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Conditional deletion of *Tgfbr2* in hypertrophic chondrocytes delays terminal chondrocyte differentiation

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ABSTRACT

Transforming growth factor β (Tgfb) signaling plays an important role in endochondral ossification. Previous studies of mice in which the Tgfb type II receptor gene (*Tgfbr2*) was deleted in the limb bud mesenchymal cells or differentiated chondrocytes showed defects in the development of the long bones or the axial skeleton, respectively. Here, we generated mouse embryos in which the *Tgfbr2* gene was ablated in hypertrophic chondrocytes. These mice exhibited delays in both the hypertrophic conversion of proliferating chondrocytes and the subsequent terminal chondrocyte differentiation. The expression domains of *Col10a1*, *Matrix metalloproteinase 13*, and *Osteopontin* were small, and the expression of *Vascular endothelial growth factor* and Platelet endothelial cell adhesion molecule was downregulated. The calcification of the bone collar in the mutant mice was markedly delayed and the periosteum was thin, possibly because of the downregulation of *Indian hedgehog* expression. We conclude that Tgfb signaling in hypertrophic chondrocytes positively regulates terminal chondrocyte differentiation, angiogenesis in calcified cartilage, and osteogenesis in the bone collar, at least partly through Indian hedgehog signaling *in vivo*.

1. Introduction

Chondrogenesis in endochondral ossification starts with the formation of the cartilage primordia through the mesenchymal condensation of chondrogenic mesenchymal cells, followed by their overt differentiation into chondrocytes. The differentiated chondrocytes first flatten and undergo a series of cell divisions along their longitudinal axes, thus forming characteristic columns of clonal cells. These proliferating chondrocytes exit the cell cycle and begin to increase in volume until they develop into hypertrophic chondrocytes. The extracellular matrix around the hypertrophic chondrocytes becomes mineralized, allowing the invasion of blood vessels, and is finally replaced by bone. This last stage of chondrogenesis is called 'terminal chondrocyte differentiation.' Terminal chondrocyte differentiation is accompanied by the differentiation of perichondrial cells into osteoblasts, followed by the deposition of a calcified bone matrix surrounding the zone of the hypertrophic chondrocytes, the so-called "bone collar." These sequential processes are coordinately controlled by several signaling molecules.

Transforming growth factor β (Tgfb) signaling regulates diverse cellular processes, including cell differentiation, proliferation, chemotaxis, and extracellular matrix production. Tgfb signals through the heteromeric serine/threonine kinases comprising its type I and type II receptors (Tgfbr1 and 2). Tgfb signaling is initiated when a ligand binds to the type II receptor on the cell surface. In human endochondral ossification, Tgfb2 is expressed in all zones of the growth plate, with the highest expression in the hypertrophic and mineralizing zones (Janssens et al., 2005). Tgfb3 is expressed in the chondrocytes of the proliferative and hypertrophic zones (Villiger and Lotz, 1992). Tgfb1 is found mainly in the proliferative and upper hypertrophic zones (Janssens et al., 2005), whereas Tgfbr1 and Tgfbr2 are expressed intensely in the hypertrophic and mineralizing zones (Grimaud et al., 2002). In previous studies, the targeted germline deletion of the *Tgfb2* gene in mice resulted in perinatal lethality (Sanford et al., 1997) and the mice presented with several skeletal defects, including cleft palate, skull ossification defects, shortened long bones, bifurcation of the sternum, and spina bifida occulta. Although more than half of the *Tgfb1*-null mice died early *in utero*, the mice that were born displayed no dysmorphic phenotype (Shull et al., 1992), whereas the *Tgfb3*-null mice had cleft palates (Kaartinen et al., 1995). This variability in the skeletal phenotypes in *Tgfb*-null mice may result from differential and overlapping expression patterns of the isoforms throughout endochondral ossification, so the other isoforms may exert compensatory effects when one of them is deleted.

Tgfbr2 is the only Tgfb receptor that is capable of binding all of the Tgfb isoforms and eliciting functional signaling. Therefore, its ablation allows the study of Tgfb signaling while avoiding the functional redundancy of the ligands. Unfortunately, the early embryonic lethality of *Tgfbr2*-null mice hinders the ability to clarify the role of Tgfb signaling in endochondral ossification (Oshima et al., 1996). The conditional inactivation of *Tgfbr2* in undifferentiated limb bud mesenchyme under the control of the Paired-related homeobox gene (Prx)-1 promoter reduced chondrocyte proliferation, accelerated

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the transition from prehypertrophic to hypertrophic cells, and delayed late hypertrophic differentiation (Seo and Serra, 2007; Spagnoli et al., 2007). The conditional inactivation of *Tgfbr2* in differentiated chondrocytes under the control of the *Col2a1* promoter caused bone malformation that was confined to the spine and the parts of the skull that developed through endochondral ossification (Baffi et al., 2004). Furthermore, mice with a dominant-negative mutant *Tgfbr2* driven by a metallothionein-like promoter displayed increased terminal chondrocyte differentiation and osteoarthritis in adulthood (Serra et al., 1997). However, the function of Tgfb signaling in hypertrophic conversion and terminal chondrocyte differentiation *in vivo* remains unclear. We recently generated *Col10a1-Cre* knock-in mice, in which the *Cre* recombinase gene was inserted into the 3′ untranslated region of the endogenous *Col10a1* gene (Kim et al., 2011). In this study, we conditionally inactivated the *Tgfbr2* gene in hypertrophic chondrocytes using *Col10a1-Cre* mice.

2. Results

2.1. Deletion of Tgfbr2 by Col10a1-Cre

To investigate the physiological role of Tgfb signaling in hypertrophic chondrocytes, we inactivated the *Tgfbr2* gene using *Col10a1-Cre* mice. The conditional *Tgfbr2*-null mutants resulting from expression of *Col10a1-Cre* (*Tgfbr2*$^{flox/flox}$; *Col10a1-Cre*), which were recovered in the expected Mendelian frequency, were viable and fertile, and exhibited a normal gross appearance compared with their littermates (Fig. 1A). When skeletal preparations from newborn mutant mice were stained with Alcian Blue and Alizarin Red, they showed no obvious abnormal skeletal phenotype (Fig. 1B and C). Immunohistochemistry indicated that Tgfbr2 protein was expressed in both proliferating and hypertrophic chondrocytes as well as in the bone collar and perichondrium. In contrast, Tgfbr2 protein was absent from the hypertrophic chondrocytes of the mutant embryos, indicating the complete inactivation of Tgfb signaling in the hypertrophic chondrocytes (Fig. 1D).

2.2. Hypertrophic conversion and terminal chondrocyte differentiation were delayed in mutant embryos

Histological analysis showed no difference between the mutant (*Tgfbr2*$^{flox/flox}$; *Col10a1-Cre*) and control (*Tgfbr2*$^{flox/flox}$) embryos until E13.5 (data not shown). In the humerus of the control embryos, the chondrocytes matured and hypertrophied in the center of the cartilage anlagen at E14.5 (Fig. 2), whereas chondrocyte hypertrophy was clearly delayed in the mutant E14.5 embryos. *In situ* hybridization showed that, in contrast to the control embryos, most cells in the center of the mutant cartilage anlagen still expressed *Col2a1*, a marker of proliferating chondrocytes, and the expression domain of *Col10a1*, a marker of hypertrophic chondrocytes, was smaller in the mutant embryos. These results indicate that the hypertrophic conversion of the proliferating chondrocytes was delayed in the mutant embryos.

2.3. Formation of the bone collar and primary ossification center was delayed in the mutant embryos

In the control embryos, bone collar formation in the perichondrium was initially detected at E15.5, and the primary ossification center emerged later. As shown in Fig. 3, the widths of the primary ossification center and the alkaline phosphatase (ALP)-stained periosteum were markedly narrower in the mutant embryos. The mineral

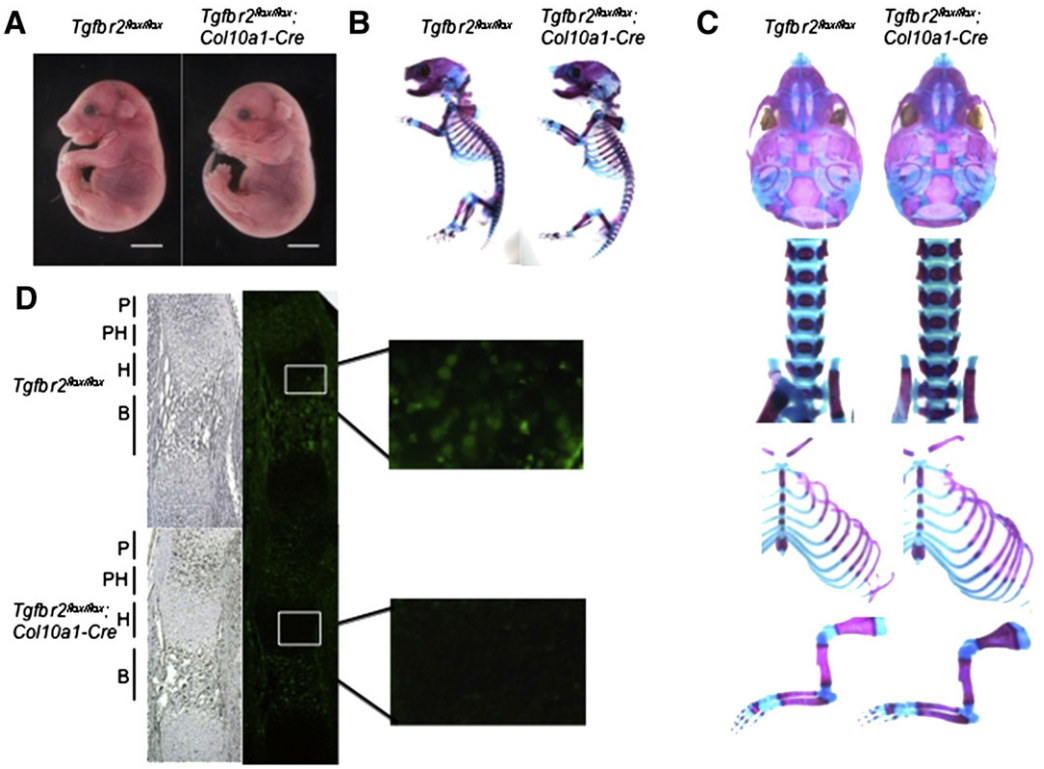

Fig. 1. Analysis of skeletal phenotypes in *Tgfbr2*$^{flox/flox}$*;Col10a1-Cre* mice. (A) Gross appearance of *Tgfbr2*$^{flox/flox}$ and *Tgfbr2*$^{flox/flox}$*;Col10a1-Cre* mice at E16.5. Bars denote 3 mm. (B) Skeletal preparation at P0 stained with Alcian Blue and Alizarin Red. (C) Detailed skeletal morphology at P0. Skull base, lumber spine, ribs, and upper extremity. (D) Immunohistochemistry analysis of Tgfbr2 protein in upper humerus at E15.5. The boxed areas of hypertrophic cartilages are enlarged. The expression of Tgfbr2 in the *Tgfbr2*$^{flox/flox}$*;Col10a1-Cre* mice is ablated. P: proliferating chondrocytes; PH: prehypertrophic chondrocytes; H: hypertrophic chondrocytes; B: bone.