**INTRODUCTION**

Bone morphogenetic proteins (BMPs) constitute the largest group of the transforming growth factor β (TGFβ) superfamily of growth factors, and are involved in a wide variety of inductive processes during embryonic development, as well as in maintenance of adult tissues (reviewed by Hogan, 1996). The large number of BMPs, their overlapping patterns of expression in many tissues, and the relatively mild phenotypes of several mouse models deficient for widely expressed BMPs, raise the issue of functional redundancy (Lyons et al., 1995; Storm and Kingsley, 1996; Solloway et al., 1998). As few receptors have been identified in proportion to the number of BMP ligands, a major question has been to understand how specificity of BMP action is achieved. One aspect of specificity is thought to be the differential affinities of the ligands for one of three type I receptors: ActRI (Acvr1), Bmpr1a and Bmpr1b (ten Dijke et al., 1994; Chen et al., 1998; Macías-Silva et al., 1998). So far only two type II receptors that can transduce BMP signals in vitro have been identified, Bmpr2 (Liu et al., 1995; Nohno et al., 1995; Rosenzweig et al., 1995) and ActRII (Acvr2), originally identified as a receptor for activins (Yamashita et al., 1995; Piek et al., 1999). The majority of Actvr2−/− mice die at birth due to palatal defects, but 20% live to be infertile adults (Matzuk et al., 1995a). The extent to which these phenotypes reflect impaired signaling through the activin versus BMP pathways is not known. Bmpr2 null mouse mutants die in utero at gastrulation (Beppu et al., 2000), demonstrating that Bmpr2 is required for primitive streak formation. The early embryonic lethality associated with loss
of Bmpr2 function precludes an analysis of the role of this receptor at later stages of development. Therefore, the generation of an allelic series consisting of impaired function alleles is needed in order to identify morphogenetic events that are sensitive to reduced levels of BMP signaling at later stages.

The potential importance of gene dosage in BMP signaling is highlighted by the recent finding that, in humans, mutations in the Bmpr2 gene, thought to lead to haploinsufficiency, cause primary pulmonary hypertension (PPH), a vascular disease that is caused by increased proliferation of endothelial and smooth muscle cells in the pulmonary arteries (Deng et al., 2000; Lane et al., 2000). We report the generation of mice carrying a modified allele of Bmpr2, Bmpr2<sup>AE2</sup>, which encodes a protein that lacks half of the extracellular ligand-binding domain. Mice homozygous for this mutant allele display a mild skeletal phenotype, which includes posterior transformation of the last thoracic vertebra, consistent with a reduction of BMP signaling in these mutants. The mutants die before birth with cardiovascular defects. As opposed to most mouse models of congenital cardiac defects, Bmpr2<sup>AE2</sup> mutants have a fully penetrant, very restricted phenotype, which is limited to the outflow tract. The defect associates an absence of septation of the outflow tract of the heart with interruption of the aortic arch, a condition known in humans as persistent truncus arteriosus type A4 (Jacobs, 2000). In addition, the semilunar valves, which prevent backflow from the aorta and pulmonary trunk into the ventricles, do not form in mutants. The signaling pathways responsible for the differentiation of the conotruncal ridges into both the conotruncal septum and the semilunar valves are very poorly understood. We show that an intact BMP signaling pathway is required for maintenance of the conotruncal ridges and formation of the semilunar valves.

**MATERIALS AND METHODS**

**Generation of Bmpr2<sup>AE2</sup> mice**

Bmpr2<sup>+</sup> clones were isolated from a 129/SvJ mouse genomic DNA BAC library (Incyte). A 13 kb BamHI fragment containing exons 2 and 3 subcloned into pBluescript II KS (Stratagene) was used to build the targeting construct. The construct was generated by replacing a 2.2 kb BglII fragment containing exon 2 with a PGKNeoA cassette. The 5′ flanking region of 6 kb EcoRI/BglII cassette, and the 3′ flanking region of 2.5 kb BglII/AsellII fragment. An MC1-thymidine kinase cassette (MC1tkpA) was placed on the 5′ end of the construct. The construct was linearized by digestion with SalI, and electroporated into RW4 ES cells (Incyte) according to established protocols (Ramirez-Solis et al., 1993). Targeted clones were injected into blastocysts by the UCLA Transgenic Mouse Facility. Chimeric mice were mated with Balb/cJ females, and albino and chinchilla offspring were analyzed by Southern blot hybridization. Chimeras were also bred to C57Bl/6J females. Phenotypic analyses were performed on embryos with mixed Balb/cJ x 129SvJ background. No differences in the cardiac phenotype were noted for the different genetic backgrounds. Bmpr2<sup>AE2</sup> mice were identified using a 600 bp 5′ external probe by Southern blot analysis of BamHI/XhoI-digested DNA prepared by tail biopsy. This strategy distinguishes a 14 kb wild-type allele from the 8 kb targeted allele (not shown). Subsequent genotyping was performed using a ~350 bp internal probe encompassing exon 3 to distinguish between a 5 kb wild-type allele from the ~1 kb targeted allele after digestion of the genomic DNA with EcoRV (Fig. 1B). The targeted allele is designated as Bmpr2<sup>AE2+/−</sup> according to the guidelines of the International Committee on Standardized Genetic Nomenclature for Mouse (The Jackson Laboratory, Bar Harbor, ME) and was abbreviated to Bmpr2<sup>AE2</sup> in this text.

**RT-PCR**

Total RNA was prepared from E12.5 embryos of each genotype using Trizol (Gibco-BRL) according to the manufacturer’s protocol. Reverse transcription was carried out with a gene-specific primer located in exon 5 (E5R, 5′-CGT CAG ACC CTG TTT CCC CGC-3′) with the ProStar kit (Stratagene). A region encompassing exon 2 was amplified by PCR with oligonucleotides E1F (5′-CTT CTT TGC TGG CCC AGG GA-3′) and E3R (5′-TGG TGT TGT GTC AGG GGG TG-3′) or E5R. As a control, a region downstream of the deletion was amplified with primers in exons 3 and 5 (E3F, 5′-GGT CTC ACA TCG TGT AGC CC-3′; E5R as above). Semi-quantitative PCR on cDNA obtained from wild-type, mutant and heterozygous littersmates was performed with oligonucleotides E1F and E5R, by collecting aliquots of each reaction after 12, 18, 22, 25, 28 and 30 cycles, run on gel, imaged, scanned and quantified. No differences in levels of expression of Bmpr2 and Bmpr2<sup>AE2</sup> were observed.

**Generation of Bmpr2 and Bmpr2<sup>AE2</sup> expression constructs and transfections**

cDNA was prepared from the above-described total RNA using Superscript II reverse transcriptase and random hexamers (Gibco-BRL). Full-length coding sequences for Bmpr2 and Bmpr2<sup>AE2</sup> were obtained by PCR using the Bmpr2<sup>+</sup> and Bmpr2<sup>AE2</sup> CDNAs, respectively, as templates, and the following primers: 5′-ctgaattc TTC BglII/AvellI and 3′-CTT CTT TGC TGG CCC AGG GA-3′. An MC1-thymidine kinase cassette (MC1tkpA) was placed on the 5′ flanks of E1<sup>I</sup>, and electroporated into the 5′<sup>I</sup> and 3′<sup>I</sup> fragments. An 13 kb B魅力II fragment containing exons 2 was digested with EcoRI/BglII and subcloned into pBluescript II KS (Stratagene). The subclones were sequenced in both strands to verify that no mutations were introduced. The sequence analysis also verified that exon 2 is absent from the gene product of the mutant allele. The EcoRI/Xbal fragments encoding Bmpr2 and Bmpr2<sup>AE2</sup> were introduced into pcDNA3 (Invitrogen) to generate pcDNA-Bmpr2 and pcDNA-Bmpr2<sup>AE2</sup>. Mv1Lu or P19 cells (ATCC) at 50-70% confluence were transfected, using the Superfect transfection reagent (Qiagen), with 0.5 μg of mss2-Lux (Liu et al., 1994; Duluisi et al., 2001), 0.5 μg of lacZ-encoding control plasmid and 0.05 μg of either pcDNA-Bmpr2 or pcDNA-Bmpr2<sup>AE2</sup> per well. After 3 hours, medium was replaced with MEM + 1% FBS + non essential amino acids. Twenty-four hours post-transfection, recombinant human BMP2 or BMP7 (Genetics Institute) was added where indicated. After an additional 24 hours, cells were lysed with Reporter lysis buffer (Promega) and luciferase activity measured with the Luciferase Assay System (Promega). Results were obtained in triplicate for each experiment and normalized to β-galactosidase activity. The results shown in Fig. 1F are normalized for four and two independent experiments for BMP2 and BMP7, respectively. Statistical significance was assessed using a t-test for correlated samples using the V assar website (http://faculty.vassar.edu/lowry/VassarStats.html).

**Ink injection**

India ink was injected into the ventricles of the hearts of two litters of E12.5 and three litters of E13.5 embryos with custom-made pulled glass pipettes and an Eppendorf micro-injector. Each embryo was genotyped a posteriori with genomic DNA made from its yolk sac. Ink was allowed to circulate, and embryos were fixed into 4% paraformaldehyde (PFA) overnight, dehydrated in an increasing series of methanol and cleared in benzyl benzoate/benzyl alcohol (2:1).

**Cleared skeletal preparation, histology and in situ hybridization**

Cleared skeletal preparations were performed as described (Hogan et
abnormal heart septation in Bmpr2 

D. E2 mutants (al., 1994). Embryos were fixed in 4% PFA and embedded in paraffin wax, sectioned (7 μm) and stained with Eosin/Hematoxylin according to standard techniques. TUNEL analysis was performed on 7 μM paraffin wax-embedded sections with Promega’s Apoptosis Detection System according to the manufacturer’s instructions. Cell proliferation was assessed by immunohistochemistry with antibodies against the Proliferating Cell Nuclear Antigen (PCNA, Zymed). Immunohistochemistry for smooth muscle actin was performed with the monoclonal anti-αSMA clone 1A4 (Sigma). Whole-mount in situ hybridization was performed as described (Hogan et al., 1994), with BM Purple (Boehringer Mannheim) as a substrate for alkaline phosphatase. For in situ hybridization on sections, the embryos were embedded into OCT medium (VWR), and 20 μm cryosections were cut and processed as described (Hogan et al., 1994). The Bmp4 probe was as described (Jones et al., 1991). The Pax3 probe is a gift from Dr P. Gruss. The probe for Ctgf was made from IMAGE clone dbEST #723742. The periostin and Tbx1 probes were cloned by RT-PCR using whole-embryo RNA as described previously (Kruzynska-Frejtag et al., 2001; Garg et al., 2001).

RESULTS

Construction of a hypomorphic allele of Bmpr2

We generated mice lacking exon 2 of the Bmpr2 gene (Fig. 1A, B). The 171 nucleotides of exon 2 code for half of the extracellular ligand-binding domain of Bmpr2 (Beppu et al., 1997). This includes three of seven cysteine residues, conserved among all type II receptors, that are required to maintain the conformation of the extracellular domain, and two of five hydrophobic amino acids thought to participate in the hydrophobic interactions that stabilize the structure (Fig. 1C) (Greenwald et al., 1999; Guimond et al., 1999).

Since the first four exons of Bmpr2 are in frame, we hypothesized that deleting exon 2 would maintain the reading frame and produce a shorter, but correctly translated, protein (Fig. 1D). To verify this, we performed RT-PCR using RNA extracted from wild type, heterozygous and homozygous mutant embryos. For all genotypes, an amplified product was obtained with primers downstream of the mutation (between exons 3 and 5), as well as with primers spanning the deletion, indicating that a stable mRNA is transcribed from the mutant allele (Fig. 1E). Semi-quantitative PCR confirmed that the levels of expression of the wild-type and mutant alleles are not significantly different (not shown). The difference in size

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**Fig. 1.** A functional truncated protein is encoded by the Bmpr2<sup>DE2</sup> allele. (A) Targeting strategy. Exon 2 of Bmpr2 was replaced by a neomycin resistance cassette. A, AarI; B, BamHI; Bg, Bg/II; E, EcoRI; Rv, EcoRV; K, KpnI; X, XbaI; Xh, Xhol. (B) Southern blot showing genotyping of pups with the internal probe. (C) Amino acid sequence comparison of the extracellular domains of mouse ActRII, TGFβRII and Bmpr2. The region encoded by exon 2 is boxed and conserved residues involved in 3D-structure formation are in red. (D) Structure of the Bmpr2 protein. Exon 1 encodes the initiator methionine. Exons 2 and 3 each encode half of the extracellular ligand-binding domain. Exon 4 encodes the transmembrane domain. The serine threonine kinase domain is encoded by exons 5-11. Exons 12-13 encode an intracytoplasmic tail of unknown function. Deletion of exon 2, which is in frame, should generate a truncated protein. (E) RT-PCR on RNA from wild type (+/+), heterozygous (+/-) and ΔE2 mutants (-/-) with primers encompassing the deletion (exons 1-5) and downstream of the deletion (exons 3-5) shows that the Bmpr2<sup>ΔE2</sup> allele is transcribed. (F) Transfection of the mutant or wild-type Bmpr2 cDNA expression plasmid confers BMP responsiveness to Mv1Lu cells. BMP signaling activity was assayed with a reporter construct that contains luciferase under the control of a BMP-responsive element from the promoter of the Msx2 gene (Daluiski et al., 2001; Liu et al., 1994).
between the mutant and wild-type PCR products corresponded to the size of exon 2. Cloning and sequencing of the full-length wild-type and Bmpr2^D/E2^ cDNAs confirmed that exon 2 was deleted and that exons 1 and 3 were in frame in the mutant.

Responsiveness to BMPs of cells transfected with the mutant Bmpr2^D/E2^ cDNA was assayed with a reporter construct that contains luciferase under the control of a BMP-responsive element from the promoter of the Msx2 gene (Liu et al., 1994; Daluiski et al., 2001). Addition of BMP2 to Mv1Lu cells led to a three- to fourfold stimulation of the BMP pathways (Fig. 1F, columns 1 and 2). Transfection with the wild-type receptor increased the level of induction to approx. fivefold (Fig. 1F, columns 3 and 4). However, in cells transfected with the Bmpr2^D/E2^ receptor, a significantly lower induction was observed (columns 5 and 6; P<0.025), showing that BMP signaling is reduced in the presence of the mutant receptor. Similar results were obtained when transfecting P19 cells under the same experimental conditions (data not shown).

BMP7, a ligand of a different subclass, was also able to induce luciferase expression in Mv1Lu cells, albeit with less efficacy (Fig. 1F, columns 1, 9 and 10). Transfection with Bmpr2 did not lead to increased mss2-Lux induction in response to 100 ng/ml BMP7 (Fig. 1F, columns 7 and 9), but a 1.4-fold increase was observed in response to 300 ng/ml BMP7 (Fig. 1F, columns 8 and 10). By contrast, cells transfected with Bmpr2^D/E2^ were unresponsive to BMP7 at either concentration (Fig. 1F, columns 11 and 12).

These results indicate that Bmpr2^D/E2^ has reduced signaling capacity compared with wild type Bmpr2. The stability, ligand binding and signal transduction properties of the mutant receptor that contribute to the defective signaling properties will be described in detail elsewhere. The diminished signaling capacity of cells overexpressing the mutant receptor compared with control cells (Fig. 1F, columns 2, 4 and 6) suggests that either Bmpr2^D/E2^ has dominant-negative properties, or that overexpression of this altered receptor leads to sequestration of BMP ligands and/or type I receptors into impaired signaling complexes. In fact, this apparent dominant negative effect in vitro when overexpressed has been observed for other Bmpr2 mutant receptors which do not have such effects in vivo, and is thought to be an artifact due to interference with intracellular trafficking in transfected cells (Rudarakanchana et al., 2002). The observations that heterozygotes exhibit no apparent defects, and that the phenotype of Bmpr2^D/E2^ mutants (see below) is much less severe than that of mice homozygous for the Bmpr2 null allele (Beppu et al., 2000), strongly argue that Bmpr2^D/E2^ is not a dominant-negative or null allele but retains some signaling capacity and thus is a hypomorphic allele.

Viability of the Bmpr2^D/E2^ mutants
Heterozygous males and females were viable and fertile, with no apparent malformations. Intercrosses of heterozygotes produced no live homozygous mutants, indicating an embryonic lethal phenotype. Up to E11.5, mutants were found in expected Mendelian ratios (39 Bmpr2^D/E2^ out of 154 embryos at E11.5). Up to this stage, mutants are alive and phenotypically indistinguishable from wild-type or heterozygous siblings (not shown). Dissection of litters at later gestational ages showed that, even within the same litter, mutants die at various stages (Fig. 2A-C), with the onset of lethality occurring between E12 and birth. Anomalies of vascularization of the yolk sac, consisting of regions without apparent vessels, were noted upon dissection (not shown). The avascular regions were larger for embryos that had died before E12.5 than for embryos that died at later stages, suggesting that yolk sac vascular anomalies could represent a major contribution to the early lethality of the mutants. This aspect of the phenotype will be reported elsewhere.

Skeletal phenotype of Bmpr2^D/E2^ mutants
BMP signaling pathways are essential for skeletal patterning and growth (Hogan, 1996), and Bmpr2 is thought to be the common receptor for all osteogenic BMPs. Therefore, the observation of a skeletal phenotype is predicted in animals with decreased BMP signaling through Bmpr2^D/E2^.

Whole-mount cleared skeletal preparations of whole litters aged E14.5-E18.5 confirmed that ossification was delayed in mutants compared with their siblings. This was observed in both endochondral and membranous bones. It was particularly severe in the lateral ossification centers of cervical vertebrae C3-C6, the fourth sternebra (not shown), the ventral processes of cervical vertebrae C1-C4 and the interparietal bone (Fig. 2D). Whether this results from a general developmental delay or from a specific defect of osteogenesis is not immediately apparent. Although there was no overt growth

Fig. 2. Skeletal phenotype of Bmpr2^D/E2^ mutants. (A–C) Three siblings from a litter dissected at E15.5 showing that mutants die at various stages of gestation. (A) wild type (WT), (B) mutant dead at E14.5, (C) mutant dead at E12, as judged by the extent of limb development. (D-F) Cleared skeletal preparations of two siblings, recovered dead at birth. Dorsal views of the base of the skulls of WT (D) and mutant (E) illustrate the major ossification delay of the interparietal bone (Ipar), and of the ventral processes of the cervical vertebrae 1-4, in particular of the atlas (At). Socc, supraoccipital bone. (F) Ventral views of the half vertebral columns of wild type (left) and mutant (right) showing the absence of the thirteenth rib in the mutant (asterisk). Thoracic (T1-T13) and lumbar (L1) vertebrae are numbered. Note the ossification delay of the ventral and lateral ossification processes of the vertebrae in the mutant.
defect, a vascular defect could conceivably result in developmental delay of the \( \text{Bmpr2}^{\Delta E2} \) mutants. However, the observation that specific skeletal elements (e.g. atlas) are more severely affected than others argues for a primary defect in skeletogenesis.

Loss of the 13th pair of ribs, associated with a posterior transformation of the 13th thoracic vertebra into a 7th lumbar vertebra, was observed in all the mutants, independent of the genetic background \((n=9\) homozygotes, Fig. 2F). Therefore, in addition to a role in skeletal growth, full activity of Bmpr2 is essential for skeletal patterning along the anteroposterior axis.

**Cardiac septation defects in \( \text{Bmpr2}^{\Delta E2/\Delta E2} \) mutants**

The onset of lethality over several days at late gestation stages suggested a cardiovascular defect. The external aspect of the heart was normal in mutants up to E12 (not shown). At later stages, two normally septated vessels, the aorta and the pulmonary trunk were seen exiting the arterial pole of the heart, but their abnormal relative position was noted (Fig. 3A,B). Closer inspection suggested that although the outflow tract was septated distally into aorta and pulmonary trunk, the region proximal to the heart was not septated. Moreover, a dimple at the apex of the ventricle, a common sign of incomplete ventricular septation, was noted in mutants (Fig. 3B).

To help visualize the anatomy of the outflow tract, we injected ink into the ventricles of embryos. At E13.5, when septation of the ventricle into a left and a right cavity is complete in wild-type embryos, injection of ink into the right ventricle of mutants immediately resulted in leakage of ink into the left ventricle, confirming a ventricular septal defect. The ink injections also documented abnormal septation of the proximal part of the outflow tract (OFT) of the heart, the conotruncus, with a single lumen and no separation between right and left vessels (Fig. 3D).

Histological examination revealed that the ventricular septal defect observed in mutants is not due to a simple developmental delay, because it is observed at all stages of gestation (e.g. Fig. 4D,1-J, I). Histological examination at E14.5 and E16.5 also confirmed that OFT septation is abnormal. Massive absence of the conal cushions (Fig. 4J,K) was visible as early as E12.5, although one of the posterior conal cushions was present (arrowhead in Fig. 4D,K) in mutants.

**Interrupted aortic arch in \( \text{Bmpr2}^{\Delta E2/\Delta E2} \) mutants**

In addition to abnormal septation of the conotruncus and a ventricular septal defect, ink staining also revealed interruption of the aortic arch (Fig. 3E-G), of varying severity, between the roots of the left common carotid and the left subclavian artery (IAA type B), resulting in extensive communication between the pulmonary trunk and the descending aorta, via the ductus arteriosus.

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**Fig. 3.** External aspect of the hearts of \( \text{Bmpr2}^{\Delta E2/\Delta E2} \) mutants. (A,B) Frontal views of wild-type (A) and \( \text{Bmpr2}^{\Delta E2/\Delta E2} \) mutant (B) hearts at E16 showing a normally septated ascending arch of the aorta (Ao) and pulmonary trunk (PT) distally, but not proximally to the heart. The black arrowhead in A indicates the semilunar valves of the PT. The pink arrowhead in B indicates a dimple at the apex of the ventricle, a common sign of incomplete ventricular septation, was noted in mutants (Fig. 3B).

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arteriosus. This regression of the aortic isthmus (which derives from the left aortic arch 4) was also observed histologically (Fig. 4H). The varying severity observed at early stages reflects a progressive regression occurring at these stages of intense remodeling of the aortic arches. The interruption of the aortic arch was complete (and fully penetrant) in animals observed at later gestation stages.

Except for occasional retro-esophageal position of the left subclavian artery (Fig. 4B), the development of neighboring tissues was normal. The pulmonary arteries stemmed normally from the pulmonary trunk (Fig. 3F,G, Fig. 4N). In particular, other tissues affected in neural crest deficiency syndromes were unaffected: thymus (Fig. 4G,H) and thyroid (not shown) were present and no tracheo-esophageal fistula was observed (Fig. 4B).

**The semilunar valves are absent in Bmpr2ΔE2/ΔE2 mutants**

Semilunar valves prevent backflow of the blood from the aortic and pulmonary trunks into the ventricles. They form at the junction of the conotruncus and the aortic sac, hypothetically by remodeling of the top part of the conotruncal ridges. No evidence for the presence of semilunar valves was detected in the outflow tract of ink-injected mutant hearts (Fig. 3C,D). Histology at various gestational stages demonstrated that semilunar valve tissue is completely absent in mutants after E12.5 (Fig. 4A-B,E-H,L). The defect was limited to the outflow tract, and the atrioventricular (future mitral and tricuspid) valves were grossly normal (Fig. 4C).

**Absent septation of the OFT results from impaired growth of the conotruncal ridges**

Two major cell types contribute to the formation of the conotruncal swellings that eventually fuse to septate the outflow tract: the endocardium and the neural crest.

Starting around day E9.5 in the mouse, the resident endocardium, under the influence of inductive signals from the underlying myocardium, undergoes an epithelio-mesenchymal transformation (EMT), which can be followed by histological analysis. At E11.5, the swellings are of maximum thickness in wild-type mice and fill the entire lumen of the outflow tract (Fig. 5A,B). In the mutants, EMT has occurred, and ridges have started to form (Fig. 5B,D), suggesting that intact BMP signaling is not required for induction of the EMT. However,
in mutants, the swellings are much thinner than in wild type, and cell density is lower (see the zones of cell-free cardiac jelly in Fig. 5B,D). No increase of cell death was observed by TUNEL analysis (not shown). However, by E12.5, the swellings are no longer apparent (Fig. 4K), suggesting that BMP signaling is involved in the continued development and/or maintenance of the conotruncal cushion tissue.

Cell proliferation rates in OFT cushions vary widely with the cell type, the stage of differentiation, and the position of the cells within the cushion (Kubalak et al., 2002). Therefore, the very different shapes and sizes of the cushions in mutants compared with wild-type embryos do not allow direct comparison of proliferation rates of each region. However, PCNA staining in myocardial cells was indistinguishable in wild-type and mutant littermates (Fig. 5E-G). Abundant PCNA-positive cells were found both in wild-type and mutant cushions, showing that, despite the reduced size of the cushions at E11.5, mesenchymal cells proliferate extensively in the mutant (Fig. 5E-G). However, as the two sections from the same mutant embryo illustrate (Fig. 5F-G), high variability was found in the extent of cell proliferation in different regions of the mutant OFT cushions, which could reflect abnormal growth regulation of these cells. This high variability in cell proliferation has also been noted within cushions of Bmp6+/−:Bmp7+/− mice (Kim et al., 2001).

Neural crest cells originating from the posterior rhombencephalon migrate through pharyngeal arches 3, 4 and 6, and contribute to the swellings (Kirby, 1999; Jiang et al., 2000). An essential role for cardiac neural crest cells in septation of the mammalian outflow tract has been inferred from studies of various mutant mouse strains (reviewed by Kirby, 1999), but the role these cells play is unknown. The best studied neural crest deletion syndrome with conotruncal abnormalities is the DiGeorge/Velo-Cardio-Facial spectrum. Most individuals with this condition harbor a large deletion of chromosome 22q11, and the phenotype is thought to be caused by the haploinsufficiency of the genes located in this region. Among the deleted genes, TBX1 is the main candidate for the

Fig. 5. Impaired OFT cushion growth in Bmpr2ΔE2ΔE2 mutants. (A-D) Sagittal sections at E11.5 show that the conotruncal ridges (arrow) form in the mutant (B), but not to the extent that they do in the wild type (A). (C,D) Higher magnifications of the boxed areas in A,B, respectively. (E-G) Immunostaining for PCNA in OFT cushions of E11.5 wild type (E) and a mutant (F,G) littermate. The arrowheads indicate the myocardium. (H) Tbx1 expression at E10.5 in a Bmpr2ΔE2ΔE2 mutant is found in the posterior part of the otic vesicle (ov), head mesenchyme (hm) and the mesenchyme surrounding the paired dorsal aortae (pm), as well as the dorsal wall of the aortic sac, with higher intensity in the posterior-most region (arrowheads) as described in wild type (Garg et al., 2001; Merscher et al., 2001; Vitelli et al., 2002). Anterior is towards the left and ventral towards the top. pal, pharyngeal arch I; Ve, ventricle; lb, left anterior limb bud. (LJ) Pax3 expression in neural crest cells (arrowhead) migrating in the neck region at E10.5 was normal in mutants (I, wild type; J, mutant). Pharyngeal arches are numbered in Roman numerals. Ve, ventricle. (K,L) Smooth muscle actin-positive neural crest cells (arrows) reach the outflow tract endocardial ridges in both the wild type (K) and mutant (L) E11.5 embryos. (M,N) At E13.5, in wild type embryos, Ctgf expression was detected in the pulmonary trunk and aorta, predominantly in the cell layers closer to the lumen, as well as in a punctate pattern in the ventricles (M). Myocardial expression is predominantly in the trabecular zone of the ventricles. Expression was not modified in mutants (N). (O,P) The expression of periostin, a BMP-regulated gene, was downregulated in the endocardial ridges of E11.5 mutants (P) compared with wild-type littermates (O). To help visualize the tissues in absence of background expression, P is an overlay of two different light exposures of the same section.
conotruncal defects observed (reviewed by Botta et al., 2001). In the Bmpr2ΔE2 mutants, the levels and sites of expression of Tbx1 were not affected (Fig. 5H). Pax3 is a transcription factor expressed in neural crest cells, including those that are fated to populate the outflow tract (Jiang et al., 2000). Pax3-deficient mice present with generalized neural crest defects including conotruncal abnormalities (Conway et al., 1997). To examine whether the septation defects in mice arise as a consequence of impaired migration of cardiac neural crest, we examined Pax3 expression. At E10.5, a stream of Pax3-expressing neural crest cells is seen migrating toward the OPT (Fig. 5I). These cells are not affected in the Bmpr2ΔE2 mutant (Fig. 5J). Although previously thought to be cells fated to the OPT, they are actually migrating toward the hypoglossal muscle (Epstein et al., 2000). In addition, smooth muscle actin (SMA)-positive cells, which represent at least a subset of the cardiac neural crest (Epstein et al., 2000), were found to reach the outflow tract in mutant as in wild-type embryos (Fig. 5K,L). These results, showing that Tbx1, Pax3 and SMA expression is unaffected, along with the absence of defects in other neural crest-derived tissues argue against a general defect in neural crest migration. However, an effect of BMP signaling on survival and/or differentiation of neural crest cells within the outflow tract is possible.

The expression of genes implicated in EMT was examined to determine whether this process is impaired in mutants. Bmp2 and Bmp4 are expressed in the myocardium underlying the cushion-forming regions and have therefore been considered candidates for an EMT-inductive myocardial signal (Lyons and Hogan, 1993). Bmp4 expression was not modified in the Bmpr2ΔE2 mutants (data not shown). Connective tissue growth factor (Ctgf) is an immediate early gene expressed in response to TGFB treatment (Kothapalli et al., 1997). TGFBs induce EMT in vitro, and are expressed in endocardial cells in regions where cushion formation takes place (reviewed by Nakajima et al., 2000). We found that Ctgf was expressed in the conotruncal ridges at day E11.5 (not shown) and in the outflow tract vessels, above the valve level, predominantly in the endothelium (Fig. 5M). Expression of Ctgf was not affected in Bmpr2ΔE2 mutants (Fig. 5N).

Finally, we tested the expression of periostin, a gene encoding an extracellular matrix protein known to be responsive to BMP signaling in osteoblasts (Ji et al., 2000). Recently, periostin was shown to be expressed at high levels in all cells of the cardiac endocardial cushions (Kruyznska-Frejtag et al., 2001). Periostin expression was greatly diminished in the OPT cushions of Bmpr2ΔE2 mutants (Fig. S5,P), while maintained at high levels in other parts of the embryo, in particular in the ventral mesenchyme (not shown).

**DISCUSSION**

Different tissues are likely sensitive to different levels of activity of the BMP signaling pathway

BMPs have been shown to elicit dose-dependent effects on patterning during gastrulation (Gurdon and Bourillot, 2001). Levels of BMP signaling are controlled through regulation of the relative concentrations of BMP ligands and antagonists such as noggin and chordin. Activation of TGFB or activin pathways can also affect BMP signaling activity in vitro via competition for shared signal transduction components (Piek et al., 1999; Candia et al., 1997). In mammals, the identification of developmental events sensitive to reduced BMP signaling has been hampered by the early embryonic lethality or apparent functional redundancy of many mutants deficient in specific BMP ligands or receptors. One approach that has proven successful in overcoming the problem of early lethality has been the generation of conditional knockouts using Cre/loxP technology. A complementary strategy is the generation of alleles with impaired function. These strategies provide different information; the Cre/loxP approach generally tests whether a specific gene product is required in a specific tissue, whereas the generation of altered alleles reveals tissues that are sensitive to changes in levels of gene activity. In this paper, we tested whether a Bmpr2 receptor with reduced function would reveal tissues whose development is sensitive to reduced levels of BMP signaling.

We found that signaling through BMP pathways is reduced in vitro in two different cell lines, with two different classes of BMP ligand, when transfected with the Bmpr2ΔE2 mutant receptor. However, in vitro elucidation of the precise mechanism(s) by which BMP signaling is decreased in the presence of the mutant receptor is hampered by the unavailability of cell lines that do not express endogenous wild-type receptor. This limitation is exemplified in studies of the effects of mutations in individuals with PPH. In this case, the molecular mechanism that lead to disease is thought to be haploinsufficiency of BMPR2 (Deng et al., 2000; Lane et al., 2000; Thomson et al., 2000; Machado et al., 2001). This conclusion is based on the finding that the altered BMPR2 alleles in many individuals with PPH encode severely truncated, nonfunctional products. Moreover, some individuals with PPH do not have mutations within the BMPR2 coding sequence, but nonetheless express BMPR2 at diminished levels (Atkinson et al., 2002). Although the above findings strongly support haploinsufficiency, an artificial dominant negative effect, similar to the one we observe, is seen in cell transfection experiments using mis-sense and frameshift BMPR2 mutations associated with PPH. This effect is most likely to be due to impaired intracellular trafficking, leading to reduced expression of both the mutant and wild-type Bmpr2 at the cell surface (Machado et al., 2001; Rudarakanchana et al., 2002). In addition to the in vitro data, several in vivo observations strongly argue that Bmpr2ΔE2 is a hypomorphic allele. Bmpr2ΔE2/ΔE2 embryos survive until midgestation, whereas null mutants die at gastrulation (Beppu et al., 2000). The dramatic difference in severity is not a result of strain-specific differences since we examined mice on several genetic backgrounds, including the 129 strain reported by Beppu and colleagues. These results indicate that Bmpr2ΔE2 retains partial activity in vivo.

The skeletal phenotype also brings strong evidence that BMP signaling is decreased in Bmpr2ΔE2 mutants. A general delay in ossification was observed in homozygous mutants, as predicted for a reduced function Bmpr2 allele. No such defects are seen in heterozygotes, arguing against a dominant-negative mode of action. Interestingly, several skeletal elements, such as the supraoccipital bone and ventral process of the atlas, are more affected than others. This result suggests that either different skeletal elements require different levels of BMP signaling for their formation, or that the reduced level of
signaling through the mutant Bmpr2 is compensated in some skeletal elements by another type II receptor.

In addition to defects in bone formation, Bmpr2ΔE2 mutants exhibit defects in vertebral patterning. The loss of the 13th thoracic vertebra, and its replacement with a lumbar vertebra, is observed in Bmpr2ΔE2 mutants as well as in follistatin-deficient mice (Matzuk et al., 1995b). Follistatin is an activin antagonist. Hence, in Bmpr2ΔE2 and follistatin mutants, the level of signaling through activin pathways is expected to be increased relative to the level of BMP signaling. Conversely, GDF11 and ActRIIB are components of activin signaling pathways, and mice deficient in these genes exhibit extra thoracic vertebrae (Oh and Li, 1997; McPherron et al., 1999; Gamer et al., 2001). Along with our results, these findings suggest that regulation of the relative levels of signaling through TGFβ/activin and BMP pathways may play a role in anteroposterior skeletal patterning.

Finally, periostin, a gene that is known to be a downstream target of the BMP pathway in other tissues (Ji et al., 2000), is downregulated in the outflow tract cushions of Bmpr2ΔE2/ΔE2 hearts compared with wild-type and heterozygous littermates. Taken together, the in vitro results, the apparently recessive nature of the Bmpr2ΔE2 mutant phenotype, the skeletal phenotypes, and the downregulation of a known target of the BMP pathway are most consistent with a hypomorphic mode of action for Bmpr2ΔE2.

The lethality of homozygotes for a probable null allele of Bmpr2 around the time of gastrulation (Beppu et al., 2000) is reminiscent of that of null mutants for BMP2 or BMP4 (Winnier et al., 1995; Zhang and Bradley, 1996), suggesting that Bmpr2 is the major receptor for these ligands during gastrulation. The fact that Bmpr2ΔE2/ΔE2 mice undergo normal gastrulation, whereas Bmpr2 null homozygotes do not, shows that the residual activity of Bmpr2ΔE2 is sufficient to transduce the effects of BMP2 and BMP4 in early development. Similarly, the fact that individuals with PPH with haploinsufficiency of Bmpr2 are viable into adulthood has suggested that 50% of normal BMP pathway activity is sufficient for humans to undergo gastrulation (Deng et al., 2000). Therefore, mesoderm formation during gastrulation does not require full BMP signaling activity. By contrast, the phenotype of the Bmpr2ΔE2ΔE2 mutants shows that morphogenesis of the cardiovascular and skeletal systems does.

Bmpr2ΔE2 mutants: a model for type A4 persistent truncus arteriosus

The cardiovascular phenotype of Bmpr2ΔE2 mutants is summarized in the cartoon in Fig. 3H-I. In Bmpr2ΔE2ΔE2 mutants, the distalmost region of the outflow tract is normally septated, suggesting that the wedge of neural crest cells that migrate into the aortic sac to form the aortico-pulmonary septum is, at least in part, present. However, the proximal part of the aortic sac and the conotruncus constitute a single outflow vessel, a condition known in humans as persistent truncus arteriosus (PTA) type A1 (as opposed to type A2 where the total length of the outflow tract is non-septated) (Jacobs, 2000).

The absence of the conal cushion tissue, which during normal embryogenesis fuses with the muscular part of the interventricular septum to complete the separation of the ventricles, results in a ventricular septal defect (VSD). Such a membranous VSD is almost always associated with PTA, where it is a hemodynamic necessity (Jacobs, 2000).

In our mutants, the PTA is associated with regression of the aortic isthmus, a tissue that derives from the left aortic arch 4. Such an association of interrupted aortic arch and PTA is also described entity in humans, known as PTA type A4 (Jacobs, 2000), for which the Bmpr2ΔE2ΔE2 mutants are the first fully penetrant animal model. The form of interrupted aortic arch (Type B), observed in Bmpr2ΔE2 mutants, with resorption between the left common carotid and subclavian arteries, is the same as that commonly observed in the DiGeorge/Velo-Cardio-Facial syndromes. However, DiGeorge syndrome is associated with a variable constellation of defects of the heart and neck region and face, including PTA and thymic hypoplasia, that is thought to result from a widespread defect of neural crest cells (Emanuel et al., 1999), a mechanism that is unlikely to cause the phenotype of Bmpr2ΔE2ΔE2 mutants (see below).

Embryological origin of the outflow tract defect: neural crest cells abnormalities or defective epithelio-mesenchymal transformation of the endocardium?

Distally, the single tube that comprises the outflow tract in early midgestation embryos is septated into the aorta and the pulmonary trunk by a structure that is purely neural crest in origin, the aortico-pulmonary septum. This is the structure that is primarily affected in DiGeorge/Velo-Cardio-Facial syndromes, resulting in type A2 PTA.

Proximally, however, septation occurs by a different, less well understood mechanism that requires at least two processes. The first of these is a transformation of endocardial cells into mesenchymal cells that populate the cardiac jelly. BMP signaling may be directly involved in the inductive interactions between myocardium and endocardium during EMT (reviewed by Nakajima et al., 2000). In vitro, BMP2 is not sufficient to trigger the onset of EMT but it can synergize the inductive effect of TGFβs. Consistent with an essential role in vivo, BMP2 and BMP4 expression in the heart is restricted to the regions of myocardium that underlie the cushion-forming regions (Lyons and Hogan, 1993; Nakajima et al., 2000). In Bmpr2ΔE2ΔE2 mutants, we observe initiation of the EMT, but, in the outflow tract, the cushions fail to progress and never reach their maximum extension. This offers genetic evidence that intact BMP signaling is not necessary for initiation of the EMT, but is required for normal growth and maintenance of the conotruncal ridges.

The second process is an invasion of the conotruncal ridges by neural crest cells that may contribute to the cell population forming the septae, and may also be involved in the proliferation and/or survival of the cells that have undergone the EMT. Several studies have shown that BMPs are required in vivo for formation and/or survival of (non-cardiac) neural crest cells (reviewed by Christiansen et al., 2000; Délot et al., 1999). However, the role of cardiac neural crest cells in mammalian endocardial cushion development is poorly understood, and investigations have long been hampered by the absence of consensus molecular markers for the subpopulation of neural crest cells that populate the outflow tract. In particular, the percentage of cells labeled by various proposed neural crest markers is highly variable, and even for a single
marker the extent of labeling varies in different lines of reporter transgenic mice (Brown et al., 2001).

The analysis of splotch (Pax3<sup>+/−</sup>) mutants, a mouse model for both total neural crest ablation in chicks and DiGeorge syndrome in humans, has suggested that the bulk number of neural crest cells migrating into the heart is a determining factor for OFT septation (Conway et al., 2000). In Bmpr2<sup>2ΔE2</sup> mutants, neural crest migration, as illustrated by Pax3 and smooth muscle actin expression, is not massively affected, consistent with defects that are different than in models of total neural crest ablation. In addition to the restriction of PTA to the conus, the adjacent tissues that develop from branchial arches, such as thyroid and thymus, appear normal in Bmpr2<sup>2ΔE2</sup> mutants. Therefore, the aortic arch remodeling defects and OFT septation defect seen in Bmpr2<sup>2ΔE2</sup> mutants are unlikely to result from widespread neural crest ablation. Moreover, cells expressing SMA are present in the OFT of Bmpr2<sup>2ΔE2</sup> mutants, indicating that at least this subset of cardiac neural crest cells migrates to the OFT. Our results are, however, consistent with a role for BMP signaling in cardiac neural crest cells. For example, BMP signaling could be necessary for cell-cell interactions between the newly formed mesenchymal cells arising as a result of EMT and the incoming neural crest cells. The pattern of expression of Bmpr2 does not offer hints as to which cell type requires intact BMP signaling, as it is ubiquitous throughout the embryo (Roelen et al., 1997) (E. C. D. and K. M. L., unpublished). Fate mapping of the cardiac neural crest (Epstein et al., 2000; Jiang et al., 2000) in Bmpr2<sup>2ΔE2</sup> mutants, as well as tissue-specific targeting of the mutation will therefore be crucial to assess whether subsets of cardiac neural crest cells have different roles in outflow tract septation and valve formation.

**Genetic control of valvulogenesis**

Semilunar valves develop at the distal end of the conotruncal ridges, hypothetically by remodeling (reviewed by Pexieder, 1995). However, in newborn mice and humans with PTA, although the septum that derives from those ridges is absent, differentiated valves are usually present (albeit with an abnormal number of leaflets). This suggests independent genetic control of septation and valve formation.

Defective semilunar valvulogenesis in Bmpr2<sup>2ΔE2</sup> mutants suggests that the duration and/or strength of BMP signals must be tightly controlled. This is highlighted by the observation of semilunar valve defects in mice deficient in other components of the BMP signaling pathway. Mice deficient for Tll1, a mammalian homologue of the Drosophila gene tolloid, which cleaves the BMP antagonist chordin (Scott et al., 1999), have dysplastic semilunar valves (Clark et al., 1999). Mice mutant for Smad6, an inhibitory intracellular mediator of BMP signaling, exhibit hyperplasia of the valves and OFT septation defects (Galvin et al., 2000). The opposing valve phenotypes of Bmpr2<sup>2ΔE2</sup> mutants and Smad6 mutants suggest that Smad6 could be a downstream antagonist of Bmpr2-mediated signaling in the endocardial ridges. More recently, double Bmp6<sup>+/−</sup>;Bmp7<sup>+/−</sup> mutants have been shown to have hypoplastic OFT cushions. However, mechanistic interpretation of this finding was difficult as the mice seem to recover at later stages, and no OFT septation abnormalities were described (Kim et al., 2001). Our results demonstrate that modulation of the levels of BMP signaling is crucial to the development of the semilunar valves, with too much signaling (Smad6 mutants) leading to hyperplasia, and too little (our results, Tll1 and Bmp6<sup>+/−</sup>;Bmp7<sup>+/−</sup>) mutants) leading to hypoplasia of the valves.

Interestingly, mutants for molecules of the EGF signaling pathway also display enlargement of the valves, restricted to the semilunar valves (Chen et al., 2000). Antagonism of the BMP pathway by EGF signaling has previously been described in vitro (Kretzschmar et al., 1997) and activation of Ras-dependent signaling suppresses EMT (Lakkis and Epstein, 1998). Thus, the opposed phenotypes of mutants deficient in EGF (which can act through Ras mediators) and Bmpr2 signaling raise the exciting possibility that valve formation could be controlled by regulating the relative levels of signal output from Ras- and BMP-dependent pathways.

In summary, our results show that the generation of Bmpr2 allele encoding a protein with altered signal transduction properties can reveal tissues that develop the development of which requires wild-type levels of BMP signaling. This approach is particularly useful for studying tissues for which Cre transgenic strains are not available. Using this approach, we find that septation and valvulogenesis of the mammalian OFT is crucially dependent upon the level of BMP signaling. The finding that the OFT septation defect is restricted in Bmpr2<sup>2ΔE2</sup> mutants to the proximal region provides further genetic evidence that mechanisms of septation of the proximal and distal OFTs are distinct. Our findings show that EMT is initiated and that at least some subpopulations of cardiac neural crest cells migrate into the OFT. However, further development of the proximal OFT is impaired, most probably due to defective cell-cell interactions that depend on BMP signaling, as suggested by the downregulation of the BMP-responsive gene periosin in the OFT. The use of Cre/loxP technology will determine which cell populations require intact BMP signaling in order to mediate these important cell-cell interactions.

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Abnormal heart septation in Bmpr2ΔE2 mutants


