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Smad4 is required for the normal organization of the cartilage growth plate

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Abstract

Smad4 is the central intracellular mediator of transforming growth factor-β (TGF-β) signals. To study the role of Smad4 in skeletal development, we introduced a conditional mutation of the gene in chondrocytes using Cre–loxP system. We showed that *Smad4* was expressed strongly in prehypertrophic and hypertrophic chondrocytes. The abrogation of *Smad4* in chondrocytes resulted in dwarfism with a severely disorganized growth plate characterized by expanded resting zone of chondrocytes, reduced chondrocyte proliferation, accelerated hypertrophic differentiation, increased apoptosis and ectopic bone collars in perichondrium. Meanwhile, *Smad4* mutant mice exhibited decreased expression of molecules in Indian hedgehog/parathyroid hormone-related protein (Ihh/PTHrP) signaling. The cultured mutant metatarsal bones failed to response to TGF-β1, while the hypertrophic differentiation was largely inhibited by Sonic hedgehog (Shh). This indicated that Ihh/PTHrP inhibited the hypertrophic differentiation of chondrocytes independent of the Smad4-mediated TGF-β signals. All these data provided the first genetic evidence demonstrating that Smad4-mediated TGF-β signals inhibit the chondrocyte hypertrophic differentiation, and are required for maintaining the normal organization of chondrocytes in the growth plate.

Keywords: Smad4; Conditional gene knockout; Endochondral ossification; Growth plate; Chondrocyte differentiation

Introduction

Endochondral ossification initiates with the condensation of mesenchymal cells, which differentiate into chondrocytes forming cartilaginous templates. The surrounding mesenchymal cells differentiate into fibroblastic cells to form the perichondrium. The cartilage tissues known as the growth plate locating at the both extremities of long bones continuously proceed through programmed proliferation, maturation, hypertrophic and finally terminal hypertrophic differentiation. Hypertrophic chondrocytes direct adjacent perichondrial cells to differentiate into osteoblasts that form a bone collar. The region of terminal hypertrophic chondrocytes is further invaded by blood vessels followed by osteoblasts and osteoclasts, which start to replace the cartilaginous extracellular matrix (ECM) with the bone ECM. The cartilage will eventually be replaced by the bone tissue through the endochondral ossification. Most importantly, this differentiation process is driven along the defined orientation and with the synchronous rate. During this process, the proliferation and differentiation of chondrocytes should be tightly regulated to maintain the normal skeletal development (Karsenty and Wagner, 2002; Kronenberg, 2003).

Members of TGF-β superfamily play important roles in the regulation of bone development. The bone morphogenetic proteins (BMPs) induce early cartilage formation (Wozney, 1989). Misexpression of the BMP antagonist, Noggin, prior to the onset of chondrogenesis resulted in the absence of formation of condensations (Pizette and Niswander, 2000), and overexpression of Noggin in the

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cartilage of the transgenic mice caused the absence of nearly all cartilages (Tsumaki et al., 2002). During the later stages of endochondral ossification, BMP genes and their receptors are expressed in specific regions of the developing cartilage elements (Vortkamp et al., 1996; Pathi et al., 1999; Zou et al., 1997; Yi et al., 2000; Daluiski et al., 2001). Several in vitro and in vivo studies have shown that BMP signaling promotes the chondrocyte proliferation and expansion of all zones of differentiation in the skeletal elements (Zou et al., 1997; Minina et al., 2001; Tsumaki et al., 2002). Notably, all these functions of BMPs have been established using in vitro culture systems or overexpression systems. Targeted disruption of *Bmps* either leads to mice with no significant phenotypes or embryonic lethality due to the functional redundancy of large number of ligands and receptors, as well as basilic roles of BMPs during early embryonic development (Kronenberg, 2003). Therefore, further studies that delete several BMP genes or BMP signaling transducer in whole animal or in specific cartilage tissues need to be done to fully understand the roles of BMPs during the endochondral ossification.

TGF-βs are also expressed abundantly during the endochondral ossification. Many studies have shown that TGF-βs play important roles in regulating chondrocyte proliferation and differentiation. Experiments on mouse embryonic metatarsal bone rudiment culture have demonstrated that TGF-β1 can inhibit chondrocyte proliferation, hypertrophic differentiation and matrix mineralization (Dieudonne et al., 1994; Serra et al., 1999). The transgenic mice expressing a dominant-negative mutation of the TGF-β type II receptor developed degenerative joint disease resembling human osteoarthritis, suggesting that TGF-β signaling can inhibit the hypertrophic differentiation of articular chondrocytes (Serra et al., 1997). However, the underlying molecular mechanism is still largely unclear.

Many data have shown that TGF-β signals interact with Ihh/PTHrP signaling at several stages to regulate the process of the endochondral bone formation. Indian hedgehog (Ihh), which is expressed in the prehypertrophic chondrocytes, can stimulate the expression of parathyroid hormone-related peptide (PTHrP) in the periarticular region. They interact in a negative feedback loop to regulate the onset of hypertrophic differentiation (Vortkamp et al., 1996; Lanske et al., 1996). Misexpression of a constitutively activated BmpR-IA during chick limb development phenocopies misexpression of Ihh, resulting in an upregulation of *Pthrp* expression and delayed chondrocyte differentiation (Vortkamp et al., 1996; Zou et al., 1997). This suggests that BMP signaling may act as the mediator between Ihh and PTHrP molecule. TGF-β2 is also suggested to act as one of the mediator signals between Ihh and PTHrP molecules (Alvarez et al., 2002). On the other hand, previous studies also suggest that the role of BMP signaling in regulating the onset of hypertrophic differentiation is independent of the Ihh/PTHrP signaling (Minina et al., 2001). All these issues need to be further clarified.

TGF-β superfamily members signal through Smad signaling pathway (Shi and Massague, 2003). There are eight Smad proteins, divided into three functional classes: the receptor-regulated Smads (Smad1, 2, 3, 5 and 8), the co-mediator Smad (Smad4) and the inhibitory Smads (Smad6 and Smad7). Smad2 and Smad3 respond to TGF-β and activin, while Smad1, 5 and 8 function in BMP signaling pathways. Receptor-regulated Smads form heterodimers with Smad4, and then translocate into the nucleus to induce or repress the expression of TGF-β target genes. Mutation analyses in mice using gene targeting have revealed multiple important functions of Smad genes in various developmental processes (Weinstein et al., 2000), including endochondral ossification. Our previous studies have shown that targeted disruption of *Smad3* resulted in a degenerative joint disease resembling human osteoarthritis. This suggests that the Smad3 is required for maintaining articular cartilage in the quiescent state by repressing chondrocyte hypertrophic differentiation (Yang et al., 1999, 2001). A missense mutation of the *Smad3* gene is found in an individual with osteoarthritis, indicating that the TGF-β/Smad3 signaling pathway is involved in the onset of osteoarthritis in human (Yao et al., 2003). Recent reports show that the Smad6/Smurf1 overexpression in cartilage delays chondrocyte hypertrophy, but does not obviously influence the chondrocyte proliferation (Horiki et al., 2004), implying the functional complexity of cytoplastic Smad signals during the bone development.

As a common mediator Smad of TGF-β signaling, Smad4 is expressed ubiquitously in all zones of the epiphyseal plate (Sakou et al., 1999), suggesting that the Smad4 may have important functions during the endochondral ossification. However, the *Smad4* deficient mice die at the early stages of embryogenesis (Sirard et al., 1998; Yang et al., 1998), which makes it difficult to access the function of Smad4 in organgenesis. Tissue specific deletion of *Smad4* begins to reveal the function of Smad4 in cerebel and mammary gland development (Zhou et al., 2003; Li et al., 2003). In order to comprehensively understand the roles of Smad4-mediated TGF-β signals in the endochondral ossification, we specifically deleted the Smad4 gene in the chondrocytes by crossing a mouse strain that carries a *Smad4* conditional allele ($Smad4^{Co}$) (Yang et al., 2002) with the chondrocyte specific *Cre* (*Col2a1-Cre*) transgenic mice (Hao et al., 2002). Our data showed that the conditional knockout of *Smad4* in chondrocytes resulted in deceased proliferation and premature hypertrophic differentiation of chondrocytes, which led to the disorganization of the growth plate. More importantly, our results suggested that Ihh/PTHrP signaling inhibited the hypertrophic differentiation of chondrocytes independent of Smad4-mediated TGF-β signals. Overall, our studies provided the genetic evidence showing that Smad4 is required for maintaining the regular arrangement and sequential differentiation of chondrocytes in the growth plate.