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BMPR-II is Dispensable for Formation of the Limb Skeleton

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Summary: Initiation of BMP signaling is dependent upon activation of Type I BMP receptor by constitutively active Type II BMP receptor. Three Type II BMP receptors have been identified; Acvr2a and Acvr2b serve as receptors for BMPs and for activin-like ligands whereas BMPR-II functions only as a BMP receptor. As BMP signaling is required for endochondral ossification and loss of either Acvr2a or Acvr2b is not associated with deficits in limb development, we hypothesized that BMPR-II would be essential for BMP signaling during skeletogenesis. We removed BMPR-II from early limb mesoderm by crossing BMPR-II floxed mice with those carrying the Prx1-Cre transgene. Mice lacking limb expression of BMPR-II have normal skeletons that could not be distinguished from control littermates. From these data, we conclude that BMPR-II is not required for endochondral ossification in the limb where loss of BMPR-II may be compensated by BMP utilization of Acvr2a and Acvr2b.

Signaling by members of the bone morphogenetic protein (BMP) family has been shown to be critical for many aspects of limb development and endochondral bone formation. BMPs transduce their signals by bringing together Type I and II serine/threonine kinase receptors to form heterotetrameric complexes. Within this complex, the constitutively active kinase domain of the Type II receptor is key to initiating the BMP signal transduction cascade: its close proximity to the kinase domain of the Type I receptor promotes Type I receptor phosphorylation (Shi and Massague, 2003). Once activated, the Type I receptor is able to phosphorylate and activate BMP-specific R-Smads, increasing their affinity for Smad4. The resulting R-Smad/Smad4 complexes travel to the nucleus where they affect a wide variety of transcriptional responses directly by binding to Smad binding elements, or indirectly through interactions with DNA-binding transcription factors and histone-modifying proteins (Miyazono et al., 2005).

Both too much BMP and too little BMP signaling result in skeletal abnormalities, consistent with the idea that BMP signaling is a highly regulated process. While many investigators have focused on the regulatory roles of BMPs, BMP antagonists, Type I BMP receptors, and BMP-specific Smads (Devlin et al., 2003; Kamiya et al., 2008; Ovchinnikov et al., 2006; Retting et al., 2009; Selever et al., 2004; Voon et al., 2005), few studies have examined the requirement for each of the three Type II BMP receptors present during skeletogenesis. It has been reported that global loss of Acvr2a or Acvr2b, Type II receptors common to both the BMP and activin/myostatin pathways has no obvious effect on formation of the embryonic skeleton (Miyazono et al., 2005; Oh and Li, 1997), allowing us to hypothesize that BMPR-II is the dominant Type II receptor utilized for skeletogenesis. Here we show the surprising result that loss of BMPR-II in the early limb has no effect on skeletal patterning or endochondral ossification.

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RESULTS AND DISCUSSION

Deletion of BMPR-II by Prx1-Cre

As global deletion of BMPR-II in mice results in embryonic lethality during gastrulation (Beppu et al., 2000), we used Cre recombinase driven by a Prx1 enhancer (Logan et al., 2002) to conditionally remove BMPR-II from limb mesenchyme. Prx1-Cre heterozygotes were mated with mice homozygous for a conditional allele of BMPR-II engineered by flanking Exons 4 and 5 (which included the transmembrane domain and a portion of the kinase domain) with lox P sites (Beppu et al., 2005) to generate BMPR-IICKO mice. We confirmed the inactivation of BMPR-II by Prx1-Cre recombination at early limb bud stages using whole mount in situ hybridization. Figure 1a,b demonstrates the efficient removal of BMPR-II transcripts from the limb mesenchyme at E11.5. BMPR-II was still detected in the limb ectoderm where the Prx1-Cre transgene is inactive. BMPR-II deletion from the limb was further verified at the protein level by western blot. As illustrated in Figure 1c, BMPR-II protein in E12 limb bud lysates was significantly reduced in the conditional knockout limbs compared to those of littermate controls.

Loss of BMPR-II Has No Effect on Early Limb Development

At E11.5, BMPR-IICKO, and Prx1-Cre+ control embryos were morphologically similar with no apparent differences in the shape, size, or pattern of the limbs (Fig. 1a,b and data not shown). Detailed histological analysis revealed no alterations in the formation or organization of the developing cartilages at E12.5 in the absence of BMPR-II (Fig. 1d,e). While quantitative polymerase chain reaction (QPCR) analysis of RNA from E12.5 BMPR-IICKO and control limb buds showed no significant changes in early cartilage markers (Sox9 and Col2a1, data not shown). To assess if loss of BMPR-II results in changes in BMP signaling, we collected limb buds from E12 embryos and analyzed them by western blot for alterations in p-Smad1/5/8 or p-Smad2/3 levels. As seen in Figure 1f, we found no changes in either BMP or TGF-β/activin signaling in BMPR-IICKO limbs when compared to control limbs. Since BMP ligands

FIG. 1. Loss of BMPR-II has no effect on early limb development. (a,b) Whole mount in situ hybridization of E11.5 control and BMPR-IICKO limb buds showing deletion of BMPR-II from limb mesenchyme. (c) Western blot analysis showing loss of BMPR-II protein in conditional knockout limbs at E12. β-actin was used as a loading control. (d,e) Alcian blue/Fast red stained sections of forelimb buds of E12.5 control and BMPR-IICKO embryos. (f) Western blot of extracts from control and BMPR-IICKO E12 limb buds (n = 3) using anti-pSmad1/5/8 antibody to evaluate BMP signaling and anti-p-Smad2/3 antibody to evaluate TGF-β signaling. Smad1 and Smad2/3 antibodies were used to detect total R-Smad protein as a control. (g) Nanostring analysis of E12 limb bud mRNA from control and BMPR-IICKO embryos showing no changes in the levels of Acrv2a or Acrv2b. Values are expressed as number of molecules per 200 ng of total RNA.
could utilize Acvr2a and Acvrb to maintain adequate signaling levels, we isolated RNA from E12 limb buds and used the Nanostring nCounter technology (Geiss et al., 2008) to examine changes in transcript levels for these receptors. As shown in Figure 1g, no significant differences were seen in the mRNA levels of Acvr2a or Acvr2b in BMPR-II\textsuperscript{CKO} limbs. From these data we conclude that Acvr2a and Acvr2b present in the early limb most likely compensate for loss of BMPR-II and are able to provide the level of BMP signaling necessary for early skeletogenesis. In addition, these data suggest that BMPR-II is not uniquely required for the first steps of limb patterning or early cartilage formation.

Deletion of BMPR-II Results in No Significant Changes in the Formation of Long Bones

Next we examined BMPR-II\textsuperscript{CKO} mice at later stages of skeletal development to see if BMPR-II was required for the subsequent steps of endochondral ossification. Alizarin Red and Alcian Blue-stained skeletons from E15.5 embryos showed no obvious changes in the shape, size, pattern, or formation of the long bones lacking BMPR-II (Fig. 2a). These observations were confirmed by histological analysis of hindlimbs from E16 BMPR-II\textsuperscript{CKO} and control embryos that showed the absence of BMPR-II did not change the timing or extent of cartilage or bone formation or impact joint morphogenesis (Fig. 2b,c and data not shown). As endothelial or pulmonary artery smooth muscle cells with BMPR-II mutations exhibit increased cell death and failed growth suppression (Morrell, 2006), we checked for similar alterations in cell proliferation or apoptosis in BMPR-II\textsuperscript{CKO} femurs but saw no differences between conditional knockout and control mice (data not shown). We performed immunohistochemistry for p-Smad1/5/8 on humeri from E16 BMPR-II\textsuperscript{CKO} and saw no changes in BMP signaling in chondrocytes, perichondrial, perisoteal, or bone cells (Fig. 2d,e). Finally, we examined BMPR-II conditional knockout mice at birth to look for any defects or fractures in the long bone. Alizarin Red and Alcian Blue stained skeletal preparation of forelimbs and hindlimbs from BMPR-II\textsuperscript{CKO} newborns (NB) showed no significant alterations in bone formation (Fig. 3a,b), and detailed histological analysis of newborn BMPR-II\textsuperscript{CKO} long bones confirmed the lack of significant changes in growth plate, trabecular bone or cortical bone in the absence of BMPR-II (Fig. 3c,h and data not shown). We verified these findings by performing in situ hybridization for chondrocyte and bone markers on humeri from NB BMPR-II\textsuperscript{CKO} mice and saw no alterations in the levels or localization of Col2a1, Col10a1, or Osteopontin (Fig. 3d-f,i-k). These data were further confirmed by using QPCR analysis of RNA from NB BMPR-II\textsuperscript{CKO} femurs (Fig. 3m). In addition, Von Kossa staining of femurs from NB mice revealed no changes in bone mineralization with loss of BMPR-II (Fig. 3g,l). Taken together, these data confirm our initial observation that loss of BMPR-II from the early limb does not impair the level of BMP signaling necessary for endochondral ossification.

Loss of Individual Type II Receptors Does Not Have Significant Effects on Endochondral Bone Formation

To confirm the finding that global loss of either of the Type II BMP receptors, Acvr2a or Acvr2b has little effect on the limb skeleton (Matzuk et al., 1995; Oh and Li, 1997), we collected femurs from Acvr2a and Acvr2b knockout mice at birth and performed a detailed histological analysis. We found, in agreement with previous studies, that loss of either Acvr2a or Acvr2b did not have a significant effect on bone or joint formation when compared to wild type controls (see Fig. 4). Taken as a whole the results obtained by analyzing the limbs of mice lacking each individual Type II BMP receptor is in keeping with the idea that these Type II receptors can compensate for one another during limb skeletogenesis and endochondral bone formation.

Our data, although surprising, are in agreement with two previous studies on the function of BMPR-II in skel-
Mice homozygous for a hypomorphic allele of BMPR-II die at mid-gestation from cardiovascular abnormalities but display only a mild axial skeletal phenotype of delayed ossification of cervical vertebrae and interparietal bone and loss of the 13th pair of ribs (Delot et al., 2003). Transgenic mice engineered to express a C-terminal truncated form of BMPR-II in osteoblast lineage cells also only exhibited a mild bone phenotype of delayed mineralization of the calvaria and vertebrae at birth (Yang et al., 2010). Taken as a whole, these data allow us to conclude that BMPR-II is not necessary for endochondral ossification and the requirement for BMP signaling may be compensated for by BMPs acting through Acvr2a and Acvr2b.

**METHODS**

**Mouse Strains**

Mice in which Exons 4 and 5 of BMPR-II was flanked with lox P sites (BMPR-II<sup>f/f</sup>) were obtained from Dr. Hideyuki Beppu, Massachusetts General Hospital, Boston, MA. The creation and genotyping of the conditional floxed BMPR-II allele has been described previously (Beppu et al., 2005). The Prx1-cre allele has also been described previously (Logan et al., 2002). BMPR-II<sup>f/f</sup> mice were mated to transgenic mice that express Cre under the control of the Prx1 promoter to create mice in which BMPR-II is deleted in the early limb mesenchyme (BMPR-II<sup>cko</sup>.

Acvr2a knockout mice were kindly provided by Dr. Martin Matzuk (Baylor College of Medicine, Houston, TX) and described previously (Matzuk et al., 1995). Acvr2b mice were kindly provided by Dr. Paul Oh (University of Florida College of Medicine, Gainesville, FL) and described previously (Oh and Li, 1997). All mouse strains used in the study were on a C57BL/6 × 129/SvEv mixed genetic background.

**Whole Mount and Section In Situ Hybridization**

Whole mount in situ hybridization was performed with digoxigenin-labeled antisense mouse BMPR-II probe (Beppu et al., 2000) as previously described (Brent et al., 2005). Section in situ hybridization with radiolabeled probes was performed as described (Bandopadhyay et al., 2006). 5'S-labeled complementary RNA probes were transcribed from plasmids encoding...
Col2a1 (Kohno et al., 1984), Col10a1 (Jacenko et al., 1993), and osteopontin (Oldberg et al., 1986).

**Skeletal Preparations**

For whole skeletal analysis, staged embryos (the day the vaginal plug was observed was considered embryonic day 0.5 - E0.5) and newborn mice were prepared and stained with Alizarin Red and Alcian Blue to identify mineralized bone and cartilage as previously described (McLeod, 1980).

**Histological and Immunohistochemical Analysis**

For histology, limbs were dissected and fixed in 4% paraformaldehyde, decalcified in EDTA/PVP when necessary, paraffin embedded, and sectioned. Slides were stained with Alcian Blue/Fast Red, Toluidine Blue and Von Kossa using standard methods. For immunofluorescence, sections were microwaved in 10 mM citrate buffer pH 6.0 for antigen retrieval, blocked with 0.5% bovine serum albumin (BSA) and incubated with phospho-Smad1/5/8 (Cell Signaling Technology) overnight at 4°C. The slides were then washed and incubated overnight at 4°C with Alexa-Fluor-555-conjugated rabbit secondary antibody (Invitrogen). Apoptosis was evaluated using the In Situ Cell Death Detection kit, POD (Roche). Cell proliferation was analyzed by proliferation cell nuclear antigen (PCNA) staining using a PCNA staining kit (Zymed). The PCNA positive or TUNEL positive cells were individually counted from at least four sections of comparable regions of the distal femur growth plate of three control and three BMPR-II\(^{CrKO}\) littermate pairs at E16.

**Western Blots**

Western blots were performed on lysates from limb buds excised from E12.5 control and BMPR-II\(^{CrKO}\) embryos. Lysates were resolved on Novex Tris Glycerine gels (Invitrogen) and transferred to Amersham Hybond ECL nitrocellulose membranes (GE Healthcare). After blocking in 10% milk, the following primary antibodies were applied overnight at 4°C: anti-C-terminal phospho-Smad1/5/8 (Cell Signaling), anti-C-terminal phospho-Smad2/3 (Cell Signaling), and anti-Smad1 (Cell Signaling) rabbit polyclonal antibodies; anti-BMPR-II (BD Biosciences), anti-Smad2/3 (BD Biosciences), and anti-β-actin (Sigma) mouse monoclonal antibodies. Primary antibodies were applied in 5% milk, except for the detection of phospho-specific isoforms which were applied in 5% BSA. Appropriate species-specific goat polyclonal secondary antibodies (anti-mouse: Kirkegaard & Perry Laboratories and anti-rabbit: Cell Signaling) were utilized. Immunoblots were developed by chemiluminescence using Western Lightning (Perkin Elmer) and exposed to Amersham Hyperfilm ECL (GE Healthcare).

**Quantitative PCR**

Total RNA was extracted from E12 embryonic limb buds and from femurs of newborn mice using Trizol reagents (Invitrogen) according to the manufacturer's protocol. RNA from E12 limb buds was analyzed using Nanostring nCounter, a novel technology able to measure directly the number of specific mRNA molecules without PCR amplification (Geiss et al., 2008). To use this ultra-sensitive technology, a CodeSet containing specific probe pairs (reporter and capture probes) for 50 genes was customized. This CodeSet was incubated at 65°C for 19 h with 200 ng of RNA. Immediately after incubation, the samples were washed and the purified target/probe complexes were immobilized in a cartridge for data collection. The results are expressed in number of molecules per 200 ng of total RNA. For Quantitative RTPCR (QPCR), cDNA was transcribed from 1 μg of total RNA from NB femurs with random primer using the Transcriptor First Strand cDNA Synthesis Kit (Roche). QPCR was performed by using the...
Roche LightCycler 480 Real-time PCR system with probe-based detection (Universal Probe Library; Roche). Values were normalized to β-actin using the 2-ΔΔCt method (Livak and Schmittgen, 2001). Experiments were performed in triplicate.

LITERATURE CITED


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