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Critical roles of the TGF-β type I receptor ALK5 in perichondrial formation and function, cartilage integrity, and osteoblast differentiation during growth plate development

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ABSTRACT

TGF-β has been implicated in the proliferation and differentiation of chondrocytes and osteoblasts. However, the *in vivo* function of TGF-β in skeletal development is unclear. In this study, we investigated the role of TGF-β signaling in growth plate development by creating mice with a conditional knockout of the TGF-β type I receptor ALK5 (ALK5^{CKO}) in skeletal progenitor cells using Dermo1-Cre mice. ALK5^{CKO} mice had short and wide long bones, reduced bone collars, and trabecular bones. In ALK5^{CKO} growth plates, chondrocytes proliferated and differentiated, but ectopic cartilaginous tissues protruded into the perichondrium. In normal growth plates, ALK5 protein was strongly expressed in perichondrial progenitor cells for osteoblasts, and in a thin chondrocyte layer located adjacent to the perichondrium in the peripheral cartilage. ALK5^{CKO} growth plates had an abnormally thin perichondrial cell layer and reduced proliferation and differentiation of osteoblasts. These defects in the perichondrium likely caused the short bones and ectopic cartilaginous protrusions. Using tamoxifen-inducible *Cre-ER*™-mediated ALK5-deficient primary calvarial cell cultures, we found that TGF-β signaling promoted osteoprogenitor proliferation, early differentiation, and commitment to the osteoblastic lineage through the selective MAPKs and Smad2/3 pathways. These results demonstrate the important roles of TGF-β signaling in perichondrium formation and differentiation, as well as in growth plate integrity during skeletal development.

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Introduction

Endochondral and intramembranous ossifications are two major processes that control skeletogenesis. In endochondral ossification, precursor mesenchymal cells condense in the areas destined to become bone and differentiate into chondrocytes. Differentiated chondrocytes proliferate and undergo further differentiation processes to mature hypertrophic chondrocytes that subsequently are replaced by bone cells (Kronenberg, 2003). Mesenchymal cells at the periphery of the condensation give rise to the perichondrium, which differentiates into osteoblasts and forms a bone collar. The perichondrium consists of the outer fibrous layer and inner osteoprogenitor cell layer (Kronenberg, 2003). In intramembranous ossification, condensed mesenchymal cells directly differentiate into osteoblasts and form bone (Nakashima and de Crombrugghe, 2003).

Transforming growth factor-β (TGF-β) and its related factors, including bone morphogenetic proteins (BMPs) and activins, regulate diverse cellular processes, such as proliferation, differentiation, apoptosis, and extracellular matrix formation during embryogenesis. TGF-β signaling is mediated by two types of transmembrane serine/threonine kinase receptors, type I (ALK5) and type II receptors, which form a heteromeric complex. In this signaling complex, following TGF-β binding to the type II receptor (TGFβRII), the type II receptor phosphorylates and activates ALK5. Activated ALK5 then induces signaling cascades through Smad-dependent and Smad-independent pathways. In the Smad-dependent pathway, the TGF-β-receptor complex activates Smad2/3, whereas the BMP-receptor complex activates Smad1/5/8 (Feng and Derynck, 2005).

TGF-β signaling has been implicated in cartilage and bone formation in a number of studies. However, this conclusion is controversial, in part because of multiple signaling cascades and redundant expression of three TGF-β isoforms (TGF-β1, -β2 and -β3). Genetic manipulations of TGF-β signaling molecules in mice have clarified some of their roles in skeletogenesis. However, since gene targeting of TGF-β signaling molecules has resulted in variable phenotypes, ranging from early embryonic lethality to normal phenotype at birth, the precise role of TGF-β signaling in skeletal development is not yet fully understood. For example, targeted germline deletions of *Tgfbr2* and *Alk5* result in early embryonic lethality because of defects in hematopoiesis and vasculogenesis

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before skeletal elements are formed (Larsson et al., 2001; Oshima et al., 1996). In contrast, Col2a1-Cre-mediated conditional inactivation of *Tgfbr2* in chondrocytes does not show obvious defects in long bone formation (Baffi et al., 2004), while Prx1-Cre-mediated *Tgfbr2* deletion in the limb mesenchyme results in short limbs and fusion of the joints of the phalanges (Seo and Serra, 2007; Spagnoli et al., 2007). A genetic deletion of *Smad3*, a known substrate for ALK5 and an important mediator of the canonical Smad-dependent pathway, displays normal phenotype at birth (Borton et al., 2001), suggesting that the TGF-β-Smad2/3 signaling may not be required for limb development. On the other hand, mice deficient in TGF-β2 suffer perinatal lethality with abnormal skeletal formation, such as reduced cranial ossification, bifurcation of the sternum, irregular and fused ribs, and shortened limbs (Sanford et al., 1997), suggesting that TGF-β signaling is indispensable for skeletogenesis.

ALK5 is one of the most prominent receptors for TGF-β superfamily members in skeletal tissues. Recent studies suggest that ALK5 may also serve as a receptor for some other TGF-β superfamily proteins, such as myostatin (GDF8) and GDF11(Andersson et al., 2006; Rebbapragada et al., 2003; Tsuchida et al., 2008; Wu et al., 2003). Deficiency of ALK5 should eliminate Smad-dependent and Smad-independent signaling for all TGF-β isoforms and other potential TGF-β superfamily proteins. In the present study, conditional knockout mice have been created in which ALK5 was inactivated in skeletal progenitor cells by Dermo1-Cre expression in mice and tamoxifen-inducible *Cre-ER*™ expression *in vitro*. This allowed us to circumvent the early embryonic lethality observed in a germline of ALK5-null mice (Larsson et al., 2001) in order to investigate the role of ALK5 in skeletogenesis. We demonstrated that ALK5 is expressed in the skeletal primordium and that Dermo1-Cre-mediated *ALK5* conditional knockout ($ALK5^{CKO}$) results in bone growth retardation, defects in perichondrium, and abnormal cartilaginous protrusions. Our studies indicate that ALK5 regulates the commitment of progenitor cells to the osteoblastic lineage, followed by osteoblast proliferation and differentiation through selective downstream pathways.

Materials and methods

Mouse lines

ALK5-floxed ($Alk5^{flox/flox}$) mice and *Dermo1-Cre* knock-in mice were kindly provided by Dr. Stefan Karlsson (Department of Molecular Medicine and Gene Therapy. Institute of Laboratory Medicine, Lund University Hospital, Lund, Sweden) (Larsson et al., 2003) and Dr. David M. Ornitz (Department of Molecular Biology and Pharmacology, Washington University Medical School, St. Louis, MI) (Yu et al., 2003), respectively. Skeletal progenitor-specific ALK5 conditional knockout ($ALK5^{CKO}$ ($Alk5^{flox/flox}$; $Dermo1^{Cre/wt}$) mice were created by crossing $Alk5^{flox/flox}$ homozygous females with $Alk5^{flox/wt}$; $Dermo1^{Cre/wt}$ double heterozygous males. ROSA26 Cre-reporter mice were created by Dr. Philippe Soriano's laboratory (Fred Hutchinson Cancer Research Center, Seattle, Washington) (Soriano, 1999) and obtained from Jackson Labs (Bar Harbor, ME). The ROSA26 promoter confers ubiquitous expression of *LacZ*. Rosa26 mice were crossed with Dermo1-Cre mice to create $Dermo1^{Cre/WT}$; *Rosa26* mice to trace Dermo1 expression. A *Cre-ER*™ mouse line (CAGG-*Cre-ER*™) created by Drs. Hayashi and McMahon (Harvard University, Cambridge, MA) (Hayashi and McMahon, 2002) was obtained from Jackson Labs. In *Cre-ER*™ mice, Cre recombinase is fused to the modified mouse estrogen receptor ER™ under the control of the chicken β-actin promoter and cytomegalovirus (CMV) enhancer, and Cre activity can be induced by tamoxifen. These two lines were crossed and double homozygous (*Rosa26*; *Cre-ER*™) mice generated. The double homozygous mice were crossed with ALK5-floxed mice to generate tamoxifen-inducible ALK5-deficient mice (ALK5-Cre-ER, $Alk5^{flox/flox}$; *Rosa26; Cre-ER*™). CreER-negative $Alk5^{flox/flox}$ and wild type mice were used to prepare control calvarial cells. The animal protocol approved by the NIDCR ACU Committee was used for maintaining and handling mice, and all animals were housed in an American Association for the Accreditation of Laboratory Animal Care-accredited mouse facility.

Reagents and chemicals

TGF-β2 and BMP-2 were obtained from R & D systems (Minneapolis, MN). SB203580, U0126 and SP600125 were purchased from Tocris Bioscience (Ellisville, MO). SIS3 was purchased from EMD Bioscience (La Jolla, CA). The enhanced chemiluminescent (ECL) blotting detection reagents were purchased from Amersham Biosciences Corp. (Piscataway, NJ). Tamoxifen and Oil Red O were purchased from Sigma, and Nile Red from Invitrogen.

Skeletal preparation

Embryos were dissected, fixed in 100% ethanol overnight, and then stained with Alcian blue, followed by Alizarin Red S, according to standard protocols (McLeod, 1980).

Metatarsal explant culture

Metatarsal rudiments were cultured as previously described (Haaijman et al., 1999). Metatarsal rudiments were dissected from embryos at E15.5 and cultured in α-minimum essential medium without nucleosides (Invitrogen) supplemented with 0.05 mg/mL ascorbic acid (Sigma), 0.05 mg/mL gentamycin (Invitrogen), 1 mM β-glycerophosphate (Sigma), and 0.2% FBS in a humidified atmosphere of 5% CO_2 in air at 37 °C. One day after starting the culture, the rudiments were incubated in 400 μL of the same medium containing 10 ng/mL of TGF-β2 (R & D), or without TGF-β2, for an additional 4 days. The explants were cultured with BrdU (bromodeoxyuridine; 10 μM) for 2.5 h at the fourth day of the culture. Stereomicroscopic photographs using Zeiss Stemi and NIH Image J software were used to measure the length of cultured explants that had been processed for histological examinations.

BrdU staining

Pregnant mice bearing E18.5 embryos were intraperitoneally injected with BrdU labeling reagent (10 μL/g body weight; Zymed Laboratories). The mice were euthanized for BrdU staining 2 h later. Metatarsal explants were cultured with BrdU (10 μM) for 2 h at day 5. The explants were fixed in paraformaldehyde at 4 °C overnight, embedded in paraffin, and cut into 5-μm sections. Incorporated BrdU was detected using a BrdU staining kit (Invitrogen).

Isolation of calvarial cells (osteoblast-enriched cells)

Calvaria of newborn ALK5-CreER mice ($Alk5^{flox/flox}$; *Rosa26; Cre-ER*™) and CreER-negative $Alk5^{flox/flox}$ and wild type mice were incubated six times for 10 min (for a total of 1 h) with 0.1% collagenase, type 1 (Worthington, Lakewood, NJ) and 0.2% dispase II (Roche Applied Science, Indianapolis, IN) in phosphate-buffered saline solution (PBS). The last two fractions were centrifuged at 1500 rpm for 5 min, resuspended in culture medium consisting of α-minimum essential medium (Invitrogen, Rockville, MD) with 10% fetal bovine serum (HyClone, Logan, UT), 100 U/mL of penicillin and 100 μg/mL of streptomycin. For cell proliferation assays, cells were seeded at a low density (2500 cells/cm^2). The next day, cells were treated with 1 μM tamoxifen, and allowed to proliferate for an additional 3 days in the presence of TGF-β2 or inhibitors. For differentiation assays, cells were seeded at a higher density (25,000/cm^2). Similarly to the proliferation assay, on the following day cells were treated with tamoxifen and