Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 310 (2007) 304–316
DEVELOPMENTAL BIOLOGY
www.elsevier.com/developmentalbiology

Deletion of *Tgfbr2* in Prx1-cre expressing mesenchyme results in defects in development of the long bones and joints

Hwa-Seon Seo, Rosa Serra *

Department of Cell Biology, University of Alabama at Birmingham, 1918 University Blvd., 660 MCLM, Birmingham, AL 35294-0005, USA

Received for publication 14 November 2006; revised 27 July 2007; accepted 30 July 2007
Available online 9 August 2007

Abstract

In this study, we address the function of Transforming Growth Factor beta (TGF-β) and its type II receptor (Tgfbr2) in limb development in vivo. Mouse embryos were generated in which the *Tgfbr2* gene was deleted in early limb mesenchyme using Prx1Cre-mediated LoxP recombination. A high level of *Tgfbr2* gene deletion was verified in limb mesenchyme by PCR between E9.5 and E10.5 days in Cre expressing mice. RT-PCR assays indicated a significant depletion of Tgfbr2 mRNA by E10.5 days as a result of Cre mediated gene deletion. Furthermore, limb mesenchyme from Cre^{+};$Tgfbr2^{f/f}$ mice placed in micromass culture did not respond to exogenously added TGF-β1 confirming the functional deletion of the receptor. However, there was an unexpected increase in the number and intensity of Alcian blue stained chondrogenic nodules in micromass cultures derived from *Tgfbr2*-deleted limbs relative to cultures from control limbs suggesting that Tgfbr2 normally limits chondrogenesis in vitro. In vivo, early limb development and chondrocyte differentiation occurred normally in Tgfbr2-depleted mice. Later in development, depletion of Tgfbr2 in limb mesenchyme resulted in short limbs and fusion of the joints in the phalanges. Alteration in the length of the long bones was primarily due to a decrease in chondrocyte proliferation after E13.5 days. In addition, the transition from prehypertrophic to hypertrophic cells was accelerated while there was a delay in late hypertrophic differentiation leading to a reduction in the length of the marrow cavity. In the joint, cartilage cells replaced interzone cells during development. Analysis of markers for joint development indicated that the joint was specified properly and that the interzone cells were initially formed but not maintained. The results suggest that Tgfbr2 is required for normal development of the skeleton and that Tgfbr2 can act to limit chondrogenesis in mesenchymal cells like the interzone.

Keywords: Micromass culture; Tgfbr2; Chondrocyte; Synovial joint; Sternum; Skull

Introduction

Transforming Growth Factor β (TGF-β) signaling regulates diverse cellular processes, such as cell growth, differentiation, proliferation, and formation of extracellular matrix during embryonic development (Dunker and Krieglstein, 2000; Mummery, 2001; Serra and Chang, 2003; Verrecchia and Mauviel, 2002). Members of the TGF-β superfamily include TGF-β isoforms (TGF-β1, 2, 3), the Activins and Inhibins, Bone Morphogenetic Protein (BMP), and Growth and Differentiation Factors (GDF). Members of the TGF-β superfamily signal through heteromeric serine threonine kinases composed of Type I and Type II receptors. TGF-β signaling is initiated when ligand binds to the type II receptor (Tgfbr2) on the cell surface (Massague, 1998). Ligand binding recruits the type I receptor (Tgfbr1) to form a heterotetrameric complex of two type I and two type II receptors. Tgfbr2 is a constitutively active serine/threonine kinase. The formation of the complex enables Tgfbr2 to phosphorylate the GS domain of Tgfbr1, activating its serine/threonine kinase. Smad proteins are downstream targets of the type I receptor kinase. Activated Smad proteins translocate into nucleus to induce cellular responses by acting as transcription factors (Feng and Derynck, 2005; Massague, 1998).

Endochondral bone is formed by a series of consecutive and strictly regulated differentiation steps (de Crombrugghe et al., 2000; Goldring et al., 2006; Lefebvre and Smits, 2005). Chondrogenesis is the earliest step for endochondral bone formation. It begins with the condensation of undifferentiated

* Corresponding author. Fax: +1 205 975 5648.
E-mail address: rserra@uab.edu (R. Serra).

0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.07.040

mesenchyme, which is the result of an increase in cell density. During this process, undifferentiated progenitor cells differentiate into chondrocytes expressing type II collagen as well as cartilage specific proteoglycans. These two cellular processes, in fact, occur almost at the same time and result in the formation of a cartilage template for future bone formation. Differentiated chondrocytes then undergo maturation and hypertrophic differentiation. Eventually, the cartilage template is replaced by bone. One of the most well characterized properties of cartilage is the elaboration of a specific extracellular matrix (ECM) that can stain with peanut agglutinin (PNA) and Alcian blue (Bagnall and Sanders, 1989; DeLise and Tuan, 2002; Stringa and Tuan, 1996).

Previously it was shown that BMPs are critical for early stages of endochondral bone formation. The majority of skeletal elements were absent in mice deficient in both BMP type I receptors, *Bmpr1a* and *Bmpr1b* (Yoon et al., 2005). It was determined that BMP is required for chondrocyte proliferation, survival, and differentiation. Based on gain-of-function studies in cell culture models, it has been suggested that TGF-β triggers chondrogenesis and that BMP maintains and promotes the differentiated phenotype (Carrington and Reddi, 1990; Chimal-Monroy and Diaz de Leon, 1997; Kulyk et al., 1989; Leonard et al., 1991; Macias et al., 1999; Roark and Greer, 1994; Verrecchia and Mauviel, 2002). The conclusions were based on the observation that treatment with TGF-β resulted in increased Alcian blue staining and expression of type II collagen even though the cells did not obtain chondrocyte morphology, meaning treatment with TGF-β resulted in a continuous sheet of Alcian blue staining and the cells remained spindle shaped (Chimal-Monroy and Diaz de Leon, 1997). In contrast, treatment with BMP or GDF5 resulted in the formation of discreet Alcian blue stained nodules containing cells with typical round chondrocyte morphology (Denker et al., 1999; Hatakeyama et al., 2004; Kulyk et al., 1989). These results led us to investigate the function of TGF-β in limb development using a loss of function model. For these experiments, the *Tgfbr2* gene was deleted in mouse limb mesenchyme using Prx1-cre mediated recombination. The expression pattern of Cre recombinase in Prx1-cre mice was previously shown using the Z/AP reporter mouse line in which the histochemical marker human placental alkaline phosphatase is transcriptionally activated following Cre-mediated recombination (Logan et al., 2002). At 9.5 days of gestation, the first expression of alkaline phosphatase reporter was shown in the forelimb mesenchyme. By E10.5 days, transgenic embryos showed complete recombination of the reporter in both forelimb and hindlimb mesenchyme. Cre recombinase activity was not detected in the apical ectodermal ridge (AER) or any of the limb ectoderm. Activity was also detected in cranial mesoderm as well as ventral mesoderm in the trunk. Here, we show maximal deletion of *Tgfbr2* exon 2 DNA in limb mesenchyme from $Prx1Cre^{+}$;$Tgfbr2^{f/f}$ mice between E9.5 and E10.5 days. Furthermore, cells from *Tgfbr2*-deleted limbs did not respond to TGF-β1 indicating functional deletion of the receptor. Nevertheless, early limb development occurred normally. That is, the mesenchyme condensed in the correct pattern and Sox9 protein was expressed normally. However, by E15.5 days, defects in the development of the limbs were apparent. The limbs were shorter than controls, the deltoid tuberosity was missing, and the joints of the phalanges were fused. Reduced limb length was shown to be primarily the result of reduced chondrocyte proliferation after E13.5 days. In the developing joint, cartilage replaced the interzone cells leading to joint fusion. Marker analysis indicated that the interzone was initially formed but was not maintained. Since interzone cells were replaced with cartilage and mesenchyme from Tgfbr2-depleted limbs grown in micromass culture formed more discreet Acian blue stained chondrogenic nodules than Tgfbr2-containing controls, we propose that Tgfbr2 normally limits chondrogenic differentiation in mesenchymal cells including the interzone. Together the results suggest that Tgfb2 is required for normal skeletal development.

Materials and methods

Mouse crosses

Mice in which exon2 of *Tgfbr2* was flanked with loxP sites ($Tgfbr2^{f/f}$) were obtained from Dr. H.L Moses, Vanderbilt University, Nasshville, TN (Chytil et al., 2002). $Tgfbr2^{f/f}$ mice were mated to transgenic mice that express Cre under the control of the Prx1 promoter (obtained from Dr. Clifford J. Tabin, Harvard Medical School, Boston, Massachusetts; Logan et al., 2002) to create a mouse in which *Tgfbr2* is deleted in early limb mesenchyme. The genotype of adult transgenic mice was determined by PCR analysis of genomic DNA isolated from tail biopsies. Embryos were genotyped using DNA isolated from one of the limb buds by proteinase K digestion. PCR for the Cre transgene was performed using two different primer sets: Cre5V (TGC TCT GTC CGT TTG CCG), Cre3V (ACT GTG TCC AGA CCA GGC), Prx1CreF (AGG AGG TAG GAG ATT GTG ATG GAG), and Prx1 CreR (ACC GGC AAA CGG ACA GAA GCA TTT). Genomic DNA was amplified for 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 60 s, and elongation for 90 s at 72 °C in reaction buffer containing 2.5 mM $MgCl_2$, 1× PCR buffer (HotMaster Taq buffer, Eppendorf), 0.2 mM dNTPs (Pharmacia, Uppsala, Sweden), 0.2 μM each primer. The loxP allele and deletion of the target gene were identified using the following primers (Fig. 5A): 8w-a (TAA ACA AGG TCC GGA GCC CA) and LA-LoxP (ACT TCT GCA AGA GGT CCC CT). Two bands can be detected with these primers. One band represents the wild-type allele (420 bp); another band represents the loxP allele (540 bp). The presence of both bands indicates a mouse that is heterozygous for the loxP allele. To detect recombination and the subsequent loss of the floxed allele (*Tgfbr2*), CldelR (AGA GTG AAG CCG TGG TAG GTG AGC TTG) was added to reactions as a third primer. Successful recombination was confirmed by the presence of a 610-bp band (Baffi et al., 2004).

RT PCR

mRNA was extracted from limbs of E9.5, 10.5, 11.5, and 12.5 days mice using the standard Trizol method. Cells were lysed with Trizol and RNA was precipitated in ethanol. RNA concentration was determined by UV spectrophotometry. For RT-PCR analysis, cDNA was synthesized from 1 μg of total mRNA using random primers. PCR for tgfb1, 2, and 3, Tgfbr2, and 18S was performed using the following primers: tgfb1 primers (forward 5′-GCT AAT GGT GGA CCG CAA CAA C-3′ and reverse 5′-CAC TGC TTC CCG AAT GTC TGA C-3′), Tgfb2 primers (forward 5′-CTT CAC CAC AAA GAC AGG AAC CTG G-3′ and reverse 5′-CCT GCT AAT GTT GTT GCC CTC CTA C-3′), tgfbr2 exon2 primers (forward 5′-TTA ACA GTG ATG TCA TGG CCA GCG-3′ and reverse 5′-AGA CTT CAT GCG GCT TCT CAC AGA-3′), tgfbr2 exon2–3 primers (5′-CCA CTT GCG ACA CCG AGA AGT-3′ and reverse 5′-GCA CAC ATG AAG AAA GTC TCG C-3′), Sox9 primers (forward 5′-GAG AAA AGC TAT GGT GAC AGA GC-3′ and reverse 5′-GTC CTC CAT GTT AAC TCT GAA GG-3′), 18S primers (forward 5′-ACG GAA GGG CAC CAC