threonine kinase receptors, leading to activation of downstream signaling mediators via two distinct mechanisms: the canonical Smad pathway and multiple noncanonical (non-Smad) pathways.[9,10] In the canonical Smad pathway, BMP signals are transduced via phosphorylation of receptor-regulated Smads (R-Smads) 1, 5, and 8 by activated type I receptors. Once phosphorylated, R-Smads associate with the common partner Smad4, translocate into the nucleus, and interact with other transcription factors to induce the expression of downstream genes. In the noncanonical pathways, BMPs activate transforming growth factor β (TGF-β)-activated kinase 1 (TAK1), leading to the activation of the mitogen-activated protein kinases (MAPKs), including p38, JNK, and extracellular signal-regulated kinases (ERKs).[11]

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Additional Supporting Information may be found in the online version of this article.

The intensity and duration of BMP signaling can be regulated both extracellularly and intracellularly. Extracellular regulation occurs via binding of antagonists, such as noggin and chordin, to BMPs, thereby preventing them from binding to BMP receptor complexes. Intracellular regulation occurs, in part, through the actions of inhibitory Smads (I-Smads) 6 and 7. Smad7 can inhibit multiple pathways, including TGFβ/activin and BMP signaling, whereas Smad6 specifically inhibits BMP signaling.[12,13] I-Smads block the phosphorylation of R-Smads by forming stable associations with activated type I receptors.[14–16] In addition, I-Smads can recruit E3 ubiquitin ligases to type I receptors, leading to ubiquitination and subsequent degradation of these receptors.[17,18] I-Smads also can bind to Smad1, thereby interfering with Smad1-Smad4 complex formation.[19] The expression of I-Smads is directly induced by BMP signaling, thus forming a negative feedback loop.[20,21]

The importance of extracellular BMP antagonists in regulating endochondral bone formation has been established. In fact, extracellular regulation by the BMP antagonist noggin is required, because *noggin*-deficient mice exhibit enlarged growth plates and joint fusions.[22] Although gain-of-function and loss-of-function studies reveal a role for I-Smads in chondrocytes in vitro,[4,20,24] in vivo studies are limited to gain-of-function studies.[25,26] Thus, whether I-Smads are required for normal endochondral bone formation is unknown. Here, we show that loss of *Smad6* in mice results in both axial and appendicular skeletal defects that can be attributed to increased BMP responsiveness in chondrocytes. Thus, Smad6 limits BMP signaling for proper skeletal development.

Materials and Methods

Generation of *Smad6*−/− mice

Generation of *Smad6*−/− mice was previously described. Briefly, targeted disruption of the *Madh6* (which encodes the Smad6 protein) was generated by insertion of a *LacZ*/neomycin resistance cassette at codon 342 of the exon encoding the MH2 domain. Embryos and mice were genotyped by PCR using the sense primer 5′-CCTTGCCATATCCTATGCTTGCG-3′ and antisense primer 5′-GCGCCGCACCGACTCACTGC-3′ to detect the wild-type (WT) allele, and the sense primer 5′-GCTTCCTCGT-GCTTTACGGTATC-3′ and antisense primer as above to detect the mutant allele. Embryos and mice were on a mixed C57BL/6J/BALB/c background.

Skeletal preparation and histology

Skeletal preparations were performed as in ref. [6]. Briefly, embryos were eviscerated and fixed in 95% EtOH overnight at 4°C, followed by Alcian Blue staining (0.01% Alcian Blue 8GX [Sigma-Aldrich, St. Louis, MO, USA, A5268] [w/v] in 95% EtOH) overnight at room temperature. Samples were then stained for Alizarin Red (0.006% Alizarin Red S [Sigma-Aldrich, A5533] [w/v] in 1% KOH) for 3 to 4 hours and cleared in a series of graded KOH in glycerol.

For histology, embryos were fixed with 10% buffered formalin (Thermo Fisher Scientific, Waltham, MA, USA, SF100) overnight at 4°C, decalcified with Immunocal (Decal Chemical Corp., Tallman, NY, USA) overnight at 4°C, embedded in paraffin, and cut at a thickness of 5–7 μm. Sections were stained with Alcian Blue (Sigma-Aldrich, A5268) and Nuclear Fast Red (Sigma-Aldrich, N8002). as in [6]. Von Kossa staining was performed by incubating sections with 1% silver nitrate under UV light for 20 minutes and then counterstaining with Nuclear Fast Red.

Immunohistochemistry and immunofluorescence staining

For immunohistochemistry (IHC) and immunofluorescence staining, paraffin-embedded tissue sections were boiled in citrate buffer pH 6.0 for 15 minutes at 95°C for antigen unmasking. Sections stained for extracellular matrix proteins (type II collagen, type X collagen, MMP-13 and osteopontin [OPN]) were digested with 1 mg/mL hyaluronidase (Sigma-Aldrich, H3506) in Dulbecco's PBS (Mediatech, Inc., Manassas, VA, USA) for 45 minutes at 37°C before antigen unmasking.

For detection using the following primary antibodies: Ihh (Santa Cruz Biotechnologies, Santa Cruz, CA, USA, sc1196), MMP-13 (Abcam, Cambridge, MA, USA, ab84594), osteopontin (Thermo Fisher Scientific, RB-9097-P0), phospho-Smad1/5/8 (Cell Signaling, Danvers, MA, USA, #9511), and Ptc1(Novus Biologicals, Littleton, CO, USA, NB100-91923), sections were quenched in 3% H_2O_2 in methanol, blocked with 0.5% blocking reagent (TSA Biotin System, Perkin Elmer, Waltham, MA, USA, NEL700A) in TBS (100 mM Tris pH 7.5, 150 mM NaCl), and incubated with primary antibody overnight at 4°C. Detection of binding was performed using the TSA Biotin System according to the manufacturer's protocol. Fluorescence detection was conducted by using either the Streptavidin–AlexaFluor -555 (Invitrogen, Carlsbad, CA, USA) secondary antibodies; sections were counterstained with DAPI (Invitrogen, D1306).

For detection of type II collagen (Abcam, ab21291) and type X collagen (Abcam, ab58632), sections were blocked and incubated with primary antibody, as described above, incubated with either the AlexaFluor-488 or -555 (Invitrogen) secondary antibodies for 30 minutes at room temperature, and then counterstained with DAPI. For detection of Smad6 (Santa Cruz Biotechnologies, sc-6034), sections were blocked, incubated with primary antibody, and quenched in 3% H_2O_2 in methanol, as described above. Sections were then incubated with biotin antigoat (Santa Cruz Biotechnologies) and Streptavidin-HRP (Perkin Elmer) secondary antibodies. Chromogenic detection was performed with the DAB Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA, USA, SK-4100) following the manufacturer's instructions; sections were counterstained with Hematoxylin QS (Vector Laboratories, H-3404).

Cell proliferation and TUNEL labeling

For detection of cell proliferation in vivo, immunofluorescence staining was performed by using either the anti-PCNA primary antibody (Thermo Fisher Scientific, #13-3900) or phospho-histone H3 (Ser10) antibody (Cell Signaling, #9701), and biotin-XX antimouse (Invitrogen) and Streptavidin-AlexaFluor-555 (Invitrogen) secondary antibodies. For TUNEL labeling, the fluorescein In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN, USA) was used according to the manufacturer's instructions.

Cell culture and luciferase reporter assays

Primary chondrocytes were isolated from costal cartilage as previously described.[28] For quantitative real-time PCR and semiquantitative RT-PCR, cells were seeded at 1×10^6 in 6-well plates and maintained for 7 or 10 days in chondrogenic media (DMEM supplemented with 10% FBS, 1% antibiotic-antimycotic [Invitrogen] and 50 μg/mL ascorbic acid). Cells were serum starved overnight with DMEM supplemented with 1% FBS and 1% antibiotic-antimycotic (Invitrogen), then stimulated the next day with 100 ng/mL BMP2 (R&D Systems, Minneapolis, MN, USA, 355-BM) for 2 hours or with an equal volume of DMSO for untreated cells. Each experiment was repeated twice (ie, from two different cell isolations).

For Western blot analysis, cells were seeded at 100×10^3 cells/well in 12-well plates and maintained for 10 days in chondrogenic media. Cells were serum starved overnight with DMEM supplemented with 1% antibiotic-antimycotic (Invitrogen), then stimulated the next day with 50 ng/mL BMP2 (R&D Systems, 355-BM) for 2, 4, 12, or 24 hours or with an equal volume of DMSO for untreated cells (0 hour). Each experiment was repeated in triplicate (ie, from three different cell isolations).

For luciferase reporter assays, cells were seeded at 100×10^3 cells/well in 12-well plates and then transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions with 0.5 μg of the 560 bp-*Msx2*-TKluc[29] and 0.05 μg of the pRL-TK (Promega, Madison, WI, USA) reporter plasmids. Cells were serum starved for 24 hours posttransfection with DMEM supplemented with 1% antibiotic-antimycotic (Invitrogen), and then stimulated the next day with 50 ng/mL BMP2 for 2 or 8 hours or with an equal volume of DMSO for untreated cells (0 hour). Firefly and Renilla luciferase activities were measured with the Dual-Luciferase Promoter Assay System (Promega). Firefly luciferase activities were obtained in triplicate for each experiment and were normalized to Renilla luciferase activities. Statistical significance was assessed via within-subjects multifactorial ANOVA using the ezANOVA statistical software (http://www.cabiatl.com/mricro/ezanova/index.html).

Quantitative real-time PCR, semiquantitative RT-PCR, and Western blot analysis

RNA was extracted using the RNeasy Kit (Qiagen, Valencia, CA, USA). Synthesis of cDNA was performed with Superscript III (Invitrogen). Quantitative real-time PCR reactions were performed with a SYBR Green Real-time PCR Master Mix (Fermentas, Glen Burnie, MD, USA) by using an Mx3005P QPCR System (Stratagene, Santa Clara, CA, USA). The primer sequences were as follows: *β-actin*: forward 5′-CTGAACCCTAAGGCCAACCG-3′, reverse 5′-GTCACGCACGATTTCCCTCTC-3′; *Ihh* (from):[30] forward 5′-GACTCATTGCCTCCCAGAACTG-3′, reverse 5′-CCAGGTAGTAGGGTCACATTGC-3′; *PPR*: forward 5′-CTGGCCATTGGGGGCACCAG-3′, reverse 5′-CGGCGCGCAGCATAAACGAC-3′; *Ptc1* (from):[31] forward 5′-CAAGTGTCGTCCGGTTTGC-3′, reverse 5′-CTGTACTCCGAGTCGGAGGAA-3′; *PTHrP* (from):[30] forward 5′-GAACATCAGCTACTGCATGACAAG-3′, reverse 5′-TCTGATTTCGGCTGTGTGGATC-3′; *Smad6*: forward 5′-ATTCTCGGCTGTCTCCTCCT-3′, reverse 5′-CCCTGAGGTAGGTCGTAGAA-3′; and type II collagen (from):[32] forward 5′-ACTGGTAAGTGGGGCAAGAC-3′, reverse 5′-CCACACCAAATTCCTGTTCA-3′. Semiquantitative RT-PCR reactions were comprised of 25–42 cycles at 95 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 1 minute. The primer sequences were as follows: *GAPDH*: forward 5′-CCAGAACATCATCCCTGCATC-3′, reverse 5′-GGTAGGAACACGGAAGGCC-3′; type II collagen: forward 5′-AATGGGCAGAGGTATAAAGATAAGGA-3′, reverse 5′-CATTCCCAGTGTCACACACACA-3′; and type X collagen: forward 5′-CAAACGGCCTCTACTCCTCTGA-3′, reverse 5′- CGATGGAATTGGGTGGAAAG-3′.

For Western blot analysis, cells were lysed in RIPA buffer (25 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 1% Na-deoxycholate, 0.1% SDS) supplemented with protease inhibitors (complete Mini Tablets, Roche Applied Science) and phosphatase inhibitors (Sigma-Aldrich, P5726). Whole cell lysates (5 μg) were run on 10% SDS-polyacrylamide gels and transferred semidry onto PVDF membranes. The membranes were blocked with 5% milk in TBS-tween (30 mM Tris pH 7.4, 300 mM NaCl, 0.2% tween-20), incubated with primary antibody (from Cell Signaling: phospho-p38 [#9215], p38 [#9212], phospho-Smad1/5/8 [#9511] or Smad5 [#9517]; Abcam: BMPR1A [#ab59947]; Sigma-Aldrich: β-actin [A5316] or tubulin [T6793]) diluted in blocking buffer overnight at 4°C, and then incubated with appropriate secondary antibody diluted in blocking buffer for 1 hour at room temperature. Binding was detected via enhanced chemiluminescence using the Amersham ECL Plus kit (GE Healthcare, Piscataway, NJ, USA).

Results

Smad6 localization in cartilage elements during development

The expression of Smad6 in the developing axial skeleton was examined by IHC on E12.5 wild-type (WT) embryos. Smad6 was expressed in the primordia of vertebrae and intervertebral discs (Fig. 1*A*), ribs (Fig. 1*B*) and sternebrae (not shown). By E14.5, Smad6 was detected in the growth plates and perichondria of vertebrae (Fig. 1*C, C′*), ribs (Fig. 1*D, D′*) and sternebrae (not shown).

In appendicular bones, Smad6 was expressed throughout the growth plate at E15.0 (Fig. 1*E*). Examination of subcellular localization revealed that Smad6 was localized in both the cytoplasm and nucleus of proliferating cells (Fig. 1*E′*), whereas it was localized predominantly in the cytoplasm of hypertrophic cells (Fig. 1*E″*). At P0, Smad6 expression persisted in the reserve and proliferative zones of growth plates, whereas higher levels were observed in the prehypertrophic and upper-hypertrophic zones; no expression was found in terminal hypertrophic chondrocytes (Fig. 1*F*). At this stage of development, Smad6 expression was localized in both the cytoplasm and the peripheral cell membrane of hypertrophic cells (Fig. 1*F′*). These results suggest that Smad6 may play a role in regulating chondrocyte proliferation and hypertrophic differentiation throughout chondrogenesis in vivo.

Skeletal defects in Smad6−/− mice

Smad6 knockout (*Smad6*−/−) mice were recovered at Mendelian ratios up to E18.5, whereas the majority of

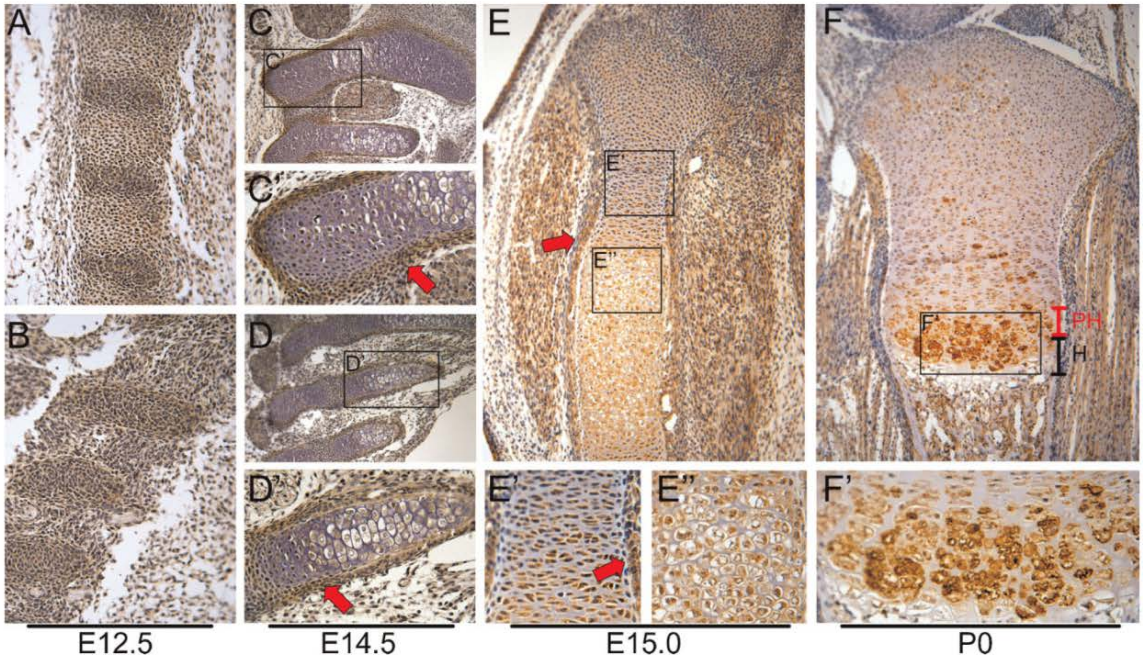

Fig. 1. Smad6 localization in cartilage during development. Immunohistochemistry of sagittal sections of WT embryos at E12.5 for Smad6 *(brown color)* shows expression in (*A*) developing vertebral bodies and intervertebral discs of lumbar vertebrae and (*B*) developing anterior ribs. Staining of sagittal sections at E14.5 shows Smad6 expression in (*C*) cervical vertebrae and (*D*) anterior ribs. Higher magnification of the (*C'*) lateral region of C1 and (*D'*) second rib shows Smad6 expression in the growth plate and perichondrium *(red arrows)*. (*E*) Smad6 expression in tibiae at E15.0. (*E'*) Smad6 is localized in the cytoplasm and nucleus of proliferative cells at E15.0. Smad6 is also expressed in the perichondrium *(red arrow)*. (*E''*) Smad6 is localized in the cytoplasm of hypertrophic cells at E15.0. (*F*) By postnatal day 0 (P0), Smad6 levels are highest in the prehypertrophic and upper-hypertrophic zones of the tibial growth plate. (*F'*) Smad6 is localized in both the cytoplasm and the peripheral cell membrane of hypertrophic cells. For all stains, no detectable staining was observed in controls, from which primary antibodies were omitted. PH, prehypertrophic; H, hypertrophic zone.

Smad6−/− mice died within 24 hours after birth (ie, *Smad6*−/− mice represented 5% of heterozygous intercross progeny [$n = 112$]). The mutant mice that did survive were slightly smaller compared with their WT littermates at P0 (Fig. 2*A*). The early lethality of *Smad6*−/− mice in our hands is in contrast to the results of Galvin and colleagues, who reported that some *Smad6*−/− mice survive to early adulthood (ie, 3–13% of heterozygous intercross progeny); but that those mice that survive to this stage die of cardiovascular defects, including cartilaginous metaplasia of the aorta.[27] The cause of the earlier lethality observed in our study is unknown, but may reflect differences in the background strain.

Analysis of whole-mount skeletal preparations at P0 revealed that *Smad6*−/− mice exhibit a domed skull and shortened snout (Fig. 2*A, B*). Analysis of the cranial base revealed that whereas the length of the cranial base in mutants was similar to that in WT littermates, the widths of both the supraoccipital bone and the posterior end of the basioccipital bone were narrow (Fig. 2*B*). These defects may contribute to the formation of the domed skull in mutants. Additionally, the mandibles exhibited more curvature and were shorter in mutants (Fig. 2*C*). No defects were found in cranial sutures (data not shown).

Loss of *Smad6* in mice led to defects in the axial skeleton. In particular, *Smad6*−/− mice exhibit a posterior transformation of the seventh cervical vertebra (C7), as evidenced by the presence of a small rib anlagen (Fig. 2*D*). Additionally, thoracic vertebral bodies are flatter in *Smad6*−/− mice compared with WT littermates (Fig. 2*E*), and bilateral ossification centers were found in lumbar vertebrae (Fig. 2*F, I*). Intervertebral discs in these regions were rounded in mutants (Fig. 2*F*). Analyses of representative disarticulated vertebrae show normal development of the first cervical vertebra (C1) in mutants (Fig. 2*G*). Formation of pedicles and transverse processes of thoracic and lumbar vertebrae also was normal in mutants (Fig. 2*H, I*). Alcian Blue staining of lumbar vertebrae shows that the bilateral ossification centers found in mutants are the result of incomplete fusion of sclerotomal cells (Fig. 2*J*).

The sternum develops by the ventral migration of two lateral mesenchymal bands that fuse at the midline. Individual sternebrae form by endochondral ossification in intercostal regions, leading to segmentation of the sternum.[33] The sterna of WT mice at P0 displayed six separate ossified sternebrae, with ribs attached to the cartilaginous intersternabrae. In contrast, *Smad6*−/− mice develop bifid sternebrae due to incomplete medial fusion (Fig. 2*K, L*). In addition, the third, fourth, and fifth sternebrae (ST3, ST4, and ST5) of mutant mice were fused (Fig. 2*K*).

Skeletal preparations at P0 showed that appendicular bones were slightly shorter in mutant mice (Fig. 2*M*), proportionate with

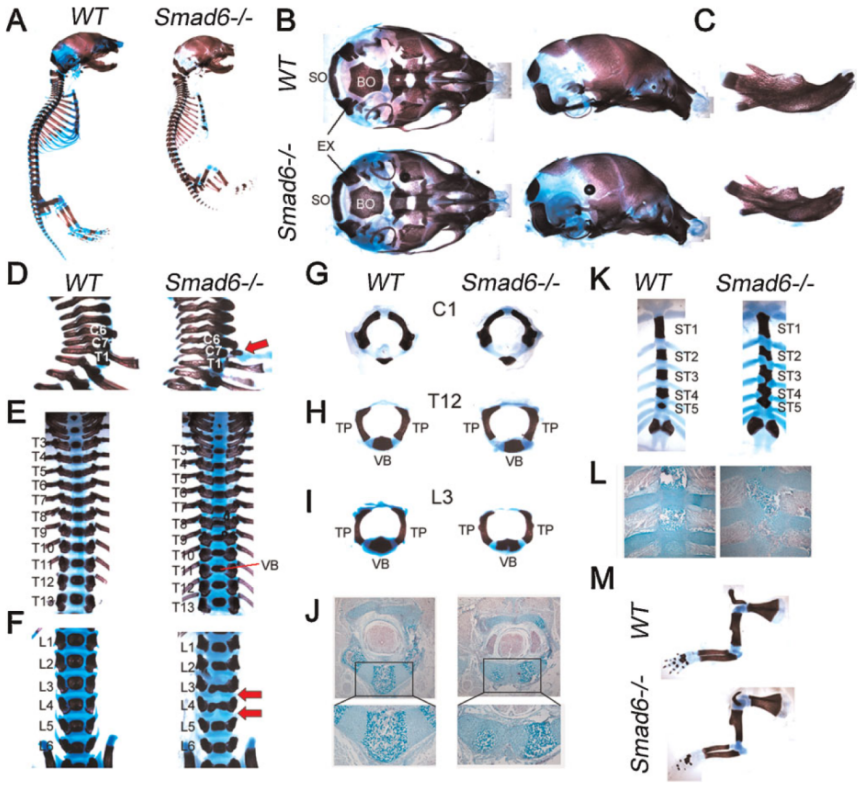

Fig. 2. Skeleton defects in *Smad6*−/− mice at P0. (*A*) Whole-mount skeletal preparations of WT and *Smad6*−/− P0 embryos. (*B*) Basal *(left)* and lateral *(right)* view of skull. Mandibles and hyoid bones have been removed. (*C*) Lateral view of mandibles. (*D*) Lateral view of cervical vertebrae. Red arrow highlights rib indicative of posterior transformation of the seventh cervical vertebra in mutants. (*E*) Dorsal view of thoracic vertebrae. (*F*) Dorsal view of lumbar vertebrae. Red arrows highlight rounded intervertebral discs in mutants. Split vertebral bodies are evident in L3 and L4 of mutants. (*G*) Anterior view of C1. (*H*) Anterior view of T12. (*I*) Anterior view of L3 shows a split vertebral body in mutants. (*J*) Alcian Blue staining of lumbar vertebra shows bilateral ossification centers in mutants, presumably because of incomplete fusion of somite pairs. (*K*) Dorsal view of sternum shows bifid sternebrae and fused ST3, ST4, and ST5 in mutants. (*L*) Alcian Blue staining of sternum shows defects in sternal band fusion in *Smad6*−/− mice. (*M*) Forelimbs of WT and *Smad6*−/− embryos at P0. EX, exoccipital; BO, basioccipital bone; SO, supraoccipital; TP, transverse process; VB, vertebral body.

the overall smaller size of *Smad6*−/− mice. Overall, these results indicate that *Smad6* regulates skeletal development.

Delayed onset of hypertrophy and increased matrix production in appendicular bones of Smad6−/− mice

Alcian Blue staining of hindlimbs was performed to examine appendicular bone formation in more detail. Whereas Alcian Blue staining revealed no defects at E13.5 in *Smad6*−/− mice (Fig. 3*A, B*), a shorter hypertrophic zone was evident by E15.0 in long bones of mutant cartilage (Fig. 3*C–F*). The shortened hypertrophic zone could result from a delay in the onset of hypertrophy, and/or retention/recruitment of chondrocytes in the proliferative pool, or defective hypertrophic differentiation. To evaluate whether the smaller hypertrophic zones at E15.0 in *Smad6*−/− cartilage resulted from defective hypertrophic differentiation, we examined the expression level of type X collagen by immunofluorescence staining. Normal levels of type X collagen were evident in both WT and mutant littermates, but the domain of type X collagen expression was reduced in mutant cartilage (Fig. 3*G, H*). These results suggest that whereas the onset of hypertrophic differentiation appears to be delayed, there is no defect in the ability of mutant chondrocytes to undergo hypertrophy.

By E16.5, distinct growth plates separated by a marrow cavity were evident in cartilage of WT and *Smad6*−/− littermates (Fig. 3*I, J*). Histologically, the growth plate appeared indistinguishable between WT and mutant littermates at E16.5 (Fig. 3*I′, J′*). Consistent with delayed onset of hypertrophy observed at E15.0, the marrow cavity was smaller in mutants (Fig. 3*K, L*). Moreover, von Kossa staining at this stage revealed defects in mineralization in mutant cartilage (Fig. 3*M, N*). Taken together, these results indicate that loss of *Smad6* leads to delayed onset of chondrocyte hypertrophy and ossification at midgestation, but once initiated, these processes can proceed normally in *Smad6*−/− mice.

By E18.5, enlarged prehypertrophic and hypertrophic zones were clearly apparent in *Smad6*−/− growth plates (Fig. 4*A–D*). Immunofluorescence staining at this stage showed that the enlarged hypertrophic zones were accompanied by increased expression domains of type X collagen (Fig. 4*E*). Moreover, the intensity of staining for type X collagen was higher in mutants

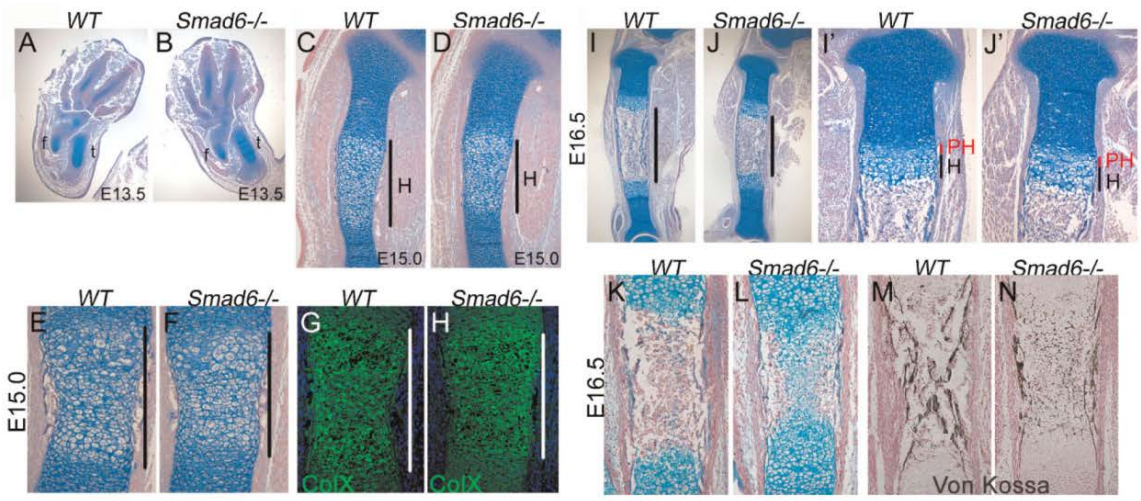

Fig. 3. Delayed onset of hypertrophic differentiation and mineralization at midgestation in *Smad6−/−* mice. (*A,B*) Formation of cartilage condensations in *Smad6−/−* limbs is similar to that in WT limbs at E13.5. (*C,D*) A shorter hypertrophic zone is evident in the *Smad6−/−* tibias at E15.0. (*E,F*) Alcian Blue staining of E15.0 femoral diaphyses. The hypertrophic zone in *Smad6−/−* mice is demarcated by black bars. (*G,H*) Immunofluorescence staining for type X collagen of E15.0 femurs. The hypertrophic zone is demarcated by white bars. No detectable staining was observed in negative controls, from which primary antibodies were omitted. (*I,J*) Shorter diaphyses are evident in the E16.5 *Smad6−/−* tibiae, as demarcated by black bars. (*I',J'*) Higher magnification of proximal tibiae at E16.5. (*K,L*) Alcian Blue staining of E16.5 tibial diaphyses. (*M,N*) Von Kossa staining of E16.5 tibiae shows defects in mineralization in *Smad6−/−* mice. f, fibula; t, tibia; PH, prehypertrophic zone; H, hypertrophic zone.

than in WT littermates, revealing increased biosynthetic activity of mutant hypertrophic chondrocytes (Fig. 4*E*, inset). Expression of osteopontin and MMP-13, markers of terminal differentiation, were examined to determine whether the enlarged expression domain of type X collagen reflected a delay in terminal maturation, in which case reduced expression of terminal differentiation markers would be expected. However, the expression domain of osteopontin, normally restricted to the lower hypertrophic zone, was expanded in mutants (Fig. 4*E*). Moreover, there was no difference in the expression domain of MMP-13, which is normally restricted to the most terminally differentiating chondrocytes (Fig. 4*E*). These results suggest that the enlarged hypertrophic zone is most likely attributed to an increase in the number of cells undergoing hypertrophic differentiation. Additionally, our results show that the pace of chondrocyte hypertrophy is not altered in mutants once differentiation is initiated.

As discussed above, analysis of type X collagen expression indicated increased matrix production in mutant hypertrophic chondrocytes. Evidence for increased activity of chondrocytes within the proliferative zone was provided by the observation that the domain of expression of type II collagen was expanded within the proliferative zone of mutant growth plates at P0 compared with WT littermates (Fig. 4*F*). Consistent with the model where loss of *Smad6* leads to increased activity of proliferative and hypertrophic chondrocytes, the levels of type II and X collagen mRNA were elevated in *Smad6*-deficient chondrocytes (Fig. 4*G*). These results show that loss of *Smad6* leads to enhanced activity of both proliferative and hypertrophic chondrocytes, associated with increased collagen production.

Increased rate of chondrocyte proliferation and apoptosis in Smad6−/− cartilage

Immunofluorescence staining for PCNA, a marker for the G1 and S phases of the cell cycle, was performed to examine whether the delay in the onset of chondrocyte differentiation at midgestation and the enlarged hypertrophic zone at late gestation result from increased chondrocyte proliferation. A trend toward a moderate increase in proliferating cells in both the reserve and proliferative zones was observed in mutant growth plates at E15.0 (Fig. 5*A, B*), but the difference was not statistically significant. Similar results were found by immunofluorescence staining for phosphohistone H3 (Ser 10), a marker for M-phase (Supplemental Fig. 1). By E18.5, a significant increase in proliferating cells was evident in the reserve zone of mutant cartilage (Fig. 5*C, D*). Apoptosis, which is normally detectable in the most terminally differentiating chondrocytes, was elevated in *Smad6−/−* growth plates (Fig. 5*E*) Therefore, the enlargement of the hypertrophic zone in mutants is not the result of prolonged retention of terminal hypertrophic chondrocytes. Rather, the data suggest that the increased pool of proliferating chondrocytes leads to the enlarged hypertrophic zone. We speculate that the increased level of apoptosis in *Smad6−/−* hypertrophic chondrocytes may be the result of the elevated biosynthetic activity of these cells, which could lead to increased cellular stress.

Increased BMP activity and responsiveness in Smad6-deficient chondrocytes

Given the known role for Smad6 as an intracellular inhibitor of BMP signaling,[14,34] we examined the effect of loss of *Smad6* on

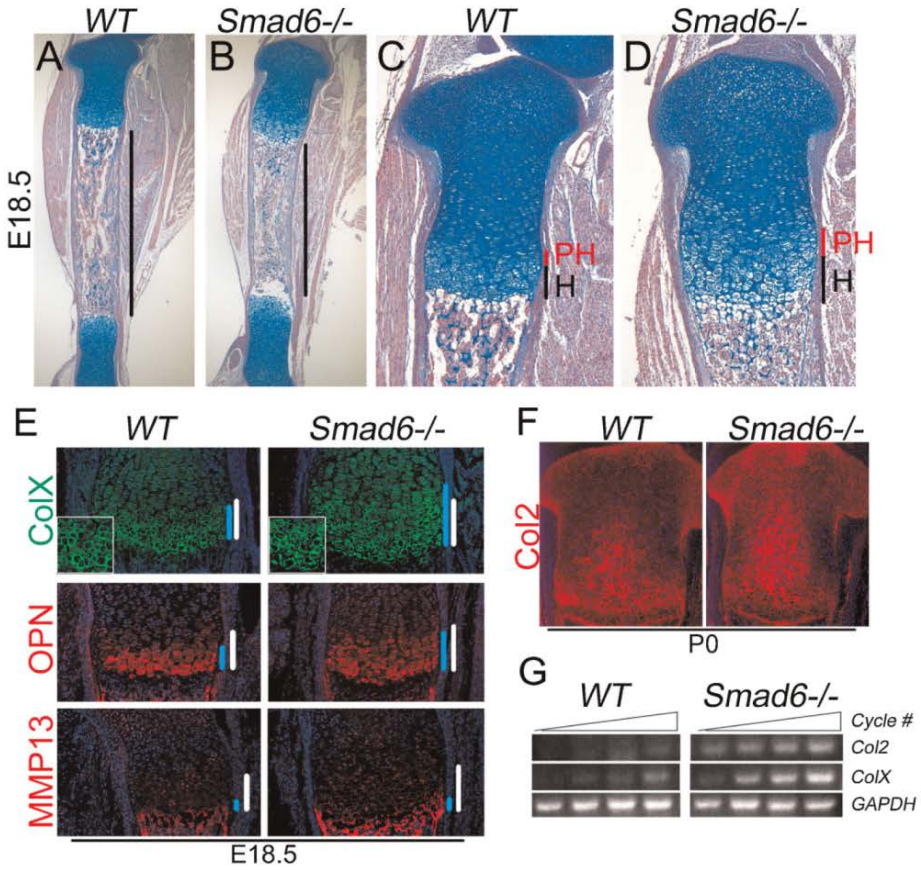

Fig. 4. Enhanced hypertrophic differentiation at late gestation in *Smad6−/−* mice. (*A,B*) Shorter diaphyses are evident in E18.5 *Smad6−/−* tibiae, as demarcated by black bars. (*C,D*) Higher magnification of proximal tibiae at E18.5. (*E*) Immunofluorescence staining of adjacent sections of E18.5 distal femurs for the hypertrophic differentiation marker, type X collagen, and terminal differentiation markers, OPN, and MMP-13. The hypertrophic zone is demarcated by white bars. The region of expression is demarcated by blue bars. No detectable staining was observed in negative controls, from which primary antibodies were omitted. (*F*) Immunofluorescence staining of tibial growth plates for type II collagen shows enhanced collagen production at P0. No detectable staining was observed in negative controls. (*G*) Semiquantitative RT-PCR analysis of RNA isolated from WT and *Smad6−/−* primary chondrocytes cultured in chondrogenic media for 7 days.

Smad1/5/8 activation in cartilage in vivo by immunofluorescence staining of growth plates. Phospho-Smad1/5/8 levels were similar in WT and mutant cartilage at E15.0 (Fig. 6*A, B*). By E18.5, phospho-Smad1/5/8 levels were significantly elevated in the resting zone of mutant cartilage (Fig. 6*C, D*). These results show that loss of *Smad6* leads to elevated canonical BMP signaling in cartilage in vivo.

Because the *Smad6−/−* mice are global knockouts, we investigated whether *Smad6* deficiency has direct effects on BMP signaling by examining Smad6 function in primary chondrocytes. Loss of *Smad6* in mutant chondrocytes was confirmed by quantitative real-time PCR analysis (Fig. 6*E*). Levels of phospho-Smad1/5/8 were elevated in *Smad6*-deficient cells cultured in chondrogenic media for 10 days as determined by Western blot analysis (Fig. 6*F*). Increased levels of the BMP receptor BMPR1A (also known as ALK3) were observed in *Smad6*-deficient cells (Fig. 6*F*), consistent with the reported function of Smad6 in promoting type I BMP receptor degradation. Taken together, these results show Smad6 is required to limit the canonical Smad pathway in chondrocytes, in part, by regulating type I BMP receptor levels.

The effects of loss of *Smad6* on the intensity and duration of canonical and noncanonical BMP signaling in chondrocytes were evaluated by treating isolated primary chondrocytes with BMP2. Elevated levels of phospho-Smad1/5/8 were observed up to 12 hours after BMP2 stimulation in *Smad6*-deficient chondrocytes (Fig. 6*G*). Similarly, phospho-p38 levels were elevated under basal conditions and for up to 12 hours poststimulation with BMP2 in mutant chondrocytes (Fig. 6*G*). We then tested whether *Smad6*-deficient chondrocytes are more responsive to BMP2 using a Smad1/5-responsive 560 bp *Msx2* promoter fragment[29] linked to luciferase (560 bp *MSX2*-luc) as a reporter. Levels of both basal and BMP2-mediated induction of the *Msx2* promoter were greater in mutant chondrocytes than that in WT cells (Fig. 6*H*). Overall, these results show that loss of *Smad6* enhances BMP2-induced activation of both canonical and noncanonical pathways, leading to increased BMP responsiveness in *Smad6*-deficient chondrocytes.

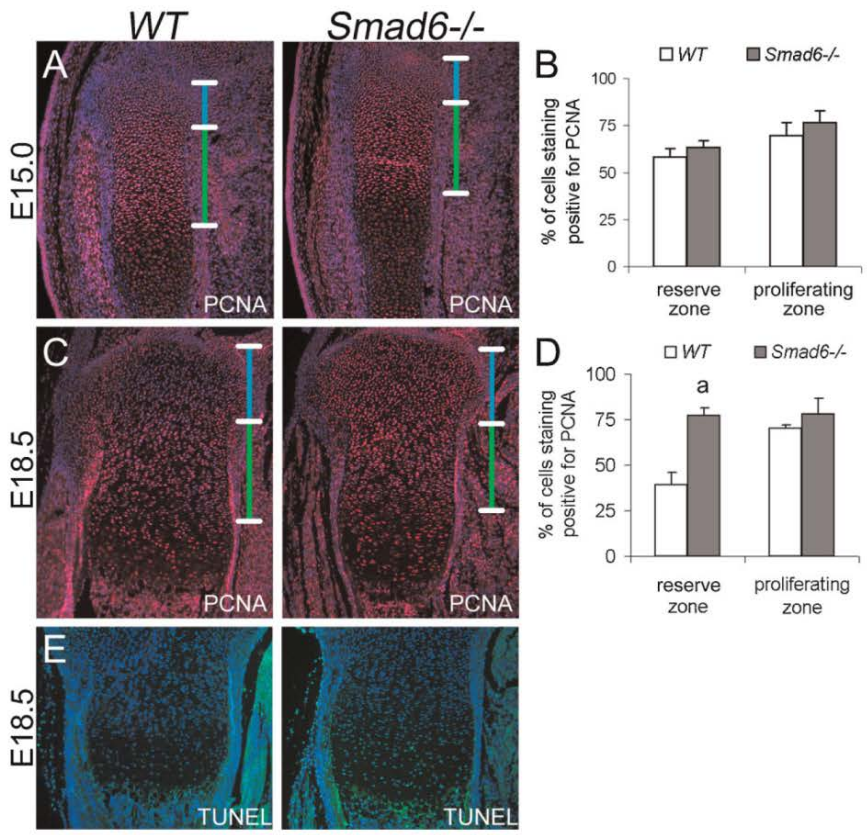

Fig. 5. Increased proliferation and apoptosis at late gestation in *Smad6−/−* mice. (*A*) Immunofluorescence staining of E15.0 proximal tibiae for the proliferation marker, PCNA. The reserve and proliferative zones are demarcated by blue and green bars, respectively. No detectable staining was observed in negative controls, from which primary antibodies were omitted. (*B*) Quantification of the rates of proliferation in WT and mutant cells at E15.0. Values are expressed as percent labeled cells. (*C*) Immunofluorescence staining of E18.5 proximal tibiae for PCNA. The reserve and proliferative zones are demarcated by blue and green bars, respectively. (*D*) Quantification of the rates of proliferation in WT and mutant cells at E18.5. Values are expressed as percent labeled cells (Student's *t*-test; $^{a}p < 0.05$). (*E*) TUNEL staining of E18.5 proximal tibiae. No detectable staining was observed in negative controls.

Increased Ihh expression and activity in Smad6-deficient chondrocytes

The Ihh/PTHrP pathway forms a negative-feedback loop to regulate the onset of chondrocyte hypertrophy. (reviewed in [1]). We have shown previously that BMP signaling through the canonical Smad pathway positively regulates Ihh signaling in chondrocytes.[6,7] To examine the consequences of *Smad6* deficiency on Ihh signaling in cartilage in vivo, we evaluated the expression of Ihh via immunofluorescence staining of growth plates. The domain of Ihh protein expression was expanded in mutant cartilage (Fig. 7*A*), demonstrating that Smad6 impacts Ihh signaling in cartilage. We then evaluated Ihh activity in vivo via immunofluorescence staining of growth plates for its downstream target, Patched 1 (Ptc1). Ptc1 was expressed at low levels in the proliferative zone and at higher levels in the prehypertrophic and periarticular regions of WT growth plates (Fig. 7*B*); in mutant growth plates, Ptc1 expression was elevated throughout the reserve and upper-proliferative zones (Fig. 7*B*).

The direct effect of loss of *Smad6* on Ihh signaling in chondrocytes was examined via analysis of *Ihh*, *PTHrP*, and PTHrP receptor (*PPR* or *PTH1R*) expression in WT and *Smad6*-deficient primary chondrocytes. Both *Ihh* and *PPR* mRNA levels were significantly increased in *Smad6*-deficient cells, whereas no difference in *PTHrP* mRNA levels was evident (Fig. 7*C*). Consistent with increased *Ihh* expression, *Ptc1* mRNA levels were elevated in mutant chondrocytes (Fig. 7*C*). Moreover, BMP-induced *Ihh* expression was markedly increased in mutant chondrocytes (Fig. 7*D*). Taken together, these in vivo *and* in vitro results show that loss of *Smad6* upregulates Ihh expression and activity, in part, due to enhanced BMP responsiveness in mutant chondrocytes.

Discussion

Mice deficient in *Smad6* exhibit cardiovascular defects, including aortic ossification resulting from cartilaginous metaplasia.[27] The skeletal phenotype of these mice, however, has not been

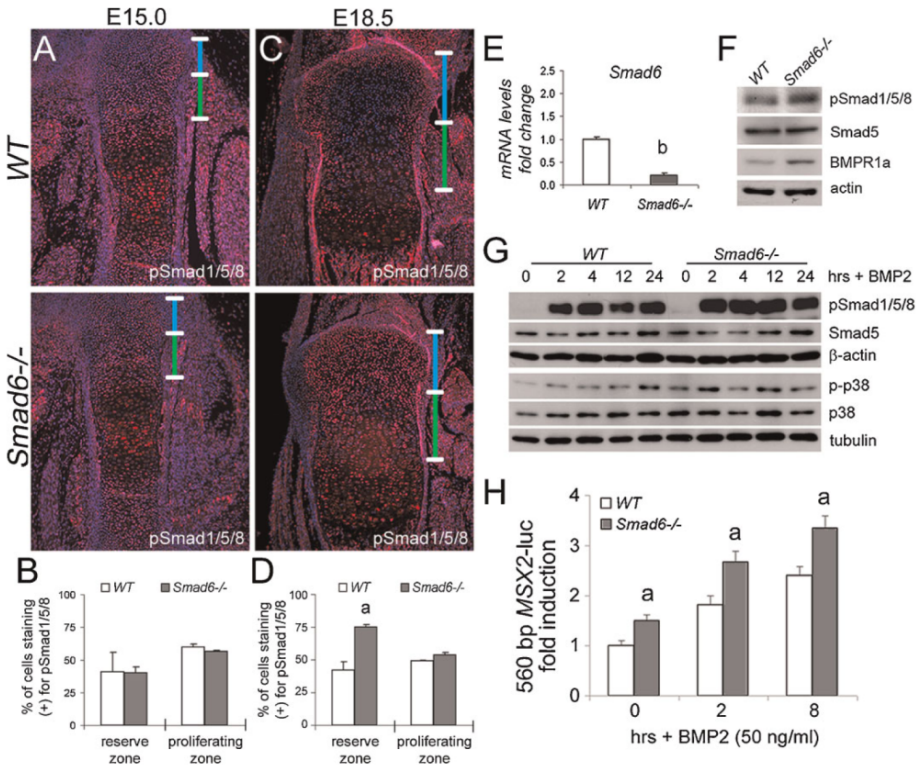

Fig. 6. Increased BMP signaling in *Smad6−/−* chondrocytes. Immunofluorescence staining of (*A*) E15.0 distal femurs and (*C*) E18.5 proximal tibiae for phosphorylated forms of Smad1/5/8 (pSmad1/5/8). The reserve and proliferative zones are demarcated by blue and green bars, respectively. No detectable staining was observed in negative controls, from which primary antibodies were omitted. Quantification of pSmad1/5/8 staining in WT and mutant cells at (*B*) E15.0 and (*D*) E18.5. Values are expressed as percent labeled cells (Student's *t*-test; $^{a}p < 0.05$). (*E*) Real-time PCR analysis of RNA isolated from WT and *Smad6−/−* primary chondrocytes cultured in chondrogenic media. Expression levels for Smad6 were normalized to ß-actin and are shown as fold change relative to WT mRNA levels. The data represent averages from triplicate reactions with the indicated s.d. and significant differences (Student's *t*-test; $^{b}p < 0.001$). (*F*) Western blot analysis of pSmad1/5/8 and BMPR1a levels in lysates isolated from WT and *Smad6−/−* primary chondrocytes cultured in chondrogenic media for 10 days. (*G*) Western blot analysis shows elevated levels of pSmad1/5/8 and phospho-p38 (p-p38) in lysates isolated from WT and *Smad6−/−* primary chondrocytes treated with BMP2 (50 ng/mL) for 0, 2, 4, 12, or 24 hours. (*H*) BMP2 induction of the 560 bp *MSX2*-luc promoter is enhanced in Smad6−/− primary chondrocytes. Cells were treated with BMP2 (50 ng/mL) for 0, 2, or 8 hours. The data represent an average from three wells with the indicated SD and significant differences (multifactorial ANOVA [Within-subjects]; $^{a}p < 0.05$ versus WT control).

investigated previously. Although it has been shown that overexpression of Smad6 in chondrocytes results in abnormal skeletal development,[25] the physiological role of Smad6 during endochondral bone formation was unknown. In this work, we found that mice deficient in *Smad6* exhibit craniofacial, axial, and appendicular skeletal defects. Elevated BMP signaling in *Smad6*-deficient chondrocytes most likely causes the mutant phenotype. Thus, the results of our study provide the first in vivo evidence that Smad6 is required to limit BMP signaling for proper skeletal development.

Smad6 is required for axial skeleton development

Loss of *Smad6* led to posterior transformation of cervical vertebrae, demonstrating a role for Smad6 in regulating anterior-posterior (A/P) patterning of the axial skeleton. BMP signaling has been shown previously to regulate A/P patterning, because mice carrying a hypomorphic type II BMP receptor exhibit anterior transformation of thoracic vertebrae.[35] Our results here suggest that the increased BMP signaling resulting from loss of *Smad6* causes posterior transformation of axial elements.

Smad6 also can interact directly with members of the *Hox* family of transcription factors,[36] which regulate axial patterning. For example, loss of either *Hoxa4*[37] or *Hoxc8*[38] in mice results in the formation of a rib anlagen at C7, similar to that found in our *Smad6−/−* mice. In vitro studies showed that Smad6 interacts with *Hoxc8* to inhibit Smad1-induced transcriptional activity,[39] whereas Smad1 antagonizes the repressor function of *Hoxc8* to promote osteopontin gene expression.[40] Thus, Smad6 may play a role in mediating cross-talk between BMP signaling and *Hox* transcription factors to specify positional identity of vertebrae along the A/P axis.

The development of the sternum also involves both Hox transcription factors and BMP signaling.[22,41,42] Defects in the fusion of sternal bands, as in *Smad6* mutants, have been found in mice with either limited[41] or elevated BMP signaling.[22] The

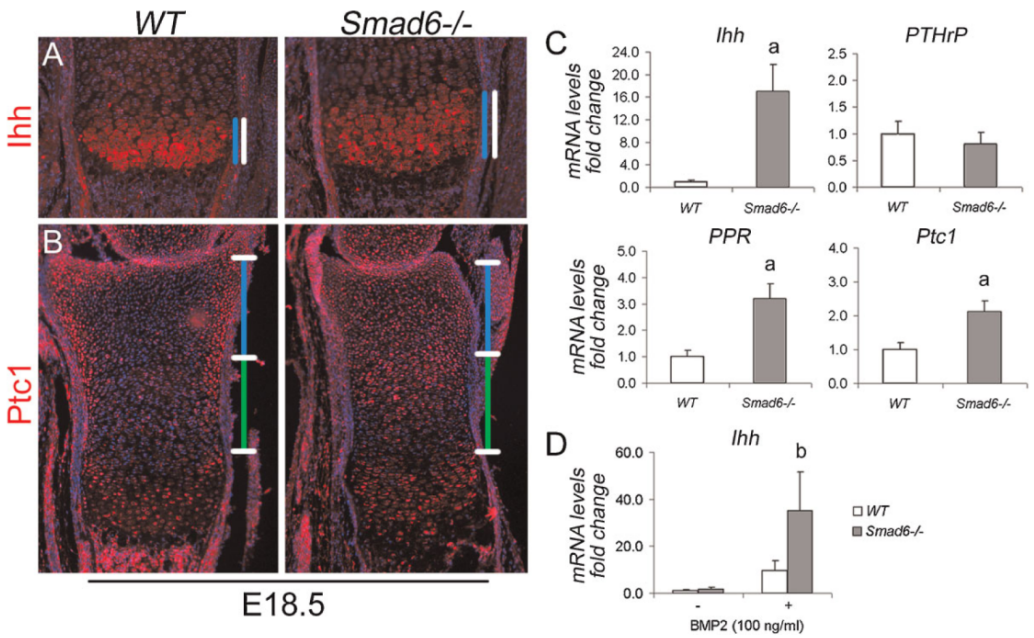

Fig. 7. Loss of Smad6 leads to increased *Ihh* expression and activity in chondrocytes. (*A*) Immunofluorescence staining of E18.5 distal tibiae for Ihh. The prehypertrophic and hypertrophic zones are demarcated by white bars. The region of expression is demarcated by blue bars. (*B*) Immunofluorescence staining of E18.5 distal tibiae for Ptc1. The reserve and proliferative zones are demarcated by blue and green bars, respectively. No detectable staining was observed in negative controls, from which primary antibodies were omitted. (*C*) Real-time PCR analysis of RNA isolated from WT and *Smad6−/−* primary chondrocytes cultured for 10 days in chondrogenic media. Expression levels for each gene of interest were normalized to ß-actin and are shown as fold change relative to WT mRNA levels. The data represent averages from triplicate reactions with the indicated SD and significant differences (Student's *t*-test; $^{a}p < 0.001$). (*D*) Real-time PCR analysis of RNA isolated from WT and *Smad6−/−* primary chondrocytes cultured in chondrogenic media for 10 days, serum-starved overnight, and then treated with BMP2 (100 ng/mL) for 2 hours. *Ihh* mRNA levels were normalized to ß-actin and are shown as fold change relative to WT mRNA levels. The data represent averages from triplicate reactions with the indicated SD and significant differences (Student's *t*-test; $^{b}p < 0.05$).

results of these studies suggest that sternum development requires strict control of BMP signaling, in part through intracellular regulation by Smad6.

Smad6 is required to limit BMP signaling during endochondral bone formation

The stage-dependent expression pattern of Smad6 parallels that of pSmad1/5 in the growth plate,[6,7] and is consistent with previous studies showing that Smad6 is a direct target of canonical BMP pathways.[43] The subcellular localization of Smad6 agrees with the reported functions of Smad6 in the cytoplasm (ie, binding to pSmad1)[19] and at the cell membrane (ie, binding to type I receptors),[19,34] and suggests that the inhibitory functions of Smad6 depend on whether the chondrocytes are undergoing proliferation or hypertrophic differentiation, as well as the stage of development. Overall, these results suggest a role for Smad6 in regulating proliferation and hypertrophic differentiation of chondrocytes in vivo.

We found that loss of *Smad6* results in stage-specific defects in endochondral bone formation. Mesenchymal condensation occurs normally in *Smad6−/−*limbs, indicating that Smad6 does not play a major role in early stages of chondrogenesis. By midgestation, delayed onset of hypertrophic differentiation is evident in *Smad6−/−* growth plates. The limb phenotype of *Smad6−/−* mice differs from that in mice in which BMP signaling is elevated as a result of *noggin*-deficiency,[22] as well as in developing limbs in which BMPs were overexpressed using transgenic approaches.[44,45] In particular, increased BMP activity in *noggin*-null mice and in limbs overexpressing BMP-2 or BMP-4 led to massive cartilage overgrowth without any increase in chondrocyte proliferation; the overgrowth was attributed to increased recruitment of progenitor cells to the chondrocyte lineage. It is thus possible that the delay in onset of hypertrophy that we observe in mutants reflects an indirect effect of loss of Smad6 in a different cell type, such as the perichondrium. Elevated BMP signaling also can enhance chondrocyte proliferation, as shown in cartilage explants treated with BMP-2.[5,46] In *Smad6−/−* growth plates, elevated BMP signaling correlated with cell cycle entry of resting chondrocytes. No difference in proliferation rates was found between proliferating chondrocytes of mutant and WT littermates. Our inability to detect increased proliferation in the proliferative zone in mutant growth plates may be because the rate of proliferation is already maximal, or because additional increases are beyond detection with PCNA or phospho-histone H3 staining. However, increased collagen production by proliferating chondrocytes was observed, indicating that Smad6 has direct effects on these cells. Taken together, it appears that noggin acts during the stage of recruitment of progenitor cells into cartilage elements, whereas

Smad6 acts at the stage of cell cycle entry of reserve chondrocytes in the growth plate. Thus, regulation of BMP signaling both intracellularly and extracellularly is required for normal endochondral bone formation.

Interestingly, delayed onset of hypertrophy, as in the *Smad6* mutants, also was found in mice overexpressing Smad6[25] and in chick limbs treated with chordin.[23] Loss of BMP signaling due to Smad6 overexpression or chordin treatment also blocks the progression of hypertrophic differentiation of chondrocytes. Correspondingly, transgenic mice expressing constitutively active BMPR1A exhibited shortened proliferative zones. The authors concluded that the growth plate phenotype resulted from accelerated hypertrophic differentiation; however, no evidence for accelerated hypertrophic differentiation was provided.[47] In the above model systems, BMP signaling is impacted in all of the cells in the growth plate. In contrast, our model reflects BMP-mediated effects on chondrocyte differentiation only in cells in which Smad6 is a physiological regulator of BMP activity. In particular, increased collagen production by both proliferating and hypertrophic chondrocytes was evident in mutant growth plates at late gestational stages. Direct actions of Smad6 on collagen production were confirmed in *Smad6*-deficient chondrocytes. Overall, our results suggest that elevated BMP signaling in the *Smad6* mutants affects the onset of hypertrophic differentiation, as well as collagen production by proliferating and hypertrophic chondrocytes.

Enhanced chondrocyte proliferation, apparent in the reserve zone of E18.5 mutant growth plates, may result from the activation of both canonical and noncanonical BMP pathways, because the main mediators of these pathways (Smad1/5/8 and TAK1) are required for chondrocyte proliferation in vivo.[6,48,49] A major consequence of canonical and noncanonical BMP pathway activation is the upregulation of Ihh expression and activity.[6,50,51] Our results show that loss of *Smad6* leads to upregulation of Ihh expression and activity, in part via enhanced BMP-responsiveness in *Smad6*-deficient chondrocytes. Given that the loss of *Ihh* leads to premature hypertrophy associated with reduced chondrocyte proliferation,[52] elevated Ihh activity evident in *Smad6*−/− mice and in *Smad6*-deficient chondrocytes is consistent with delayed onset of hypertrophic differentiation.

PPR mediates chondrocyte proliferation to regulate the onset of hypertrophic differentiation, as evidenced by accelerated differentiation of chondrocytes in *PPR*−/− mice.[53] We found that *PPR* expression is elevated in *Smad6*-deficient chondrocytes. This finding also is consistent with our conclusion that loss of *Smad6* leads to delayed onset of hypertrophic differentiation. The transcription factors that directly regulate *PPR* expression in chondrocytes are unknown. It is possible that the canonical BMP pathway mediates *PPR* expression in chondrocytes, given that *PPR* expression is reduced in mice lacking Smads1 and 5.[6]

Smad6 inhibits BMP signaling intracellularly by associating with type I BMP receptors,[19,34] thereby facilitating Smurf1-mediated receptor degradation.[17] Our in vivo and in vitro studies show that loss of *Smad6* results in elevated canonical BMP signaling, in part, by regulating type I receptor levels in chondrocytes. The enhanced activation of both the canonical and noncanonical pathways by BMP2 in *Smad6*-deficient chondrocytes is consistent with the increased levels of BMPR1A. Smad6 also can limit BMP signaling by associating with receptor-activated Smad1, thereby inhibiting its association with Smad4.[19] Thus, increased BMP signaling in *Smad6*-deficient chondrocytes also may be a result of increased availability of Smad1.

In summary, we have identified a role for Smad6 as a regulator of multiple stages of chondrogenesis, including specification of A/P fate, entry and exit of resting chondrocytes into the proliferative pool, and ECM synthesis. We also show that Smad6 executes these functions, in part, through BMP-mediated regulation of Ihh expression and activity. Additional studies will be required to test whether Smad6 and the remaining inhibitory Smad, Smad7, have overlapping functions in the growth plate.

Disclosures

All the authors state that they have no conflicts of interest.

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Authors' roles: Kristine Estrada, Kelsey Retting, and Alana Chin all contributed to the design of experiments, as well as the acquisition and analysis of data. Kristine Estrada prepared all of the figures. Kristine Estrada, Alana Chin, and Karen Lyons contributed to the drafting and revising of the manuscript. All authors approved the final version of the submitted manuscript.

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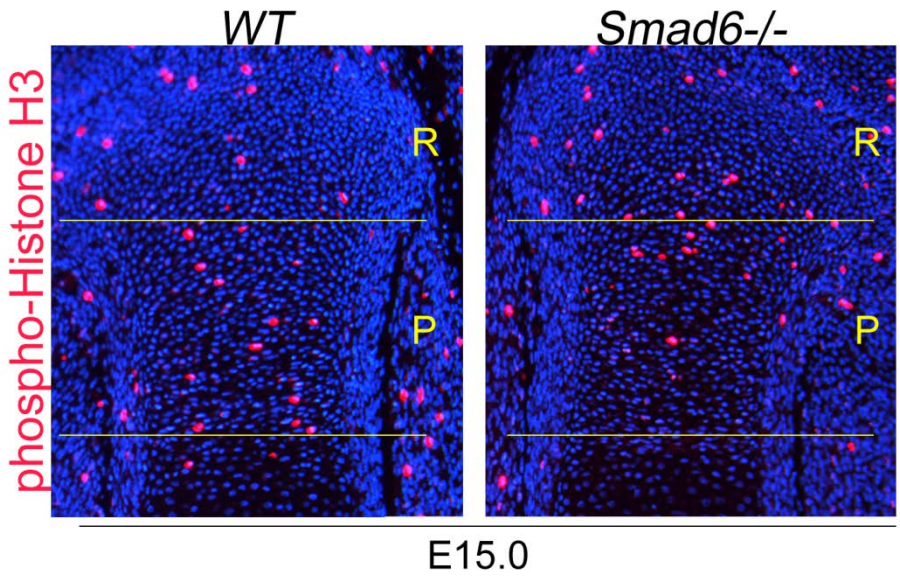
Supplemental Data



Supplemental Figure 1. Analysis of proliferation rates via phospho-histone H3 staining. Immunofluorescence staining of E15.0 proximal tibiae for phospho-histone H3. No detectable staining was observed in negative controls, from which primary antibodies were omitted. R, reserve zone; P, proliferative zone.

CHAPTER 4:

Smad7 regulates terminal maturation of chondrocytes during cartilage development

Preface

In vivo studies in which Smad7 was overexpressed have shown that Smad7 can regulate multiple stages of endochondral bone formation, in part, by inhibiting BMP-activated p38 mitogen-activated protein kinase pathways (Iwai et al., 2008). However, in these studies, Smad7 was expressed in regions of the growth plate that do not normally express Smad7. Moreover, overexpression results in non-physiological expression levels. Thus, it is still unknown whether Smad7 is required for endochondral bone formation. Additionally, whether Smad7 inhibits TGFβ signaling during cartilage development remains unclear. Hence, our work provides the first *in vivo* evidence that Smad7 inhibits both canonical and noncanonical BMP and TGFβ pathways in cartilage during development.

Preliminary phenotypic analyses of the growth plate were performed by Kelsey Retting. Additional histological and immunohistochemical stains were performed (with my guidance) by several undergraduate researchers, Fuad Elkhoury, Christina Hong, Alana Chin and Tony Chien. Another undergraduate researcher, Mathew Hilton, helped generate images of the whole-mount skeletal preparations. I extended the study by determining whether the development of the hypocellular core in *Smad7*-/- growth plates could result from an impaired response to hypoxic stress. I prepared all of the figures and wrote the initial draft of the manuscript. This manuscript is in preparation for submission to *Developmental Biology*.

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Iwai, T., Murai, J., Yoshikawa, H., Tsumaki, N., 2008. Smad7 Inhibits chondrocyte differentiation at multiple steps during endochondral bone formation and down-regulates p38 MAPK pathways. J Biol Chem. 283**,** 27154-64.

Original Article

Smad7 regulates terminal maturation of chondrocytes during cartilage development

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Abstract

Bone morphogenetic protein (BMP) signaling pathways play critical roles in regulating multiple aspects of endochondral bone formation. On the other hand, transforming growth factor-beta (TGFβ) pathways promote chondrocyte proliferation, but inhibit chondrocyte maturation. *In vivo* studies in which Smad7 was overexpressed in chondrocytes at various stages of endochondral bone formation have demonstrated that Smad7 has the potential to regulate these processes, in part, by inhibiting BMP-activated p38 mitogen-activated protein kinase pathways. However, whether Smad7 is actually required for endochondral bone formation remains unclear. Moreover, whether Smad7 regulates TGFβ signaling during cartilage development is still unknown. In this study, we found that Smad7 is required for both axial and appendicular skeletal development. *Smad7-/-* mice exhibit posterior transformation of the seventh cervical and sixth lumbar vertebrae. In appendicular bones, loss of *Smad7* leads to defects in terminal maturation of chondrocytes owing to the retention of cells in a prehypertrophic state. These defects correlated with aberrant type X collagen expression and secretion, likely due to upregulation of both BMP and TGFβ signaling in *Smad7* mutant growth plates. Moreover, *Smad7-/-* mice develop hypocellular cores in the medial growth plates, associated with elevated HIF1α levels, ER stress, and cell death. Thus, Smad7 may also be required to mediate cell stress responses in the growth plate during development.

Highlights: ▶ Smad7 regulates anterior/posterior patterning of the axial skeleton. ▶ Upregulation of BMP and TGFβ signaling in *Smad7-/-* growth plates leads to defects in terminal maturation of chondrocytes in appendicular bones. ▶ Cellular responses to hypoxic stress are mediated by Smad7.

Key words: BMP; TGFβ; Smad; Mice; chondrocytes; hypoxia; ER stress

Introduction

Endochondral ossification is the process by which the majority of the vertebrate skeleton, including the vertebral column of the axial skeleton and the long bones of the appendicular skeleton, is formed. This multi-step process begins with the condensation of mesenchymal cells, which then differentiate into chondrocytes that rapidly proliferate and actively secrete extracellular matrix proteins (e.g. collagen, fibronectin and aggrecan) to form cartilage primordia. The proliferating chondrocytes in the center of the cartilage primordium exit the cell cycle and consequently mature into prehypertrophic and hypertrophic chondrocytes. Terminally hypertrophic chondrocytes undergo apoptosis, and the surrounding cartilaginous matrix is invaded by blood vessels, osteoblasts and osteoclasts that degrade and replace cartilage with bone.

Endochondral ossification is tightly regulated by local paracrine factors, including bone morphogenetic proteins (BMPs) and transforming growth factor β (TGFβ). Cellular responses to BMPs and TGFβ are mediated by canonical (Smad) and noncanonical (non-Smad) pathways (Derynck and Zhang, 2003; Massague, 1998). Both pathways are initiated upon ligand binding via activation of the type I receptors by type II receptors. In the canonical pathway, activated type I receptors phosphorylate receptor-regulated Smads (R-Smads 1/5/8 for BMP signaling; R-Smads 2/3 for TGFβ signaling), which then form complexes with the common-partner Smad4. R-Smad/Smad4 complexes translocate into the nucleus to regulate the expression of target genes. In the noncanonical pathways, BMPs and TGFβs transduce their signals via the mitogen-activated protein (MAP) kinases (ERK, JNK, p38) by activating TGFβ-activating kinase (TAK1) (Moriguchi et al., 1996; Yamaguchi et al., 1995). Other non-Smad pathways include activation of PI3K and Src kinase (Heldin et al., 2009).

Downregulation of BMP and TGFβ signaling is mediated extracellularly through binding of ligand antagonists, such as chordin and noggin, and intracellularly through attenuation of R-Smad activity by inhibitory Smads (I-Smad) 6 and 7. I-Smads antagonize BMP/TGFβ signaling by recruiting E3 ubiquitin ligases to type I receptors, causing degradation of type I receptors via the ubiquitin-proteasome pathway (Inoue and Imamura, 2008; Murakami et al., 2003). In addition, I-Smads interfere with R-Smad phosphorylation by competitively binding to activated type I receptors (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997). While Smad6 specifically inhibits the BMP pathway, Smad7 can inhibit both the BMP and TGFβ pathways (reviewed in (Massague et al., 2005)). I-Smad expression is induced directly by BMP and TGFβ, thus forming an auto-inhibitory feedback mechanism.

Genetic analyses in mice demonstrate that the majority of BMP signaling in cartilage during development occurs via the canonical pathway, as evidenced by severe chondrodysplasia in mice with a combined loss of either the two major type I BMP receptors (Yoon et al., 2005) or R-Smads (Retting et al., 2009) in chondrocytes. The precise role of TGFβ signaling in endochondral ossification is less clear, as variable phenotypes have been reported in mice with targeted inactivation of TGFβ signaling components. For example, mice with conditional deletion of the type II TGFβ receptor (TGFβRII) in chondrocytes exhibit defects in axial but not in appendicular development (Baffi et al., 2004). In addition, appendicular bones were normal in mice deficient in *Smad3* at one month after birth (Yang et al., 2001), suggesting that canonical TGFβ signaling may not be required for appendicular development. On the other hand, both axial and appendicular skeletal development was impaired in mice with conditional deletion of the type I TGFβ receptor (ALK5) in skeletal progenitor cells (Matsunobu et al., 2009), demonstrating that TGFβ is indispensable for endochondral ossification.

Gain- and loss-of function studies *in vivo* (Estrada et al.; Horiki et al., 2004) and *in vitro* (Li et al., 2003; Scharstuhl et al., 2003) reveal important roles for Smad6 in regulating chondrocyte function during development. Although *in vivo* studies in which Smad7 was overexpressed in chondrocytes at various steps of differentiation show that Smad7 has the capacity to limit BMP signaling during cartilage development (Iwai et al., 2008), these observations do not define the actual physiological roles of Smad7 in regulating BMP signaling in developing cartilage. Moreover, whether Smad7 regulates TGFβ signaling in chondrocytes during cartilage development remains unclear. Here, we show that loss of *Smad7* in mice leads to axial and appendicular skeletal defects resulting, in part, from increased BMP and TGFβ responsiveness in chondrocytes. Hence, Smad7 is required for proper regulation of BMP and TGFβ signaling during cartilage development.

Materials and Methods

Generation of Smad7 knockout mice. *Smad7* knockout (*Smad7-/-*) mice were generated as previously described (Li et al., 2006a). Briefly, targeted disruption of the first exon of *Smad7* was generated via insertion of a neomycin resistance cassette. Embryos and mice were genotyped by PCR using the sense primer 5'- CCCTCCTGCTGTGCAAAGTGTTC -3' and anti-sense primer 5'- GCATGTCTATTCAGTAGAAGGATAAG -3' to detect the wild-type allele, and the sense primer 5'- GCTTCCTCGTGCTTTACGGTATC -3' and anti-sense primer as above to detect the mutant allele. Embryos and mice were on a mixed C57BL/6J/CD1 background. To obtain Smad7-deficient embryos, *Smad7* heterozygous male and female mice were mated and wild-type littermates were used as controls.

Skeletal preparation and histology. Skeletal preparations were performed as in (Estrada et al.). In brief, embryos were eviscerated and fixed in 95 % EtOH overnight at 4 °C, followed by Alcian Blue staining (0.01 % Alcian Blue 8GX (Sigma-Aldrich, A5268) (w/v) in 95 % EtOH) overnight at room temperature. Samples were then stained for Alizarin Red (0.006 % Alizarin Red S (Sigma-Aldrich, A5533) (w/v) in 1 % KOH) for 3-4 hours and cleared in a series of graded KOH in glycerol.

For histology, embryos were fixed with 10 % buffered formalin (Fisher Scientific, SF100) overnight at 4 °C, decalcified with Immunocal (Decal Chemical Corp., Tallman, NY, USA) overnight at 4 °C, embedded in paraffin, and cut at a thickness of 5-7 μm. For Alcian blue staining, sections were stained with Alcian Blue (Sigma-Aldrich, A5268) and Nuclear Fast Red (Sigma-Aldrich, N8002) as in (Estrada et al.). For Safranin-O staining, sections were stained according to standard protocols (Rosenberg, 1971).

***Immunohistochemical and immunoflourescence staining*.** Antigen retrieval was performed by either boiling in citrate buffer pH 6.0 for 15 min at 95 °C or incubating in 1 mg/ml hyaluronidase (Sigma-Aldrich, H3506) in Dulbecco's PBS (Mediatech, Inc., Manassas, VA, USA) for 1 hour at 37 °C.

For detection using the following primary antibodies: GRP78/BiP (Cell Signaling, #3177), HIF1α (Santa Cruz Biotechnologies, sc10790), Ihh (Santa Cruz Biotechnologies, sc1196), MMP-13 (Abcam, ab84594), osteopontin (Thermo Scientific, RB-9097-P0), phospho-Smad1/5/8 (Cell Signaling, #9511), phospho-Smad2 (Cell Signaling, #3108), phospho-TAK1 (Cell Signaling, #4508) and Ptc1(Novus Biologicals, NB100-91923), sections were quenched in 3 % H_2O_2 in methanol, blocked with 0.5 % blocking reagent (TSA™ Biotin System, Perkin Elmer, NEL700A) in TBS (100 mM Tris pH 7.5, 150 mM NaCl), and incubated with primary

antibody overnight at 4 °C. Detection of binding was performed using the TSA™ Biotin System according to the manufacturer's protocol. Fluorescent detection was conducted by using the Streptavidin-AlexaFluor -555 (Invitrogen) secondary antibodies; sections were counterstained with DAPI (Invitrogen, D1306).

For detection of Type II Collagen (Abcam, ab21291) and Type X Collagen (Abcam, ab58632), sections were blocked and incubated with primary antibody, as described above, incubated with either the AlexaFluor-488 or -555 (Invitrogen) secondary antibodies for 30 min at room temperature, and then counterstained with DAPI. For detection of Smad7 (Thermo Scientific, PA1-41506), sections were blocked, incubated with primary antibody, and quenched in 3 % H_2O_2 in methanol, as described above. Sections were then incubated with biotin-XX anti-rabbit (Invitrogen, B2770) and Streptavidin-HRP (Perkin Elmer) secondary antibodies. Chromogenic detection was performed with the DAB Peroxidase Substrate Kit (Vector Laboratories, SK-4100) following the manufacturer's instructions; sections were counterstained with Hematoxylin QS (Vector Laboratories, H-3404).

Cell proliferation and TUNEL labeling. For detection of cell proliferation *in vivo*, immunofluorescence staining was performed using either anti-PCNA primary antibody (Zymed, #13-3900) or phospho-histone H3 (Ser10) antibody (Cell Signaling, #9701), and biotin-XX anti-mouse (Invitrogen) and Streptavidin-AlexaFluor-555 (Invitrogen) secondary antibodies. For TUNEL labeling, the fluorescein *In Situ* Cell Death Detection Kit (Roche Applied Sciences) was used according to the manufacturer's instructions.

Cell culture. Primary chondrocytes were isolated from costal cartilage, as previously described (Estrada et al.), and were seeded at 3 x 10^6 cells/well in 12-well plates. For quantitative real-time PCR, cells were maintained for 2-8.5 days in chondrogenic media (DMEM supplemented with

10% FBS, 1% antibiotic-antimycotic (Invitrogen) and 50 μg/mL ascorbic acid). Each experiment was repeated twice (i.e. from two different cell isolations). For Western blot analyses, cells were maintained for 3 days in chondrogenic media. Cells were serum starved overnight with DMEM supplemented with 1% antibiotic-antimycotic, then stimulated the next day with either 50 ng/ml BMP2 (R & D Systems, 355-BM) or 5 ng/ml TGFβ1 (R & D Systems, 240-B) for 2, 4, 8, 12 or 24 hours or with equal volume of DMSO for untreated cells (0 hour). Each experiment was repeated in triplicate (i.e. from three different cell isolations).

Quantitative real-time PCR and Western blot analyses. RNA was extracted using the RNeasy Kit (Qiagen, Valencia, CA, USA). Synthesis of cDNA was performed with the First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD, USA). Quantitative real-time PCR reactions were performed with a SYBR Green Real-time PCR Master Mix (Fermentas) by using a Mx3005P QPCR System (Stratagene, Santa Clara, CA, USA). The primer sequences were as follows: *β-actin*: forward 5'-CTGAACCCTAAGGCCAACCG-3', reverse 5'-GTCACGCACGATTTCCCTCTC-3'; *MMP-13* (from (Li et al., 2006b)): forward 5'- TTT GAG AAC ACG GGG AAG AC -3', reverse 5'- ACT TTG TTG CCA ATT CCA GG -3'; *type II collagen* (from (Clark et al., 2009)): forward 5'-ACTGGTAAGTGGGGCAAGAC-3', reverse 5'-CCACACCAAATTCCTGTTCA-3'; and *type X collagen* (from (Li et al., 2006b)): forward 5'- ACCCCAAGGACCTAAAGGAA -3', reverse 5'- CCCCAGGATACCCTGTTTTT -3'.

For Western blot analysis, cells were lysed in RIPA buffer (25 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 1% Na-deoxycholate, 0.1% SDS) supplemented with protease inhibitors (complete Mini Tablets, Roche Applied Science) and phosphatase inhibitors (Sigma-Aldrich, P5726). Whole cell lysates (3-5 μg) were run on 10% SDS-polyacrylamide gels and transferred

semidry onto PVDF membranes. The membranes were blocked with 5% milk in TBS-tween (30 mM Tris pH 7.4, 300 mM NaCl, 0.2% tween-20), incubated with primary antibody (from Cell Signaling: phospho-p38 [#9215], p38 [#9212], phospho-Smad1/5/8 [#9511], phospho-Smad2 [#3108], Smad2 [#3122] or Smad5 [#9517]; Sigma-Aldrich: β-actin [A5316] or tubulin [T6793]) diluted in blocking buffer overnight at 4°C, and then incubated with appropriate secondary antibody diluted in blocking buffer for 1 hour at room temperature. Binding was detected via enhanced chemiluminescence using the Amersham ECL Plus kit (GE Healthcare, Piscataway, NJ, USA).

Results

Smad7 localization in the growth plate during development. Immunohistochemical staining was performed to evaluate the spatial and temporal expression of Smad7 in developing appendicular bones. Smad7 was expressed at high levels in prehypertrophic and hypertrophic cells at both E15.0 and P0 (Fig 1A,B), while low levels were observed in the reserve and proliferative zones (Fig. 1B). At both stages, Smad7 was localized in the cytoplasm. These results suggest that Smad7 may play a role in regulating the onset of hypertrophic differentiation, as well as terminal maturation of chondrocytes *in vivo*.

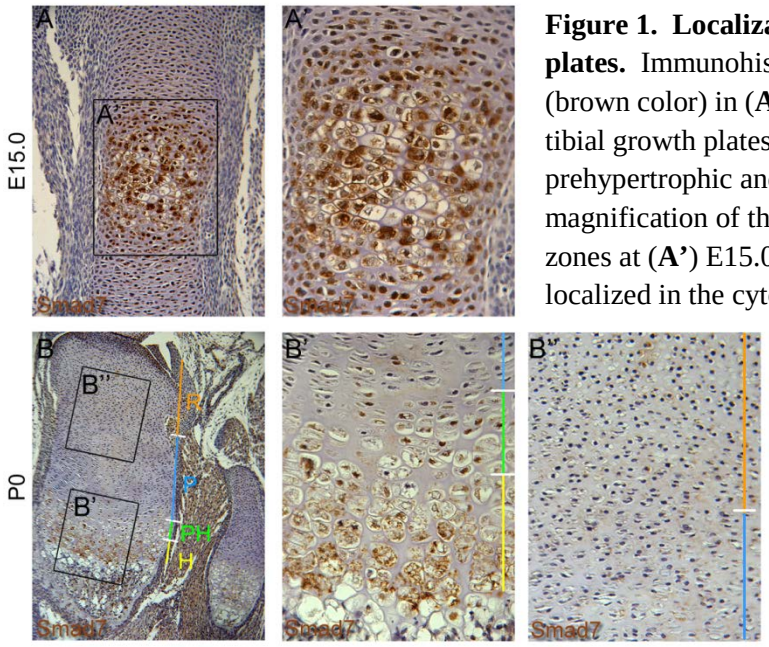

Figure 1. Localization of Smad7 in wild-type growth plates. Immunohistochemical staining for Smad7 (brown color) in (**A**) wild-type femurs at E15.0 and (**B**) tibial growth plates at P0 demonstrates expression in prehypertrophic and hypertrophic chondrocytes. Higher magnification of the prehypertrophic and hypertrophic zones at (**A'**) E15.0 and (**B'**) P0 shows that Smad7 is localized in the cytoplasm of hypertrophic cells. (**B''**) Higher magnification of the reserve and proliferative zones at P0. R, reserve zone; P, proliferative zone; PH, prehypertrophic zone; H, hypertrophic zone.

Skeletal defects in Smad7-/- mice. *Smad7-/-* embryos were recovered at the expected Mendelian ratios up to E18.5. By the time of weaning (21 days after birth), *Smad7-/-* mice were represented only 5 % of heterozygous intercross progeny (n = 134). As early as E12.5, *Smad7-/-* embryos appeared smaller compared to wild-type littermates (data not shown) and moderate dwarfism in mutant mice was maintained postnatally (Fig. 2A). In addition to growth defects, the *Smad7-/-* mice that survived to weaning were severely dehydrated and malnourished. Thus, these mice were euthanized in accordance with the policies of UCLA's Animal Research Committee. The observations that *Smad7-/-* embryos and mice were underrepresented and smaller are consistent with the reported findings of Li and colleagues (Li et al., 2006a), who demonstrated that *Smad7-/-* mice exhibit defective immune response. The difference between the percentage of underrepresented homozygous mice reported by Li and colleagues (15 %) and our findings (5 %) may reflect differences in background strain or vivarium conditions.

Examination of whole-mount skeletal preparations at postnatal day 0 (P0) revealed several skeletal abnormalities in *Smad7-/-* mice. In particular, defects in the ossification of the

frontal and parietal calvarial bones were observed in mutant mice, likely contributing to the widened frontal and sagittal sutures in mutants (Fig. 2B). No defects in the development and ossification of the chondrocranium were detected (data not shown).

Loss of *Smad7* in mice led to defects in the axial skeleton. At P0, six ossified sternebrae separated by cartilaginous intersternebrae were found in wild-type mice. In contrast, *Smad7-/-* mice exhibited a fusion of the fourth (ST4) and fifth (ST5) sternebrae (Fig. 2C). *Smad7-/-* mice also developed a posterior transformation of the seventh cervical vertebra (C7), as evidenced by the presence of a small rib rudiment at C7 (Fig. 2D).

While patterning and development of thoracic vertebrae were normal (Fig. 2E), defects in patterning of lumbar vertebrae were observed. Particularly, posterior transformation of the sixth lumbar vertebra (L6) was evident in mutants (Fig. 2F), as evidenced by the formation of sacro-iliac joints, normally found in the first sacral vertebra (S1), at L6 in *Smad7-/-* mice (Fig. 2F, arrowheads).

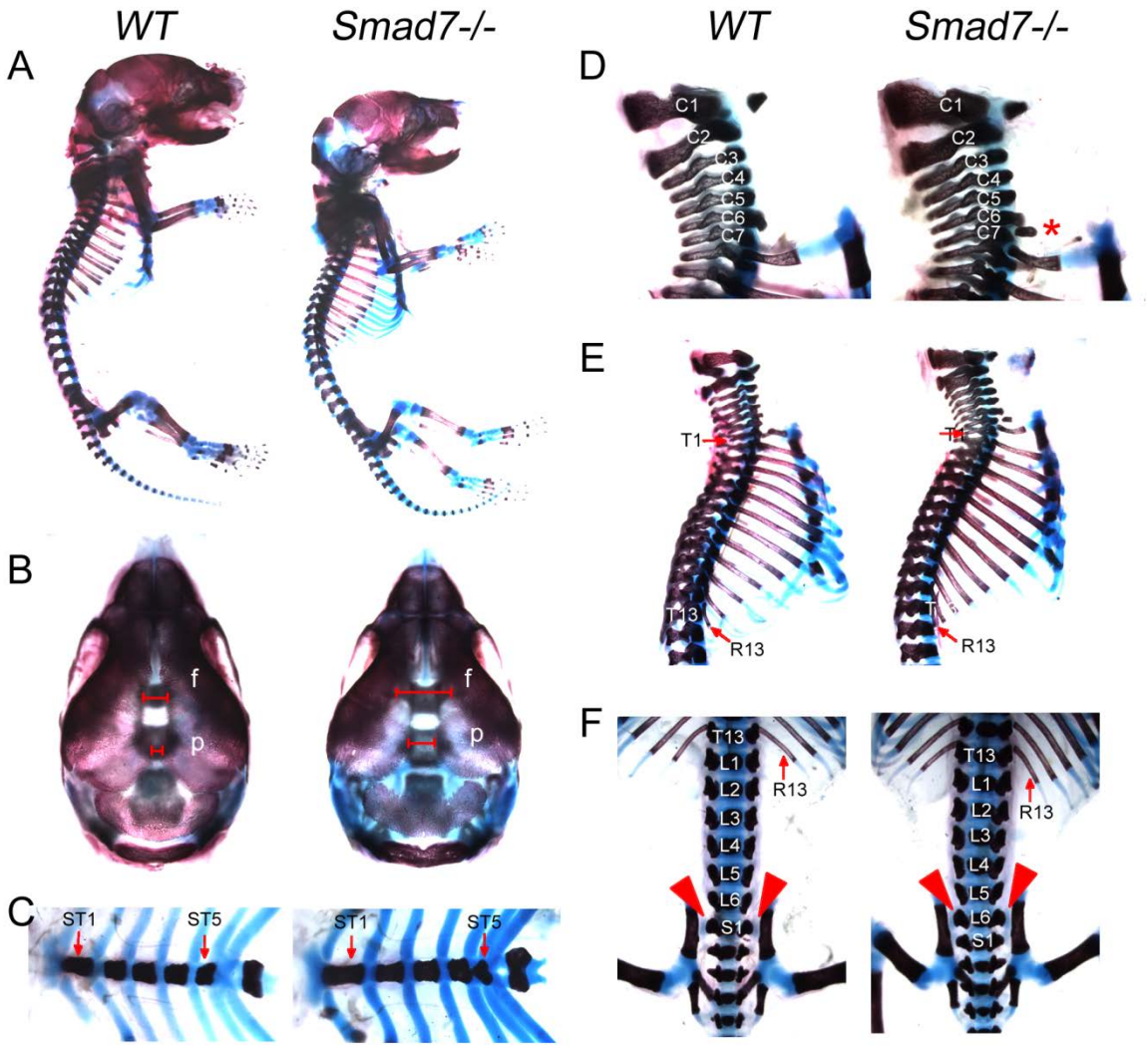

Figure 2. Skeletal defects in *Smad7-/-* mice at P0. (**A**) Whole mount skeletal preparations of wild-type (*WT*) and *Smad7-/-* mice at P0. Cartilage is stained by Alcian Blue; bone is stained by Alizarin Red S. (**B**) Anterior view of the skull. Red brackets highlight wider frontal and sagittal sutures in mutants. (**C**) Ventral view of the sternum shows fused ST4 and ST5 in mutants. (**D**) Lateral view of cervical vertebrae. Red asterisk highlights rib anlagen indicative of posterior transformation of the seventh cervical vertebra (C7) in mutants. (**E**) Lateral view of thoracic vertebrae. (**F**) Dorsal view of lumbar vertebrae. Red arrow heads highlight sacro-iliac joints at L6 in mutants. f, frontal bone; p, parietal bone.

Hypocellularity and shortened hypertrophic zones in Smad7-/- growth plates. Histological analyses of hindlimbs throughout development were performed to further examine the development of appendicular bones. Alcian blue staining revealed no differences between the growth plates of wild-type and *Smad7-/-* embryos at E15.5 (Fig. 3A). By this stage of development, vessels from the perichondrium invade the hypertrophic zone to facilitate the replacement of cartilage by bone. While vascular invasion was evident in wild-type embryos by E15.5, delayed vascular invasion was observed in *Smad7-/-* embryos at this stage (Fig. 3B). By E17.5, however, ossification of mutant long bones was observed (Fig. 3C). Thus, the delay in vascular invasion may reflect an overall delay in development of mutant embryos.

Chondrocytes in the proliferative zone normally appear flattened and are organized into well-defined columns. Safranin-O staining at E17.5 revealed enlarged, atypical proliferating cells in the medial growth plates of *Smad7-/-* embryos (Fig. 3C). The aberrant morphology of proliferating chondrocytes persisted through P0, resulting in hypocellularity in the medial growth plates of mutants (Fig. 3D). Moreover, the hypertrophic zones were much shorter in *Smad7-/-* growth plates (Fig. 3D', D").

Given that some *Smad7-/-* mice can survive up to three weeks after birth, alcian blue staining was performed on postnatal growth plates to see whether additional defects develop postnatally. By P7, the hypocellular core diminished, but the shorter hypertrophic zones persisted in *Smad7-/-* growth plates (Fig. 3E). By P17, the prehypertrophic zones were expanded at the expense of the hypertrophic zones in mutants (Fig. 3F). Overall, these results show that loss of *Smad7* significantly impacts chondrogenesis during development. Furthermore, the effect of loss of *Smad7* on chondrocyte hypertrophy during development persists after birth, and this defect in hypertrophy is not a consequence of a generalized growth delay.

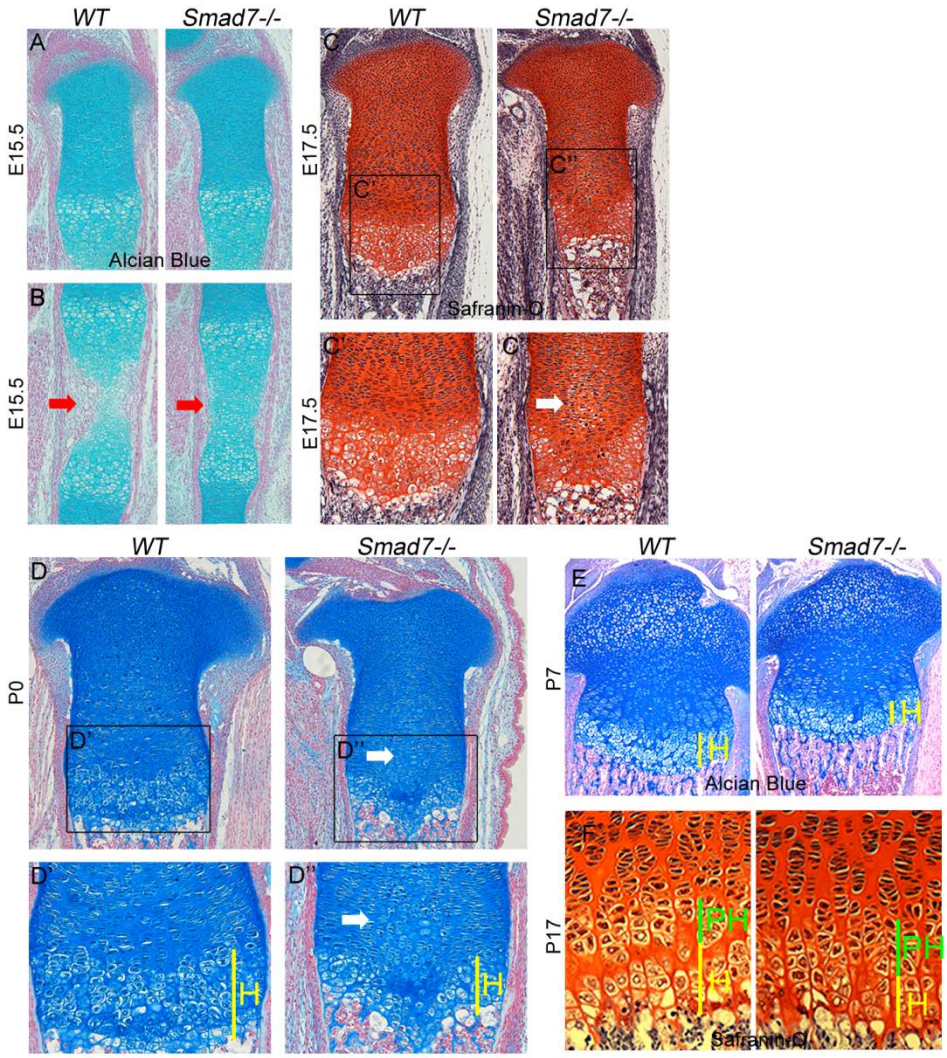

Figure 3. Hypocellularity and shortened hypertrophic zones in *Smad7-/-* growth plates. (**A, B**) Alcian blue and nuclear fast red staining of *WT* and *Smad7-/-* tibiae at E15.5. Red arrows highlight delayed vascular invasion in mutant growth plates. (**C**) Safranin-O, Fast Green, and hematoxylin staining of *WT* and *Smad7-/-* tibial growth plates at E17.5. Higher magnification of (**C'**) *WT* and (**C''**) *Smad7-/-* growth plates at E17.5. White arrow highlights enlarged proliferating cells in mutant growth plates. (**D**) Alcian blue and nuclear fast red staining of *WT* and *Smad7-/-* tibiae at P0. Higher magnification of (**D'**) *WT* and (**D''**) *Smad7-/-* growth plates at P0, highlighting shortened hypertrophic zones in mutant growth plates. White arrow highlights enlarged proliferating cells in mutant growth plates. (**E**) Alcian blue and nuclear fast red staining of *WT* and *Smad7-/-* tibiae at P7. (**F**) Safranin-O, Fast Green, and hematoxylin staining of *WT* and *Smad7-/-* tibial growth plates at P17, highlighting expanded prehypertrophic and shortened hypertrophic zones in mutant growth plates. PH, prehypertrophic zone; H, hypertrophic zone.

Smad7 deficiency leads to impaired cell cycle progression, as well as increased cell death in the medial growth plate. The shorter hypertrophic zones in *Smad7-/-* mice may reflect defects in chondrocyte proliferation. We assessed this possibility via immunofluorescence staining for proliferation markers. Immunofluorescence staining for phospho-histone H3, a marker for M-phase of the cell cycle, revealed no differences between the numbers of cells entering the cell cycle in wild-type and *Smad7-/-* growth plates at E16.5 (Fig. 4A, B). However, immunofluorescence staining for PCNA, a marker for cells in the late G1 through S-phases of the cell cycle, revealed increased levels in mutant growth plates at E17.5 (Fig. 4C-E). In particular, increased levels were evident in the prehypertrophic zones of *Smad7-/-* growth plates. Hence, loss of *Smad7* leads to a decreased number of cells exiting the cell cycle, thereby retaining chondrocytes in a prehypertrophic state.

Apoptosis is normally detected in the lower hypertrophic zone, which consists of terminally differentiating chondrocytes. TUNEL labeling was performed to detect any defects in apoptosis in mutant chondrocytes at P0. Apoptotic cells were found in the medial region of the proliferative zone in *Smad7-/-* growth plates (Fig. 4F, G), correlating with the region in which the hypocellular core developed in mutants. Moreover, a decrease in the number of apoptotic cells were found in the lower hypertrophic zones of mutant growth plates (Fig. 4F', G').

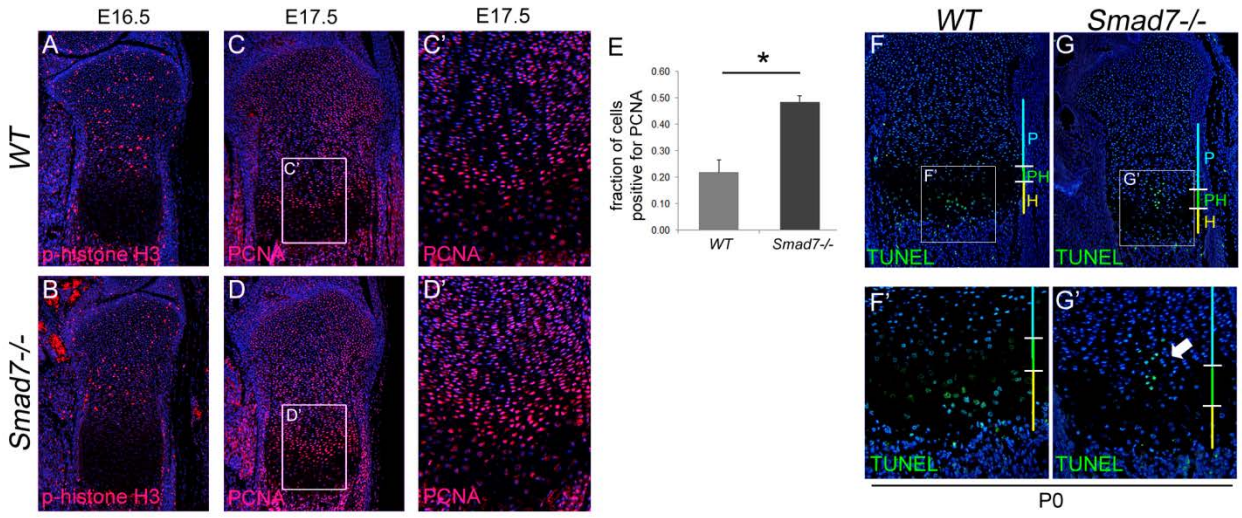

Figure 4. Impaired cell cycle progression and increased cell death in medial growth plates of *Smad7-/-* mice. (**A, B**) Immunofluorescence staining of E16.5 tibial growth plates for Phospho-histone H3. (**C, D**) Immunofluorescence staining of E17.5 tibial growth plates for the proliferation marker, PCNA. Higher magnification of PCNA staining in (**C'**) *WT* and (**D'**) *Smad7-/-* growth plates at E17.5. (**E**) Quantification of the number of cells positive for PCNA. Values are expressed as fraction of labeled cells in field of view shown in (**C'**, **D'**). Student's *t*-test; $^{*}p < 0.05$. (**F, G**) TUNEL staining of P0 proximal tibiae. Higher magnification of TUNEL staining in (**F'**) *WT* and (**G'**) *Smad7-/-* growth plates. Arrow highlights increased cell death in medial growth plates of mutant mice. P, proliferative zone; PH, prehypertrophic zone; H, hypertrophic zone.

Loss of Smad7 leads to defects in terminal chondrocyte maturation. Alterations in chondrocyte maturation (i.e. from proliferation to terminal hypertrophic differentiation) were assessed to determine whether loss of *Smad7* regulates these processes. First, immunofluorescence staining for type II collagen, normally expressed by proliferating chondrocytes, was performed to investigate whether the development of the hypocellular core in mutant growth plates (Fig. 5A, B) was attributed to defects in the activity of proliferating chondrocytes. Increased intracellular retention of type II collagen, co-localizing with the regions of hypocellularity and increased cell death, was observed in the medial growth plates of mutants at P0 (Fig. 5C, D).

Type X collagen is normally expressed by chondrocytes in the early phase of hypertrophic differentiation. We found that the domain of type X collagen expression was shorter and was limited to the upper hypertrophic zone in *Smad7-/-* growth plates at P0 (Fig. 5 E, F). No expression was evident in the lower part of the hypertrophic zone of mutants. Moreover, type X collagen was localized intracellularly in *Smad7-/-* growth plates. These results suggest that mutant chondrocytes are able to enter the hypertrophic differentiation process, but the chondrocytes that enter this process are defective in their ability to secrete type X collagen.

Given that *Smad7-/-* mice are global knockouts, impaired secretion of types II and X collagen may be secondary to other defects. For example, impaired cellular response to hypoxia could lead to abnormal collagen synthesis and secretion (Pfander et al., 2004; Thoms and Murphy, 2010). Thus, the direct effects of loss of *Smad7* on *Col2a1* and *Col10a1* expression were investigated via quantitative real-time PCR analyses of mRNA isolated from primary chondrocytes cultured in chondrogenic media for an 8.5-day time course. *Col2a1* and *Col10a1* mRNA levels were elevated *Smad7*-deficient chondrocytes relative to controls at all of the time points (Fig. 5G, H). This increase in mRNA levels most likely represents a compensatory

response to the impaired secretion of types II and X collagen. These results also demonstrate that Smad7 acts directly on chondrocytes to control the expression and secretion of types II and X collagen. Overall, the *in vivo* and *in vitro* results suggest that mutant chondrocytes undergo accelerated onset of hypertrophic differentiation, but the chondrocytes that enter this process are impaired in their ability to undergo terminal maturation.

Alternatively, the shortened hypertrophic zone in mutants (Fig. 5J, L) could be due to accelerated maturation. If this was the case, reduced expression of type X collagen would be associated with increased expression of the terminal maturation markers, osteopontin (OPN) and MMP-13. However, immunofluorescence staining for OPN at P0 revealed reduced levels in the hypertrophic zones of *Smad7-/-* mice (Fig. 5M, N). In addition, MMP-13 levels were decreased at the ossification front of *Smad7-/-* growth plates (Fig. 5O, P). Real-time PCR analyses of MMP-13 levels in isolated primary chondrocytes cultured in chondrogenic media for an 8.5-day time course revealed reduced levels at 8.5 days in mutant chondrocytes relative to controls (Fig. 5Q). Taken together, the data indicate that loss of *Smad7* leads to retention of chondrocytes in a prehypertrophic state, as well as to an inability of chondrocytes to undergo terminal maturation, ultimately leading to enlarged prehypertrophic and shortened hypertrophic zones in *Smad7*-/- growth plates.

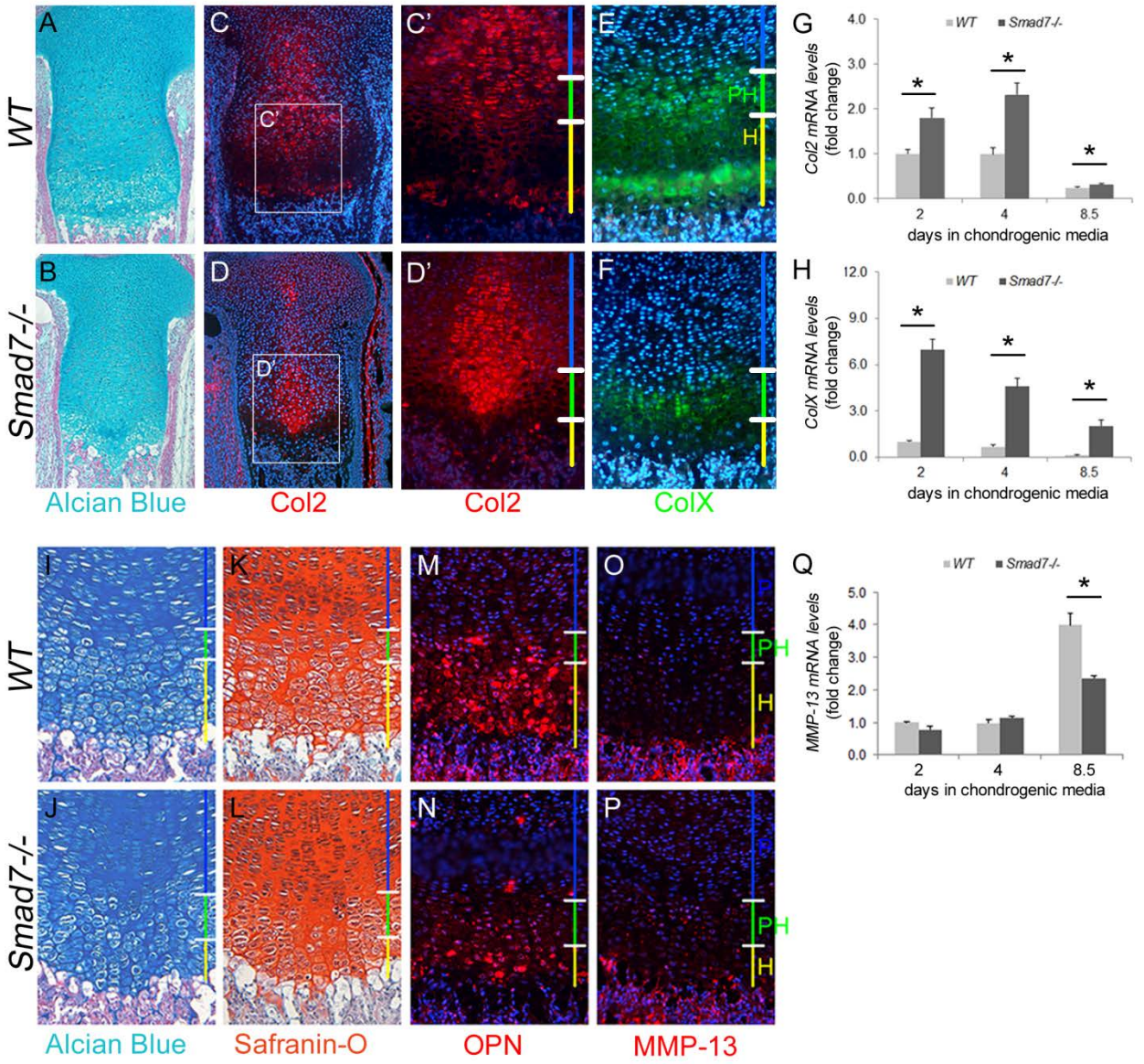

Figure 5. Defects in terminal maturation of chondrocytes in *Smad7-/-* growth plates at P0. (**A, B, I, J**) Alcian blue and nuclear fast red staining of P0 proximal tibiae. (**C, D**) Immunofluorescence staining of tibial growth plates for type II collagen (Col2). Higher magnification of Col2 staining in (**C'**) *WT* and (**D'**) *Smad7-/-* growth plates. (**E, F**) Immunofluorescence staining of tibial growth plates for type X collagen (ColX). Real-time PCR analysis of (**G**) *Col2* and (**H**) *ColX* mRNA levels in *WT* and *Smad7-/-* primary chondrocytes cultured in chondrogenic media for 2, 4, or 8.5 days. The data represent averages from triplicate reactions and are expressed as fold change ± s.d. Student's *t*-test; $^{*}p < 0.05$. (**K, L**) Safranin-O, Fast Green, and hematoxylin staining of of P0 proximal tibiae. Immunofluorescence staining of tibial growth plates for late terminal markers: (**M, N**) osteopontin (Opn) and (**O, P**) MMP-13. (**Q**) Real-time PCR analysis of MMP-13 mRNA levels in *WT* and *Smad7-/-* primary chondrocytes cultured in chondrogenic media for 2, 4, or 8.5 days. The data represent averages from triplicate reactions and are expressed as fold change ± s.d. Student's *t*-test; $^{*}p < 0.05$.

Smad7 deficiency does not severely impact Ihh signaling in P0 growth plates. It is well established that the Ihh and PTHrP pathways interact to form a negative feedback loop to limit the number of chondrocytes that undergo hypertrophic differentiation (Kronenberg, 2003). We thus assessed the effects of loss of *Smad7* on Ihh signaling to determine whether the enlarged prehypertrophic zones in *Smad7*-/- growth plates result from aberrant Ihh signaling. Immunofluorescence staining for Ihh revealed that the domain of Ihh localization spanned the proliferative, prehypertrophic, and hypertrophic zones of wild-type and *Smad7*-/- growth plates at P0, and that the domain of Ihh localization was similar in both genotypes (Fig. 6A). Ihh pathway activity was evaluated via immunofluorescence staining for its downstream target, Patched 1 (Ptc1). Ptc1 was expressed at low levels in the proliferative zone and at higher levels in the prehypertrophic/hypertrophic zones and periarticular regions of wild-type growth plates at P0 (Fig. 6B). Similar expression patterns were observed in mutant growth plates (Fig. 6B). As expected, based on the shorter hypertrophic zones in *Smad7* mutants, a shorter domain of Ptc1 expression was observed in the hypertrophic zones of *Smad7*-/- mice. These results indicate that loss of *Smad7* does not severely impact Ihh signaling, and that the retention of prehypertrophic chondrocytes in *Smad7*-deficient mice is most likely not due to impaired Ihh signaling.

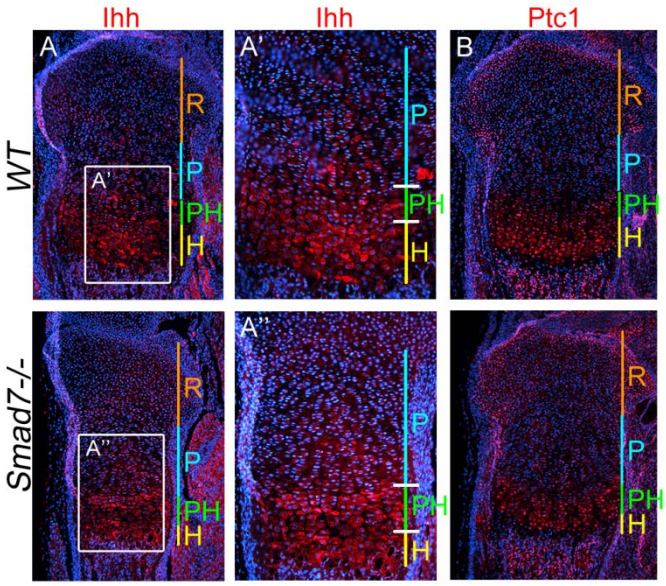

Figure 6. *Smad7*-deficiency does not severely impact Ihh signaling in P0 growth plates. Immunofluorescence staining of tibial growth plates for (**A**) Ihh and its downstream target, (**B**) Ptc1. Higher magnification of Ihh staining in (**A'**) *WT* and (**A''**) *Smad7-/-* growth plates. R, reserve zone; P, proliferative zone; PH, prehypertrophic zone; H, hypertrophic zone.

TGFβ and BMP pathways are elevated in Smad7-/- growth plates and isolated primary chondrocytes. Smad7 is known to be a key intracellular antagonist of both the BMP and TGFβ pathways (Hanyu et al., 2001; Ishisaki et al., 1999; Mochizuki et al., 2004). Thus, we examined the effect of loss of *Smad7* on the activation of both the canonical and noncanonical BMP and TGFβ pathways in cartilage. At E17.5, immunofluorescence staining for the activated canonical TGFβ mediator, phospho-Smad2, revealed increased levels in the proliferative zones of *Smad7-/-* growth plates (Fig. 7A). Similarly, immunofluorescence staining for the activated canonical BMP mediators, phospho-Smad1/5/8, revealed increased in levels in the proliferative zones, as well as in the prehypertrophic and hypertrophic zones of mutant growth plates (Fig. 7C).

By P17, the phospho-Smad2 expression domain was expanded in mutant growth plates, coinciding with the enlarged prehypertrophic zone (Fig. 7B). Moreover, phospho-Smad2 levels appeared to be increased on a per cell basis in mutants. The phospho-Smad1/5/8 expression

domain was expanded throughout both the prehypertrophic and hypertrophic zones (Fig. 7D). Thus, loss of *Smad7* leads to activation of both BMP and TGFβ canonical pathways.

The most upstream regulator of noncanonical BMP and TGFβ signaling, TAK1, is widely expressed in developing cartilage and then becomes restricted to the prehypertrophic and hypertrophic zones postnatally (Shim et al., 2009). By P0, we found that activated TAK1, phospho-TAK1, was localized primarily in the prehypertrophic zones of wild-type growth plates (Fig. 7E). We also found that the expression domain of phospho-TAK1 was expanded in *Smad7-/-* growth plates (Fig. 7E). Taken together, these results indicate that both canonical and noncanonical BMP and TGFβ signaling are elevated in *Smad7-/-* growth plates.

Primary chondrocytes were isolated to examine the direct effects of loss of *Smad7* on BMP and TGFβ responsiveness *in vitro*. First, *Smad7* deficiency was confirmed via real-time PCR analysis for *Smad7* levels in wild-type and mutant isolated chondrocytes (Fig. 7F). The extent and duration of canonical and noncanonical TGFβ signaling were evaluated via treatment of primary chondrocytes with TGFβ1. Levels of phospho-Smad2 were greater at 2 hours post-stimulation, and increased levels were maintained up to 24 hours post-stimulation in *Smad7*-deficient chondrocytes compared to levels in wild-type controls (Fig. 7G). Likewise, phospho-p38 levels were elevated under basal conditions and for up to 24 hours post-stimulation in mutant chondrocytes (Fig. 7G). The extent and duration of canonical BMP signaling was then evaluated with treatment of primary chondrocytes with BMP2. Basal levels of phosphor-Smad1/5/8 were elevated in mutant chondrocytes. In addition, levels of phospho-Smad1/5/8 were greater at 2 hours post-stimulation and increased levels were maintained up to 12 hours post-stimulation in *Smad7*-deficient chondrocytes (Fig. 7H). Overall, these results show that loss

of *Smad7* leads to increased basal levels of canonical and noncanonical BMP signaling, as well as increased responsiveness to exogenous TGFβ and BMP in mutant chondrocytes.

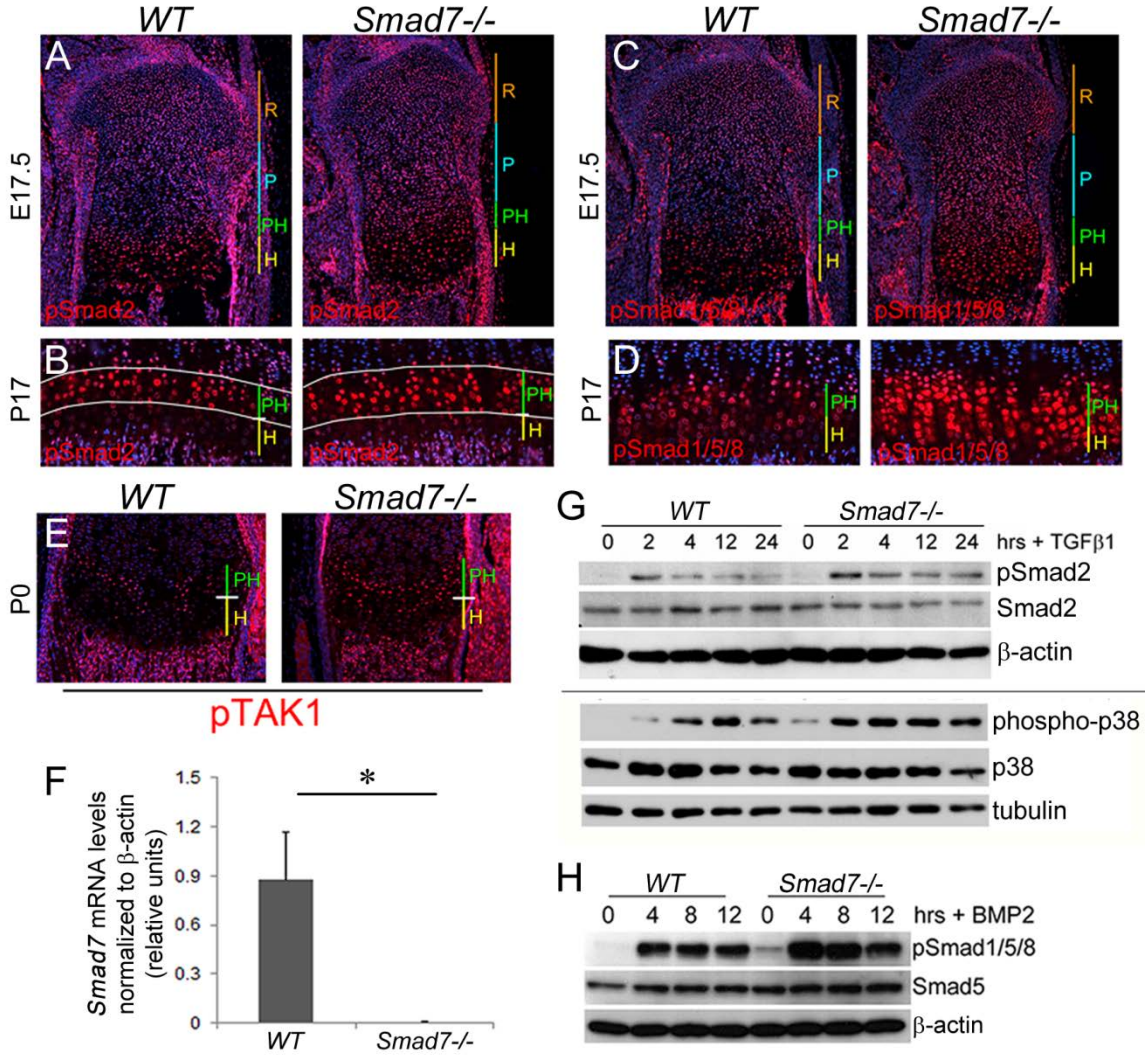

Figure 7. TGFβ and BMP pathways are elevated in *Smad7-/-* growth plates and isolated primary chondrocytes. Immunofluorescence staining of tibial growth plates for phospho-Smad2 (pSmad2) at (**A**) E17.5 and (**B**) P17. Immunofluorescence staining of tibial growth plates for phospho-Smad1/5/8 (pSmad1/5/8) at (**C**) E17.5 and (**D**) P17. (**E**) Immunofluorescence staining of P0 tibial growth plates for phospho-TAK1 (pTAK1). (***F***) Real-time PCR analysis of RNA isolated from WT and *Smad7-/-* primary chondrocytes cultured in chondrogenic media. Expression levels for *Smad7* were normalized to *β-actin* and are shown as fold change relative to *WT* mRNA levels. The data represent averages from three independent samples with the indicated s.d. and significant differences (Student's *t*-test; $^{*}p < 0.05$). (**G**) Western blot analysis shows elevated levels of pSmad2 and phosphor-p38 in lysates isolated from *WT* and *Smad7-/-* primary chondrocytes treated with TGFβ1 (5 ng/ml) for 0, 2, 4, 8, 12 hours. (**H**) Western blot analysis shows elevated levels of pSmad1/5/8 in lysates isolated from *WT* and *Smad7-/-* primary chondrocytes treated with BMP2 (50 ng/ml) for 0, 4, 8, 12 hours.

Loss of Smad7 leads to increased cell stress responses in the growth plate. The growth plate functions at lower oxygen tension than most tissues. Schipani and colleagues have shown that the hypoxic regions of the growth plate are localized to the medial regions of the reserve and proliferative zones (Schipani et al., 2001). Of note, hypocellularity and increased cell death in *Smad7-/-* growth plates occurred in the hypoxic core of the proliferative zone. Thus, this particular phenotype may result from the inability of mutant chondrocytes to properly adapt to the hypoxic environment. Immunofluorescence staining for HIF1α, the main activator of the hypoxic stress response, was performed at E17.5 to determine whether loss of *Smad7* impacts the hypoxic stress response at the stage of development in which hypocellularity arises in mutant embryos. Increased levels of HIF1α were found throughout the proliferative zones of *Smad7-/-* embryos, with the highest levels in the lower proliferative/upper prehypertrophic zones (Fig. 8A). These results indicate Smad7 mediates the hypoxic stress response via HIF1α, and that loss of *Smad7* leads to upregulation of HIF1α in developing cartilage.

Hypoxia can also activate the unfolded protein response as a consequence of endoplasmic reticulum (ER) stress (Wouters and Koritzinsky, 2008). To evaluate whether loss of *Smad7* impacts the ER stress response, we performed immunofluorescence staining for the master regulator of ER stress, GRP78/BiP, at P0. While GRP78/BiP was found at low levels in the prehypertrophic and hypertrophic zones, and at higher levels in the ossification front in wild-type growth plates, increased GRP78/BiP levels were evident in the medial region of the proliferative zones in mutants (Fig. 8B). Taken together, hypocellularity and increased cell death may be attributed to abnormal stress responses in mutant growth plates.

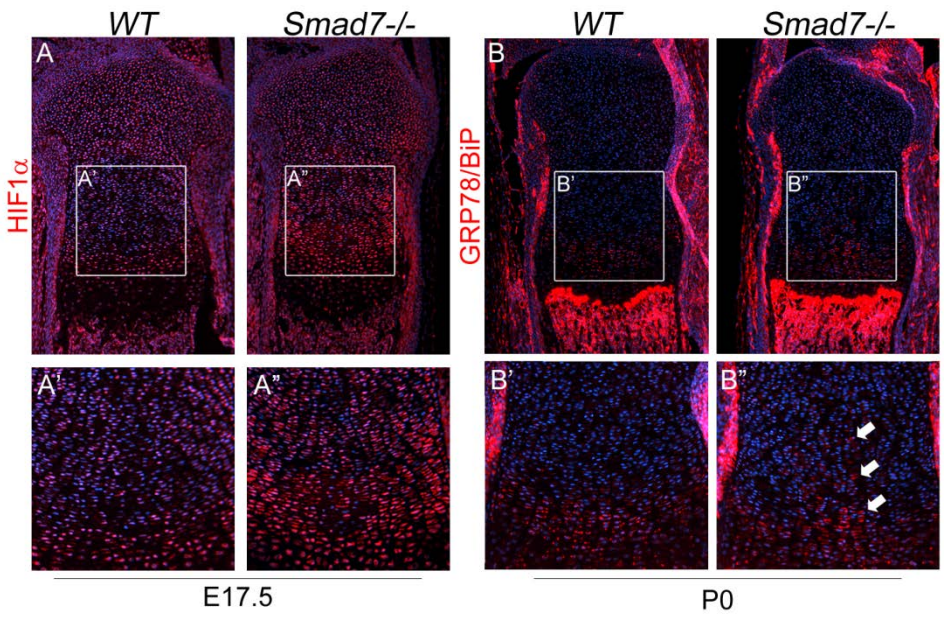

Figure 8. Loss of *Smad7* leads to increased cell stress responses in the growth plate. (**A**) Immunofluorescence staining of tibial growth plates for HIF1α at E17.5. Higher magnification of HIF1α staining in (**A'**) *WT* and (**A''**) *Smad7-/-* growth plates. (**B**) Immunofluorescence staining of tibial growth plates for the ER stress marker, GRP78/BiP, at P0. Higher magnification of GRP78/BiP staining in (**B'**) *WT* and (**B''**) *Smad7-/-* growth plates. White arrow highlights increased levels in the medial region of the proliferative zones in mutant growth plates.

Discussion

Overexpression of Smad7 in murine chondrocytes at various stages of development has been shown to adversely affect all stages of endochondral bone formation, in part by attenuating noncanonical BMP signaling (Iwai et al., 2008). These studies demonstrate that Smad7 has the potential to regulate BMP signaling in cartilage during development. However, given that overexpression studies result in non-physiological expression levels and often do not recapitulate actual expression patterns, it is still unknown whether Smad7 is actually required for endochondral bone formation. In this study, we show that Smad7 regulates both axial and appendicular skeletal development. In particular, mice deficient in Smad7 exhibit defects in anterior/posterior (A/P) patterning, as evidenced by posterior transformation of cervical and lumbar vertebrae. In appendicular bones, loss of *Smad7* results in the retention of chondrocytes in a prehypertrophic state, leading to an expanded prehypertrophic zone at the expense of the hypertrophic zone in mutant growth plates. These defects were attributed, in part, to elevated BMP and TGFβ signaling in *Smad7*-deficient chondrocytes. Thus, our results provide the first *in vivo* evidence that Smad7 plays a role in limiting both BMP and TGFβ signaling during endochondral bone formation.

Smad7 and Smad6 have overlapping functions in anterior/posterior patterning. Mice deficient in *Smad7* exhibit posterior transformation of the seventh cervical vertebrae and of the sixth lumbar vertebrae. These results demonstrate a role for Smad7 in regulating A/P patterning of the axial skeleton. Similarly, mice deficient in *Smad6* exhibit posterior transformation of the seventh cervical vertebrae (Estrada et al., 2011); lumbar patterning, however, was normal in *Smad6-/-* mice. Thus, Smad7 and Smad6 appear to have similar functions in A/P patterning, although Smad7 has plays a wider role.

BMP and TGFβ signaling have been shown to differentially affect the expression pattern, as well as the activity of members of the *Hox* family of transcription factors, which are known to regulate vertebral axial patterning. For example, mice deficient in the type I TGFβ receptor, *ALK5,* exhibit normal levels of *Hoxc8* (expressed in the paraxial mesoderm) as compared to wild-type littermates, while the expression of the posterior determinant, *Hoxc10,* was absent in *ALK5* mutants (Andersson et al., 2006). Smad1, which transduces BMP signals, has been shown to interact with Hoxc8 to regulate osteopontin gene expression (Shi et al., 1999). The difference in the regulation of A/P patterning of the axial skeleton between *Smad6-/-* and *Smad7-/-* mice may be due to differences in the signaling pathways mediated by either Smad6 or Smad7. That is, *Smad6-/-* mice may exhibit A/P patterning defects attributed to alterations in BMP signaling, while defects in *Smad7-/-* mice may reflect alterations in both BMP and TGFβ signaling. Alternatively, Smad7 may be a more potent inhibitor of BMP signaling than Smad6. For example, Smad6 inhibits signaling from the BMP receptors ALK3 and ALK6 in preference to ALK2 (Goto et al., 2007). In contrast, Smad7 inhibits signaling induced by all of these three ALK receptors (Goto et al., 2007), as well as ALK1 (Valdimarsdottir et al., 2006). Further studies are required to determine the mechanisms by which Smad6 and Smad7 regulates the expression and activity of Hox transcription factors.

Smad7 limits BMP and TGFβ signaling during cartilage development. We found that loss of *Smad7* led to the retention of chondrocytes in a prehypertrophic state, consequently resulting in an expanded prehypertrophic zone at the expense of the hypertrophic zone. These findings are consistent with *in vivo* studies showing that TGFβ signaling prevents hypertrophic differentiation. Loss of responsiveness to TGFβ in mice due to either the expression of a kinase-dead TGFβ type II receptor (Serra et al., 1997) or deficiency in *Smad3* results in

accelerated hypertrophic differentiation (Yang et al., 2001) associated with expanded domains of type X expression. Moreover, *in vitro* studies have shown that Smad3 plays important roles in suppressing chondrocyte maturation (Li et al., 2006b).

However, increased *Col10a1* expression in isolated *Smad7-/-* chondrocytes suggests that TGFβ signaling is not the only pathway that is contributing to the mutant phenotype. It is well established that BMP signaling promotes hypertrophic differentiation partly by stimulating *Col10a1* expression. In particular, *in vitro* studies utilizing mouse and chick chondrocytes have shown that BMP2 and BMP6 induce *Col10a1* and *Ihh* expression (Grimsrud et al., 1999; Grimsrud et al., 2001; Valcourt et al., 2002). Similarly, treatment of limb explants with BMP2 results in expanded expression domains of type X collagen (Minina et al., 2001). Noncanonical signaling via TAK1 and p38 MAPK pathways has also been shown to promote hypertrophic differentiation of chondrocytes (Gunnell et al., 2010; Shim et al., 2009; Stanton et al., 2004). Given that *Smad7*-deficient chondrocytes were found to be more responsive to BMP and TGFβ, the growth plate phenotype of *Smad7-/-* mice likely results from upregulation of both canonical and noncanonical BMP and TGFβ pathways in chondrocytes.

Smad7 mediates cell stress responses in the growth plate. A striking aspect of the *Smad7-/-* growth plate phenotype is the manifestation of a hypocellular core in the medial growth plate at mid-gestation. This finding was accompanied by impaired secretion of type II collagen by chondrocytes, as well as increased cell death. Interestingly, these observations were specifically localized to the most hypoxic region of the growth plate, as previously reported by Schipani and colleagues (Schipani et al., 2001). Impaired adaptation of cells to a hypoxic environment has been shown to be detrimental to chondrocyte survival. Specifically, cartilage-specific deletion of HIF1α , the main mediator of the hypoxic response, results in cell death in the center of the

growth plate (Schipani et al., 2001). Thus, we examined whether loss of *Smad7* leads to an impaired response to hypoxia by analyzing levels of HIF1α in the growth plate at the stage in which the hypocellular core became apparent. Contrary to our hypothesis, we found increased levels of HIF1α in *Smad7-/-* growth plates.

Chondrocytes are highly synthetic cells and are required to constantly synthesize extracellular matrix. HIF1α has been shown to be essential in regulating extracellular matrix synthesis in chondrocytes (Pfander et al., 2003). Moreover, increased matrix deposition associated with severe hypocellularity throughout the growth plate was observed in mice with elevated HIF1α due to von Hippel Landau (VHL) deficiency in chondrocytes (Pfander et al., 2004). Thus, increased HIF1α levels may also contribute to the development of the hypocellular core, as increased HIF1α can lead to enhanced matrix synthesis, rendering chondrocytes more susceptible to ER stress. In fact, hypocellularity owing to increased activation of HIF1α signaling and ER stress was found in mice cartilage-specific deletion of the tumor suppressor PTEN (Yang et al., 2008). ER stress in these mice was characterized by the presence of engorged ER, as well as upregulation BiP mRNA and protein levels preceded by increased stabilization of HIF1α. The authors concluded that overactivation of HIF1α signaling was responsible for the emergence of ER stress in *PTEN*-deficient chondrocytes. Given that we found increased levels of the ER stress marker, BiP, in *Smad7-/-* medial growth plates, it is possible that the observed hypocellular phenotype may result from ER stress, in part, due to elevated HIF1α levels.

Conclusions

In summary, we have determined that Smad7 plays an important role the development of the axial and appendicular skeleton. Loss of *Smad7* leads to defects in anterior/posterior pattern specification, as well as in terminal maturation of chondrocytes. We also show that Smad7 regulates these processes by inhibiting both BMP and TGFβ signaling. Finally, we show for the first time that Smad7 regulates stress pathways in the growth plate. Additional studies are required to determine which signaling pathways impacted by Smad7 are responsible for elevated HIF1α levels in chondrocytes.

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CHAPTER 5:

Conclusions and Future Directions

Summary

The studies presented in this dissertation describe the first investigations into the physiological roles of inhibitory Smads 6 and 7 in endochondral bone formation. Prior to our investigations, *in vivo* analyses were limited to overexpression studies, which showed that inhibitory Smads 6 and 7 have the potential to limit BMP-mediated canonical and noncanonical pathways (Horiki et al., 2004; Iwai et al., 2008). However, these overexpression studies have obvious limitations, namely I-Smads are expressed in all chondrocyte subpopulations at non-physiological levels. The results presented in this dissertation show that I-Smads are required for normal endochondral bone formation. While both Smad6 and Smad7 are essential in limiting BMP signaling during cartilage development, Smad7 has additional functions as it can also limit TGFβ signaling. Understanding the mechanisms underlying endochondral bone formation is important as these processes are recapitulated in pathological conditions, such as osteoarthritis. Thus, our findings may have important implications for understanding the mechanisms underlying the pathogenesis of osteoarthritis. Detailed conclusions, as well as future studies, are presented below.

I-Smads have similar functions in regulating anterior-posterior (A/P) patterning of the axial skeleton. Analyses of skeletal preparations at P0 revealed that both *Smad6-/-* and *Smad7-/-* mice exhibit defects in A/P patterning. In particular, posterior transformation of the seventh cervical (C7) vertebrae was evident in both mouse lines. Moreover, preliminary skeletal

preparations from compound Smad6 and Smad7 mutant embryos at E18.5 revealed that compound mutants also exhibit posterior transformation at C7. In fact, the rib anlagen found in *Smad6-/-; Smad7+/-* embryos was much longer and extended further towards the sternum than that found in single mutants, and a complete joint was formed between the ribs at C7 and T1 in *Smad6+/-;Smad7-/-* embryos. Additional defects, however, were found in *Smad7-/-* mice. Specifically, *Smad7-/-* mice exhibit posterior transformation of the sixth lumbar vertebrae. These results indicate that I-Smads have similar functions in regulating A/P patterning, and that Smad7 plays a wider role.

BMP and TGFβ signaling have been shown to regulate A/P patterning. For example, mice carrying a hypomorphic type II BMP receptor exhibit anterior transformation of thoracic vertebrae (Delot et al., 2003). *In vitro* studies have shown that Smad6 preferentially inhibits BMP signaling, while Smad7 can inhibit both BMP and TGFβ pathways (reviewed in Chapters 1 and 2). Hence, additional defects in A/P patterning evident in *Smad7-/-* mice may reflect alterations in both BMP and TGFβ signaling, while A/P patterning defects in *Smad6-/-* mice reflect alterations only in BMP signaling. Alternatively, Smad7 may be a more potent inhibitor of BMP signaling than Smad6. For example, Smad7 can inhibit signaling via multiple BMP type I receptors (ALK1-3, and ALK6), while Smad6 inhibits signaling from ALK3 and ALK6 (Goto et al., 2007; Valdimarsdottir et al., 2006).

Members of the *Hox* family of transcription factors are well known as regulators of vertebral axial patterning. BMP and TGFβ signaling have been shown to regulate the expression and activity of *Hox* transcription factors (Andersson et al., 2006; Shi et al., 1999). Thus, it is possible that the A/P patterning defects reflect alterations in BMP- and/or TGFβ-mediated *Hox* transcription factor expression or activity. Future studies include the investigation of the

mechanisms by which Smad6 and Smad7 regulates the expression and activity of *Hox* transcription factors.

Smad6 limits BMP signaling pathways, while Smad7 limits both BMP and TGFβ pathways during cartilage development. Histological analyses of appendicular growth plates of *Smad6-/-* and *Smad7-/-* mice revealed dissimilar growth plate phenotypes. At P0, enlarged prehypertrophic and hypertrophic zones were evident in *Smad6-/-* growth plates, while enlarged prehypertrophic and shorter hypertrophic zones were evident in *Smad7-/-* growth plates. Based on what is known about the regulation of cartilage development by BMP and TGFβ signaling pathways, and the preference of I-Smads in inhibiting these pathways, we propose that the growth plate phenotype of *Smad6-/-* mice results from upregulation of canonical and noncanonical BMP signaling, while the growth plate phenotype of *Smad7-/-* mice results from upregulation of both canonical and noncanonical BMP and TGFβ pathways.

Future studies include the investigation of the differential effects of canonical and noncanonical pathways on endochondral bone formation in *Smad6-/-* or *Smad7-/-* mice. That is, *ex vivo* cultures of limb explants isolated from either *Smad6-/-* or *Smad7-/-* mice treated with BMP2 or TGFβ1 in the presence or absence of inhibitors (noggin, SB431542 (ALK5), or SB203580 (p38)) may give insight into the individual roles of canonical and noncanonical pathways in regulating proliferation and hypertrophic differentiation of Smad6 and Smad7 mutant chondrocytes. Such studies have been conducted in wild-type limb explants (Minina et al., 2002; Minina et al., 2001; Mukherjee et al., 2005; Yang et al., 2001), but not in explants from *Smad6-/-* or *Smad7-/-* mice.

Smad7 has been shown to mediate other signaling pathways, such as Wnt/β-catenin signaling and NFκB pathways. These pathways have been shown to play roles in regulating endochondral bone formation (Chen et al., 2008; Feng et al., 2003; Mak et al., 2006; Wu et al., 2007), but whether Smad7 is required to inhibit these pathways during cartilage development is unknown. Thus, future studies will be to investigate the role of Smad7 in regulating these pathways, as dysregulation of these pathways may also contribute to the *Smad7*-/- growth plate phenotype.

Smad7 mediates cell stress responses in the growth plate. Histological analyses of *Smad7-/-* growth plates show that mutant growth plates develop a hypocellular core at mid-gestation. This finding as associated with impaired type II collagen secretion, as well as increased cell death. Because these findings were specifically localized to the most hypoxic region of the growth plate, as previously reported by Schipani and colleagues (Schipani et al., 2001), we hypothesized that the manifestation of a hypocellular core in *Smad7-/-* growth plates may be due to impaired adaptation of chondrocytes to a hypoxic environment. In accordance with our hypothesis, loss of HIF1α, the main mediator of the hypoxic response, impairs chondrocyte survival, as evidenced by hypocellularity due to massive cell death in the medial growth plates of mutants (Schipani et al., 2001). Therefore, we investigated whether loss of *Smad7* altered levels of HIF1α in mutant growth plates. Contrary to our hypothesis, we found increased levels of HIF1α in *Smad7*-/- growth plates. As mentioned in the discussion section of Chapter 4, it is possible that elevated HIF1α levels could also contribute to the development of the hypocellular core, as HIF1α can lead to enhanced extracellular matrix synthesis (e.g. type II collagen), rendering chondrocytes

more susceptible to ER stress. Correspondingly, we found increased levels of the ER stress marker, BiP, in *Smad7-/-* growth plates.

Future studies will be to investigate the effects of loss of *Smad7* on HIF1α activity. This can be completed immunostaining for markers of HIF1α activity (VEGF, PGK-1, etc.). Furthermore, future experiments will include the investigation of signaling pathways mediated by Smad7 that could be responsible for elevated HIF1α levels in mutant growth plates. Candidate pathways include canonical and noncanonical TGFβ signaling, as well as NFκB pathways, given that these pathways have been shown to regulate HIF1α levels and activity in other cell types (McMahon et al., 2006; Rius et al., 2008). These studies are paramount, as the role of Smad7 in regulating the hypoxic stress response has never been investigated.

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