The type I BMP receptor BMPRIB is required for chondrogenesis in the mouse limb

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SUMMARY

Mice carrying a targeted disruption of BmprIB were generated by homologous recombination in embryonic stem cells. BmprIB−/− mice are viable and, in spite of the widespread expression of BMPRIB throughout the developing skeleton, exhibit defects that are largely restricted to the appendicular skeleton. Using molecular markers, we show that the initial formation of the digital rays occurs normally in null mutants, but proliferation of prechondrogenic cells and chondrocyte differentiation in the phalangeal region are markedly reduced. Our results suggest that BMPRIB-mediated signaling is required for cell proliferation after commitment to the chondrogenic lineage. Analyses of BmprIB and Gdf5 single mutants, as well as BmprIB; Gdf5 double mutants suggest that GDF5 is a ligand for BMPRIB in vivo. BmprIB; Bmp7 double mutants were constructed in order to examine whether BMPRIB has overlapping functions with other type I BMP receptors. BmprIB; Bmp7 double mutants exhibit severe appendicular skeletal defects, suggesting that BMPRIB and Bmp7 act in distinct, but overlapping pathways. These results also demonstrate that in the absence of BMPRIB, Bmp7 plays an essential role in appendicular skeletal development. Therefore, rather than having a unique role, BMPRIB has broadly overlapping functions with other BMP receptors during skeletal development.

Key words: BMP, BMPRIB, Cartilage, Mutation, Mouse

INTRODUCTION

Specificity of BMP action is thought to be achieved in part by differential affinities of distinct ligands for one of four type I receptors: TSR-I (ALK1), ACTRI (ALK2), BMPRIA (ALK3) and BMPRIB (ALK6) (Hoodness et al., 1996; Kretzschmar et al., 1997; Chen et al., 1998a; Macías-Silva et al., 1998; Chen and Massagué, 1999). BMPRIA and BMPRIB are more closely related to each other than to other type I BMP receptors (ten Dijke et al., 1994), raising the possibility of functional redundancy. This possibility is supported by the fact that, although BmprIB has a more limited distribution and is not detected until about E9.5, it is coexpressed with BmprIA in many tissues of the developing mouse embryo (Dewulf et al., 1995). Moreover, BMPRIA, BMPRIB and ActRI activate the same downstream signaling components, Smad1 and Smad5 (Hoodness et al., 1996; Kretzschmar et al., 1997; Macías-Silva et al., 1998).

However, several lines of evidence suggest that BmprIA and BmprIB may have distinct functions in skeletal development. First, BMPRIA and BMPRIB bind to specific BMP ligands with differing affinities (Liu et al., 1995, ten Dijke et al., 1994, Nishitoh et al., 1996). Because loss of BmprIA results in early embryonic lethality (Mishina et al., 1995), it has not been possible to distinguish between unique or redundant functions for BmprIA and BmprIB at later stages of development. Second, BmprIA and BmprIB are expressed in broadly overlapping, but not identical, patterns in developing appendicular, axial and craniofacial elements (Francis et al., 1994; Yamaji et al., 1994; Dewulf et al., 1995; Kawakami et al., 1996; Zou et al., 1997). Finally, expression of constitutively active (CA) forms of BMPRIA and BMPRIB promote chondrogenesis in vivo. BMPRIA and BMPRIB act synergistically, as has been demonstrated for the two type I BMP receptors in Drosophila, Thickveins and Saxophone (Neul and Ferguson, 1998; Nguyen et al., 1998). In order to investigate the role of BmprIB in embryonic development, we generated a null allele by homologous recombination in embryonic stem (ES) cells. Homozygous null mice are viable, but mutants exhibit skeletal defects. In spite of widespread expression of BmprIB mRNA throughout the skeleton, defects are essentially restricted to appendicular elements. Analysis of mutant limbs reveals impaired proliferation and differentiation of cells committed to the
chondrogenic lineage. We show that BMPRIB is activated by multiple BMPs in the developing skeleton and provide genetic evidence that GDF5 is one of these ligands in vivo. We also find, in contrast to previous studies utilizing dominant-negative forms of BMP receptors, that BMPRIB does not mediate cellular responses that are fundamentally distinct from those controlled by other type I BMP receptors, but rather has overlapping functions with at least one other receptor during skeletal development.

MATERIALS AND METHODS

Generation of BmprIB<sup>−/−</sup> mice

BmprIB clones were isolated from a 129/Sv mouse genomic DNA λphage library (Stratagene). The targeting construct was generated by replacing a 2.8 kb HindIII-BglIII fragment containing the initiating methionine within exon 1 with the neomycin resistance gene under the control of the PGK promoter (PGKneo). An MC1-thymidine kinase cassette (MC1tkpA) was placed 4.4 kb downstream of the neo cassette. Plasmid sequences were released by digestion with SalI. The targeting vector was electroporated into CCE ES cells according to established protocols (Ramírez-Solis et al., 1993). Targeted clones were injected into blastocysts by the UCLA Transgenic Mouse Facility. Chimeric mice were mated with C57Bl/6J females, and agouti offspring were analyzed by Southern blot hybridization. Chimeras were also bred to 129/Sv females. The phenotypes on the 129/Sv and the mixed (129/Sv × C57Bl/6J) backgrounds were the same, and further analyses were performed on the mixed background. BmprIB<sup>−/−</sup> mice were genotyped by Southern blot analysis of XbaI-digested genomic DNA using the external probe indicated in Fig. 1. Deletion of exon 1 was confirmed by RT-PCR using total RNA from newborn pups or adult brain. The primers within the 5′ untranslated exon and exon 2 were 5′ GCC TGA A TC ACA ACC A TT TGG and 5′ AGG AGG A T 3′.

In situ hybridizations

In situ hybridizations in whole mount and on 20 μm frozen sections were performed as described (Hogan et al., 1994). The BmprIB probe was generated by subcloning a 1 kb partial mouse cDNA obtained from the IMAGE consortium (clone ID 350367) into pBluescript (Stratagene), linearizing with EcoRI, and reverse transcribing with T7 polymerase. The 900 bp Gdf5 probe was amplified by PCR from an E17.5 mouse library (Clontech) using primers within the coding region of the Gdf5 gene (5′ CAG GAA TGT CTT TAG GCC 3′, and 5′ ACA GTC TTG TCA TCC TGG 3′). The amplified product was subcloned into pCR2.1 (Invitrogen). A Sox9 partial cDNA clone was digested with HindIII-BglII, EcoRI-HindIII, and XbaI. The amplified product was subcloned into pCR2.1. The targeting vector replaces exon 1 and flanking sequences with the type locus, exon 1 is indicated as a box. The external probe used for Southern analysis is shown as a line under the wild-type locus. The targeting vector replaces exon 1 and flanking sequences with the neomycin-resistance gene (PGKneo) in the same transcriptional orientation. Lines below the targeting vector indicate the expected lengths of XbaI fragments detected by the external probe. B, BglII; H, HindIII; N, NotI; S, SalI; X, XbaI. (f) Example of Southern blot analyses XbaI-digested DNAs of a litter obtained from an intercross of BmprIB<sup>−/−</sup> mice. (g) RT-PCR analysis of cDNA prepared from normal and mutant newborn pups. Left panel, primers spanning exon 1 showing amplification of a smaller PCR product consistent with loss of exon 1 in BmprIB<sup>−/−</sup> and BmprIB<sup>−/−</sup> mice. Right panel, no amplification is seen in BmprIB<sup>−/−</sup> mutants using a primer specific for exon 1, followed by Southern blot analysis.

Fig. 1. Expression of BmprIB in the developing skeletal system and targeted disruption of the BmprIB gene. (a-d) Expression of BmprIB in limbs at E10.5, E11.5, E13.5 and E15.5, respectively. (a) At E10.5, BmprIB is expressed in migrating sclerotome, gut mesenchyme and mesenchymal cells at the core of the limb bud (arrow); sc, scrolotome. (b) At E11.5, transcripts are present in mesenchyme of the fetus. (c) At E13.5, BmprIB is highly expressed in digital rays. (d) By E15.5, BmprIB transcripts are localized to articular surfaces and perichondrium lining the joint cavities. (e) Targeted disruption of the BmprIB gene to create the BmprIB<sup>tm1kml</sup> allele. In the wild-type locus, exon 1 is indicated as a box. The external probe used for Southern analysis is shown as a line under the wild-type locus. The targeting vector replaces exon 1 and flanking sequences with the neomycin-resistance gene (PGKneo) in the same transcriptional orientation. Lines below the targeting vector indicate the expected lengths of XbaI fragments detected by the external probe. B, BglII; H, HindIII; N, NotI; S, SalI; X, XbaI. (f) Example of Southern blot analyses XbaI-digested DNAs of a litter obtained from an intercross of BmprIB<sup>−/−</sup> mice. (g) RT-PCR analysis of cDNA prepared from normal and mutant newborn pups. Left panel, primers spanning exon 1 showing amplification of a smaller PCR product consistent with loss of exon 1 in BmprIB<sup>−/−</sup> and BmprIB<sup>−/−</sup> mice. Right panel, no amplification is seen in BmprIB<sup>−/−</sup> mutants using a primer specific for exon 1, followed by Southern blot analysis.
623BMP receptor IB (BmprIB) gene disruption obtained from the IMAGE consortium (clone ID 659759); the 1200 bp fragment was subcloned into pBluescript (Stratagene). The aggrecan and pro-alpha 1(II) collagen partial cDNA clones were gifts from A. J. Celeste and E. Vuorio, respectively. In situ hybridization with a \[^{35}\text{S} \]UTP-labeled BmprIB antisense RNA probe was performed as described (Lyons et al., 1995).

Cell proliferation and apoptosis
BrdU labeling was performed using the In Situ Cell Proliferation (Boehringer Mannheim) kit according to manufacturer’s protocols.

Briefly, BrdU was injected intraperitonally into pregnant mice 1 hour before death. Embryos were fixed and embedded in paraffin. 7 µm sections were subjected to nonradioactive in situ hybridization using

Fig. 2. Limb defects in adult and newborn BmprIB\(^{-/-}\) and Gdf5\(^{bp-J-}\)/- mice. (a-c) X-ray autoradiographs of hindlimbs from adult wild-type (a), BmprIB\(^{-/-}\) (b), and Gdf5\(^{bp-J-}\)/- (c) mice. The proximal and middle phalanges are fused and reduced in BmprIB\(^{-/-}\) and Gdf5\(^{bp-J-}\)/- mice. (d) Bingh of appendicular skeletal elements in adult wild-type and BmprIB\(^{-/-}\) mice. (e-g) Radioulnar joints from wild-type (e), BmprIB\(^{-/-}\) (f) and Gdf5\(^{bp-J-}\)/- (g) mice. The joints of the BmprIB\(^{-/-}\) and Gdf5\(^{bp-J-}\)/- mice have dislocated. (h-j) Cleared skeletal preparations of hindlimbs from newborn wild-type (h), BmprIB\(^{-/-}\) (i) and Gdf5\(^{bp-J-}\)/- (j) mice. The calcaneus is indicated by an arrowhead. dp, distal phalanx; h, humerus; mc, metacarpal; mp, middle phalanx; mt, metatarsal; pp, proximal phalanx; r, radius; t, tibia; u, ulna.

Fig. 3. Expression of Sox9 and Gdf5 in forelimbs from normal (left limb in each panel) and BmprIB mutant (right limb in each panel) embryos by whole-mount in situ hybridization. (a,b) Sox9 expression in E12.5 and E13.5 limbs, respectively. Sox9 levels are severely reduced in the presumptive phalangeal region in BmprIB mutants by E13.5. Arrowheads in b denote the primordium of the metacarpal/phalangeal joint. Arrow in b marks the primordium of the interphalangeal joint. (c-e), Gdf5 expression in E12.0, 13.0 and 13.5 limbs, respectively. By E13.0, an expanded domain of Gdf5 expression is seen in BmprIB mutants and, by E13.5, a single band of Gdf5-expressing cells is present in the phalangeal region of the digits. (e) Arrowheads and arrow are as described in b.
the Gdf5 probe. This allowed unequivocal identification of the phalangeal region in developing limbs. Sections containing the primordia of the metacarpal and phalanges were used immediately for immunodetection of BrdU. Sections were then fixed and counterstained with Hematoxylin. To determine the rate of cell proliferation, photomicrographs were taken at a 20× magnification and cells within the phalangeal region of the digital ray corresponding to digits II or III were counted. The analysis was done including (data not shown) or excluding the perichondrium. Cells in three (E12.5) or five (E13.5) adjacent sections, each spanning 40 μm, from three independent wild-type and BmprIB+/− embryos were scored. Five adjacent sections of interdigital mesenchyme from three E12.5 embryos were scored as an internal control. The percentage of labeled nuclei was determined using Student's t-test.

To confirm that gene targeting altered the BmprIB locus in the predicted fashion, total RNA from individual embryos was analyzed using RT-PCR. Correct targeting would produce an allele that lacks exon 1. This modification is expected to produce a null or at least a severe hypomorphic allele for the following reasons. First, the initiating methionine and the leader sequence are deleted. Furthermore, the next methionine codon, located on exon 2 is out of frame and its utilization would result in a 9 amino acid product. Finally, although utilization of an in-frame methionine located further downstream is possible, the predicted peptide would lack a leader sequence.

A transcript lacking the 162 bp exon 1 is predicted to be a product of the mutated locus because transcription of the BmprIB gene is initiated from a 5′ untranslated exon (data not shown). As expected for loss of exon 1, a smaller PCR product is seen by RT-PCR analysis in BmprIB+/− and BmprIB−/− mice using primers that span the 5′ untranslated region (5′ UT) and exon 2 (Fig. 1g). To confirm that exon 1 is deleted, primers specific to exon 1 (E1int.) and exon 4 (E4) were used for RT-PCR analysis. No amplification product was detected following Southern blot analysis (Fig. 1g). In summary, the targeted allele is most likely null.

BmprIB−/− mice have limb abnormalities

Despite the widespread expression of BmprIB in the developing central nervous system, stomach, gut and lung epithelia, and kidney (Dewulf et al., 1995), no defects were apparent in these tissues. However, an essential role for BmprIB in skeletal development was confirmed by the observation that mutants exhibit forelimb and hindlimb defects. No phenotypic differences are observed on the hybrid (129Sv × C57Bl/6) and inbred 129Sv backgrounds. In adult BmprIB−/− mice, the proximal interphalangeal joint is absent, and the phalanges are replaced by a single rudimentary element, while the distal phalanges are unaffected (Fig. 2a,b). The lengths of the radius, ulna and tibia are normal, but the metacarpals/metatarsals are reduced, and several carpal/tarsal bones are affected in mutants (Fig. 2d, data not shown). These abnormalities are fully penetrant. As described below, the appendicular defects in BmprIB−/− mice resemble those seen in mice homozygous for the Gdf55bp−/− null allele of the Gdf5 locus (Grünberg and Lee, 1973; Storn et al., 1994). BmprIB mutants sustain frequent (28% of adults, 7/25) elbow joint dislocations (Fig. 2e,f). No defects are observed in craniofacial elements (data not shown) and, although BmprIB is highly expressed in sclerotomal cells (Fig. 1a), axial defects are confined to a reduced 4th sternabra in 33% (3/9) of neonatal mutants (data not shown).

To identify the developmental processes affected by loss of BmprIB, cleared skeletal preparations were made from

RESULTS

Localization of BmprIB mRNA during skeletal development

Expression of BmprIB in developing chick and mouse limbs at midgestation stages has been reported (Dewulf et al., 1995; Zou et al., 1997; Kawakami et al., 1996; Yamaji et al., 1994; Merino et al., 1998). We examined earlier and later stages, and detected low levels of expression in mesenchymal cells within the core of the developing limb bud at E10.5. At E10.5, BmprIB transcripts are present in sclerotomal and gut mesenchyme (Fig. 1a). At E11.5, BmprIB expression can be detected in bands of mesenchymal cells, which may represent the primordia of the long bones, prior to the appearance of condensed precartilaginous mesenchyme (Fig. 1b). By E13.5, BmprIB is highly expressed within the condensed mesenchyme of the digital ray (Fig. 1c; see also Fig. 5) and, by E15.5, transcripts are restricted to the perichondrium and articular surfaces (Fig. 1d).

Targeted disruption of the BmprIB gene

To investigate BmprIB function, we generated a vector that replaces 2.8 kb of BmprIB, including the first coding exon, with a neomycin-resistance expression cassette (Fig. 1e). Correct targeting deletes this exon, which encodes the translation initiation codon, signal peptide and 40% of the extracellular domain. A correctly targeted ES clone was identified by Southern blot analysis and introduced into the mouse germline.

The mutant allele was outcrossed to the C57Bl/6 strain, or maintained on an inbred 129Sv background. Heterozygous mice appeared normal and were intercrossed to obtain homozygous mutant mice. BmprIB−/− mice were obtained in Mendelian ratios (Fig. 1f).
neonatal wild-type (n=21) and mutant (n=16) littermates. No abnormalities were detected in heterozygotes but, in mutants, chondrogenesis is severely impaired in the region of the proximal and middle phalanges. Additional defects are seen in the calcaneus, in which there is an overall reduction in length and in the size of the ossification center (Fig. 2h,i).

**Molecular analysis of BmprIB<sup>−/−</sup> mutants**

The failure in phalanx formation in BmprIB<sup>−/−</sup> mice may reflect a role for BmprIB in the specification of prechondrogenic mesenchyme, or in the subsequent proliferation, differentiation and/or survival of cells committed to the chondrogenic lineage. To investigate these possibilities, histological analyses and in situ hybridizations using markers of mesodermal patterning and differentiation were performed.

We examined expression of Sox9, a gene required for chondrogenesis (Bi et al., 1999). Sox9 is expressed within precartilaginous condensations, as well as in their condensing progenitor cells (Wright et al., 1995). Expression declines in differentiating chondrocytes, but low levels persist in these cells, and Sox9 has been shown to directly regulate expression of α1(II) collagen (Bell et al., 1997). To determine whether BmprIB mutants exhibit defects in specification of cells to the chondrogenic lineage, expression of Sox9 was compared in wild-type and mutant limbs. At E12.5, the highest levels of Sox9 expression are found in the region of the future terminal phalanges and lower levels are found in the region of the metacarpal/phalangeal joint (Fig. 3a). No differences in Sox9 expression are observed in wild-type and BmprIB<sup>−/−</sup> limbs up to and including E12.5, indicating that the prechondrogenic limb mesenchyme is specified and is able to form condensations up to this time (Fig. 3a).

By E13.5, alterations in Sox9 expression can be detected. In normal mice Sox9 is expressed at highest levels in the distal cells of the digital ray and in the region of increased cell density corresponding to the future interphalangeal joint. Moderate transcript levels persist in the phalangeal region (Fig. 3b). In BmprIB<sup>−/−</sup> mice, Sox9 expression is markedly reduced in the presumptive phalanges and there is no evidence of interphalangeal joint formation (Fig. 3b). These results show that loss of BmprIB does not prevent specification or initial condensation of prechondrogenic mesenchyme in the early limb bud, but BmprIB is required for further morphogenesis of the phalangeal region of the digital ray.

To examine the role of BmprIB in more detail, the gene encoding the TGFβ superfamily member Gdf5 was used as a marker. Gdf5 is expressed in regions of future joint formation, and later becomes restricted to articular surfaces and synovial capsules. Moreover, Gdf5 is essential for digit and joint formation (Strom et al., 1994; Storm and Kingsley 1996). No differences in Gdf5 expression patterns are observed through E12.5 in mutant and wild-type mice (Fig. 3c). By E13.0, the domain of Gdf5 expression in the digital ray is expanded in BmprIB mutants relative to the wild-type pattern (Fig. 3d). By E13.5, Gdf5 expression is restricted in wild-type mice to the presumptive joint interzone and the cells that will form the synovial capsules (Fig. 3e, Storm and Kingsley 1996). In contrast, in BmprIB<sup>−/−</sup> limbs, a single elongated domain of Gdf5 expression is observed in the region of the digital ray corresponding to the proximal and middle phalanges. The high level of Gdf5 expression in the future synovial capsule of the interphalangeal joint in normal mice is also greatly reduced in BmprIB mutants (Fig. 3e). These studies show that BmprIB activity is not required for the specification of mesenchyme to the prechondrogenic lineage, but is required for phalangeal development at or prior to E13.0.

**Defective chondrocyte differentiation and reduced cell density in BmprIB mutants**

The absence of Alcian blue staining in the defective phalanges of newborn BmprIB mutants suggests that chondrocyte differentiation is impaired. To investigate this, we compared the patterns of aggrecan and Gdf5 expression in midgestation embryos. In normal limbs, aggrecan expression parallels that of α1(II) collagen; aggrecan is first detected in prechondroblasts and later in differentiating chondroblasts (Mallien-Gerin et al., 1988). By E13.5, aggrecan is expressed throughout the digits of wild-type embryos (Fig. 4a), but no expression can be detected in the phalangeal region of mutants (Fig. 4b), which is identified by the domain of Gdf5 expression (Fig. 4c). Aggrecan expression is normal in the metacarpals of mutants, demonstrating that the differentiation defect is restricted to the phalanges. Identical results were obtained for α1(II) collagen; no expression was detected in the phalanges of mutant limbs at E13.5, despite apparently normal levels of expression in other skeletal elements within the autopod (Fig. 4d).

The differentiation defect seen in mutants may reflect a primary requirement for BmprIB in chondrocyte maturation, or may be a secondary consequence of an earlier requirement, either for the formation of mesenchymal condensations, and/or for proliferation. For example, the ability of prechondrogenic mesenchyme to differentiate into chondroblasts is dependent on cell density (Takahashi et al., 1998). Thus, the absence of aggrecan and reduction in Sox9 expression in the phalangeal regions of E13.5 mutant limbs may be a result of reduced cell density. Similarly, the broader domain of Gdf5 expression in mutant limbs could be due to a reduction in the number of prechondrogenic cells within the phalanges, resulting in a failure to physically restrict the Gdf5-expressing cells to the joint interzone during development.

To determine whether cell density in the digital ray is affected in BmprIB mutants, wild-type and mutant limbs were examined histologically. Sections through E12.5 forelimb phalanges reveal no differences in the lengths or widths of the digital rays (data not shown). However, by E13.5, the phalanges of BmprIB<sup>−/−</sup> mice exhibit decreased cell density and defective proximal phalanx formation (Fig. 4e). In the wild-type digit, metatarsal and phalangeal articular surfaces can be distinguished, and cells within the metatarsal and phalanx have acquired the morphology associated with chondrogenic differentiation (Hall, 1978). In contrast, in the mutant digit, a joint surface has formed on the metatarsal and phalangeal cells show no evidence of differentiation (Fig. 4e). Decreased cell density and failure to undergo differentiation within the phalanges persists in mutants at least until birth. In newborn mutants, a residual population of undifferentiated cells persists in the region between the metatarsal and phalanx (Fig. 4f).
Reduced cell proliferation in mutant phalanges

To determine the cause(s) of the decreased cell density in Bmpr1B mutants, we estimated the relative contributions of increased apoptosis and/or decreased proliferation to the phenotype. No differences were observed in levels of apoptosis in E12.0-E13.5 embryos as determined by TUNEL labeling (data not shown), suggesting that increased cell death is not a major cause for the reduction in cell density in Bmpr1B-/- mice. We therefore examined the effect of the mutation on proliferation by monitoring bromodeoxyuridine (BrdU) incorporation. As an internal control, cell proliferation was also examined in interdigital mesenchyme of mutant and wild-type limbs. E12.5 is the earliest stage at which it is possible to distinguish phalangeal from metacarpal precursors within the digital ray (see Materials and Methods). Although no morphological differences can be detected at E12.5, the percentage of proliferating cells is reduced by 33% in the region of the presumptive phalanges in mutants (Fig. 4g; Table 1). By E13.5, a striking 62% reduction in the level of cell proliferation in the phalangeal region is seen in mutants. The metacarpals of E13.5 mutants exhibit decreased proliferation, but the difference is not statistically significant (Fig. 4g; Table 1). These results show that Bmpr1B is required for the proliferation of phalangeal prechondrogenic cells beginning at early stages of limb development.

### Table 1. Cell proliferation in the digital ray and interdigital mesenchyme

<table>
<thead>
<tr>
<th>Age</th>
<th>Wild type</th>
<th>Bmpr1B-/-</th>
<th>Inhibition (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>E12.5 phalangeal mesenchyme</td>
<td>38.3±10.8</td>
<td>25.6±9.4</td>
<td>33</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>E12.5 interdigital mesenchyme</td>
<td>56.3±6.7</td>
<td>56.9±6.9</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>E13.5 phalangeal mesenchyme</td>
<td>34.0±8.1</td>
<td>12.9±8.5</td>
<td>62</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>E13.5 metacarpal mesenchyme</td>
<td>3.4±2.3</td>
<td>2.4±2.0</td>
<td>n.s.</td>
<td></td>
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Embryos were labeled in vivo with BrdU and sections were subjected to nonradioactive in situ hybridization with the Gdf5 probe in order to identify the phalangeal region of the digital ray, followed by immunohistochemistry to detect BrdU, as described in Materials and Methods. As an internal control, labeled cells in E12.5 interdigital mesenchyme were scored. The fraction of BrdU-labeled cells was determined by dividing the number of labeled nuclei by the total number. Data are expressed as the mean ± s.e.m.
Overlapping functions for BMPRIB and other receptors during skeletal development

The restriction of the BmprIB−/− skeletal phenotype to a subset of appendicular elements could be due to limited patterns of expression of the relevant BMP ligand(s), and/or to the ability of a second receptor to compensate for loss of BmprIB. BMP2, BMP4, BMP7 and GDF5 bind and activate BMPRIB in vitro (Liu et al., 1995; ten Dijke et al., 1994; Nishitoh et al., 1996) and are expressed in the developing skeletal system (Lyons et al., 1995; Jones et al., 1991; Storm et al., 1994). GDF5 binds to multiple receptors, but activates BMPRIB most efficiently (Nishitoh et al., 1996) in vitro. Therefore, we investigated the possibility that GDF5 acts through BMPRIB in vivo. Gdf5 and BmprIB are expressed in adjacent tissues during limb development, suggesting that GDF5 may act in a paracrine manner as a ligand for BMPRIB in vivo (Fig. 5). To explore this possibility further, we compared BmprIB−/− mice to mice homozygous for the brachypodism J (Gdf5 bp-J) mutation, which encodes a defective Gdf5 allele (Storm et al., 1994). In both Gdf5 bp-J−/− and BmprIB−/− mice, the proximal and middle phalanges are reduced and fused, the metatarsals and metacarpals are shorter, the distal phalanges are unaffected, and both mutant strains sustain frequent joint dislocations (Fig. 2a-e; Grünberg and Lee 1973). This

Fig. 5. Expression of Gdf5 and BmprIB in midgestation limbs. (a) Gdf5 and (b) BmprIB expression at E12.5 in adjacent sections. (c) Overlay of Gdf5 and BmprIB, with Gdf5 expression shown in green. (d) Gdf5 and (e) BmprIB expression at E13.5 in adjacent sections. (f) Overlay showing Gdf5 expression in green. Gdf5 and BmprIB are expressed in nonoverlapping, but adjacent regions of the limb. (Liu et al., 1995; ten Dijke et al., 1994; Nishitoh et al., 1996) and are expressed in the developing skeletal system (Lyons et al., 1995; Jones et al., 1991; Storm et al., 1994).

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phenotypic correspondence, in addition to adjacent patterns of expression (Fig. 5) and in vitro data (Nishitoh et al., 1996) suggests that GDF5 may activate BMPRIB in the developing phalanges.

Although the phalanges of Gdf5bp-J−/− mice closely resemble those of BmprIB mutants, other limb elements are more severely affected in Gdf5bp-J−/− mice, suggesting that GDF5 cannot act exclusively through BMPRIB in the limb (Fig. 2). To test whether GDF5 is the only ligand that activates BMPRIB in vivo, we constructed BmprIB; Gdf5bp-J compound heterozygous and double null mutants. If BMPRIB is activated exclusively by GDF5, we would not expect to observe defects in BmprIB+/−; Gdf5bp-J−/− double mutants that are not seen in Gdf5bp-J−/− or BmprIB−/− mutants. A detailed description of the phenotypes of these mice will be presented elsewhere but several informative features are discussed below.

Newborn Gdf5bp-J+/+ (n=13) and BmprIB+−/− (n=10) mice are normal, except that 46% (6/13) of Gdf5bp-J+/− mice exhibit delayed ossification of the middle phalanx (data not shown). In BmprIB+/−; Gdf5bp-J−/− mice the penetrance of the delayed ossification of the middle phalanx increases to 100% (n=8). (Fig. 6a,b). This result is consistent with a role for GDF5 as an activator of BMPRIB in vivo, but may also reflect a requirement for BMPRIB and GDF5 in separate signaling pathways.

Overall, the limbs of BmprIB; Gdf5bp-J−/− double null mutants closely resemble Gdf5bp-J−/− mice (Fig. 6a,c,d), consistent with the possibility that BMPRIB is activated primarily by GDF5 in the limb. However, GDF5 and BMPRIB may act in separate pathways as well, because double mutants exhibit subtle defects not seen in either of the single mutants. For example, in the forelimbs, metacarpals I and V of newborn BmprIB; Gdf5bp-J−/− double null mice are reduced to a greater extent than either of the single mutant strains (Fig. 6c-e), and additional nonadditive fusions/reductions of sternal and tarsal elements are found (data not shown).

In summary, analyses of BmprIB; Gdf5bp-J compound heterozygous and double null mutants provide evidence consistent with a role for GDF5 in the activation of BMPRIB. However, these studies strongly suggest that GDF5 can act through other receptors, and that BMPRIB may be activated by other ligands.

Bmp7 and Bmprib participate in parallel pathways during skeletal development

BMPRIA, BMPRIB and ActRI are broadly expressed in the developing skeletal system (Dewulf et al., 1995; Shuto et al., 1997) and transduce similar intracellular signals (ten Dijke et al., 1994; Liu et al., 1995; Macias-Silva et al., 1998), raising the possibility that they have overlapping functions. Because ActRI, BMPRIB and, to a lesser extent, BMPRIA, are activated by BMP7 in vitro, we examined the phenotypes of Bmp7; BmprIB compound heterozygotes and double mutants. BMP7 is expressed in early limb bud mesenchyme, and later becomes restricted to interdigital tissue (Lyons et al., 1995). Bmp7−/− mice have normal limbs (Fig. 6f,i), with the exception of ≈25% incidence of unilateral preaxial polydactyly (Dudley et al., 1995; Luo et al., 1995). If BMPRIB and an additional receptor(s) activated by BMP7 have overlapping functions, nonadditive limb defects might be expected in double mutants, due to elimination of BMP7 signaling through ActRI, BMPRIA and/or an as yet unidentified receptor for BMP7.

BmprIB+/−; Bmp7+/− compound heterozygotes (n=6) appear normal (Fig. 6g). However, dramatic synergistic effects are seen in BmprIB; Bmp7 double mutants (Fig. 6h-j). Within the forelimb autopod, every element except the distal phalanx is severely reduced or absent. The ulna is nearly absent and the radius is shortened. Additional reductions in the length of the humerus and the width of the dorsal margin of the scapula are apparent in the double mutant (Fig. 6j). Similar defects are seen in the hindlimbs (data not shown). A more detailed description of the Bmp7/BmprIB compound and double mutant phenotypes will be presented elsewhere. These results demonstrate that BmprIB serves some essential and some redundant functions in the limb and, in the absence of signal transduction through BMPRIB, BMP7 signaling through other receptors is essential for endochondral skeletal development.

DISCUSSION

Several lines of evidence, outlined in the introduction, suggested that BMPRIB is essential for chondrogenesis throughout the developing skeletal system. In order to examine the role of BmprIB in vivo, we generated mice deficient in BmprIB. Our results confirm an essential role for this receptor and show that its absence leads to multiple abnormalities of the appendicular skeleton, including brachydactyly, reductions in metacarpals/metatarsals and joint defects. However, the restriction of skeletal defects to a subset of appendicular elements argues that BmprIB does not mediate a signal fundamentally distinct from that of other BMP type I receptors, and that multiple type I receptors have overlapping functions during chondrogenesis.

BmprIB is not essential for specification of prechondrogenic mesenchyme

BmprIB is expressed in prechondrogenic mesenchyme and its expression precedes chondrogenesis induced by exogenous administration of TGFBs and BMPs (Fig. 1, Yamaji et al., 1994; Kawakami et al., 1996; Zou et al., 1997; Merino et al., 1998). Our analysis shows that the initial specification of mesenchyme to the chondrogenic lineage in digital rays appears to be normal, as judged by the expression of a variety of markers (Fig. 3). However, BmprIB may play a role in specification that is shared by other type I BMP receptors expressed in early mesenchyme. BmprIA is expressed throughout limb mesenchyme (Kawakami et al., 1996; Zou et al., 1997) and transcripts for ActRI have been detected in skeletal tissues by RT-PCR (Shuto et al., 1997). The possibility of functional redundancy at the stage of specification to the chondrogenic lineage is currently under investigation.

BmprIB is required for the proliferation of prechondrogenic mesenchyme

BmprIB is essential for the proliferation of cells within the phalangeal region of the digital ray, which normally expresses high levels of BmprIB (Fig. 4). Decreased proliferation is observed by E12.5, showing that BmprIB activity is essential at early stages of chondrogenesis once the digital ray has formed. This result is consistent with studies demonstrating
that expression of CA BMPRIB promotes cell proliferation (Zou et al., 1997). The reduced proliferative capacity in BmprIB mutants may reflect a primary role for BMP signaling in regulation of the cell cycle. Alternatively, BmprIB may affect proliferation by influencing cell adhesiveness or extracellular matrix production.

BMPs regulate cell survival in the limb (Dudley et al., 1995; Luo et al., 1995; Macias et al., 1997) and expression of DN BMPRIB results in increased levels of apoptosis in limb mesenchyme (Zou and Niswander, 1996). Although we cannot rule out a role for BmprIB in cell survival, increased levels of apoptosis do not appear to contribute significantly to the BmprIB−/− phenotype since TUNEL studies did not reveal any differences between wild-type and mutant mice. In contrast, Baur et al. (2000) observe increased levels of apoptosis in mice homozygous for the BmprIBTg regulatory allele.

An additional role for BMPRIB in the induction or maintenance of chondrocyte differentiation is suggested by the observations that phalangeal cells in BmprIB mutants do not express aggregan or αl(II) collagen, are not stained by Alcian blue and do not assume a differentiated morphology (Fig. 4). BmprIB is highly expressed in digital rays at E13.5 (Fig. 1) and these cells are undergoing chondrocytic differentiation (Fig. 4), raising the possibility that BmprIB directly regulates differentiation. Studies showing that expression of DN BMPRIB downregulates expression of αl(II) collagen and aggregan support a direct role for BMPRIB in the maintenance of the differentiated phenotype (Enomoto-Iwamoto et al., 1998). Alternatively, the differentiation defect observed in BmprIB−/− mice could be a secondary consequence of decreased cell density (Takahashi et al., 1998 and refs. therein).

**BMPRIB has redundant functions with other type I receptors**

A major conclusion of this study is that BMPRIB has overlapping functions with other type I receptors during skeletal development. Several lines of evidence support this conclusion. First, in spite of its widespread pattern of expression, loss of BMPRIB affects only a subset of skeletal elements. Second, the phenotype of BmprIB; Bmp7 double mutants strongly suggests that multiple type I receptors have overlapping functions in chondrogenesis.

GDF5 is a potential ligand for BMPRIB and binds more strongly to this receptor than to BMPRIA or ActRI (Nishitoh et al., 1996). The similar phalangeal defects seen in BmprIB−/− and bp/bp mice (Fig. 2) and the patterns of BmprIB and Gdf5 expression (Fig. 5) are consistent with the possibility that BMPRIB and GDF5 act in a single essential pathway in the phalanx. One potential target of BMPRIB signaling may be GDF5 itself since ectopic expression of GDF5 in developing limbs leads to downregulation of endogenous Gdf5 expression (Merino et al., 1999; Storm and Kingsley, 1999). This result is consistent with the expanded domain of Gdf5 expression observed in both Gdf5bp-j−/− mutants (Storm and Kingsley, 1999) and BmprIB−/− mutants (Fig. 3). The phenotypes of BmprIB; Gdf5bp-j compound heterozygotes and double mutants (Fig. 6) support the possibility that GDF5 activates BMPRIB in vivo, but suggest that GDF5 and BMPRIB have some overlapping functions and therefore must act in parallel pathways as well (see also Baur et al., 2000).

That BMPRIB and other type I receptors are likely to have overlapping functions is particularly clear in the case of BmprIB; Bmp7 double null mutants, where every element in the appendicular skeleton is affected to some extent (Fig. 6). Bmp7 can bind to and activate multiple type I BMP receptors (ten Dijke et al., 1994; Liu et al., 1995; Macías-Silva et al., 1998; Kawabata et al., 1998), but binds to BMPRIB and ActRI much more efficiently than to BMPRIA (ten Dijke et al., 1994). Further experiments are underway to clarify the nature of the genetic interactions revealed in BmprIB/Bmp7 double mutants.

**Conclusions**

The widespread expression of BMPRIB throughout the skeletal system and studies utilizing DN forms of BMP receptors suggested that BMPRIB plays a unique and essential role in chondrogenesis. The experiments reported here provide in vivo evidence for considerable functional redundancy among type I BMP receptors during chondrogenesis. Although our results do not directly address whether different receptors activate distinct intracellular pathways, they suggest that BMPRIB and other receptors elicit many shared cellular responses during chondrogenesis. Although BMPRIA, BMPRIB and ActRI all transduce their signals through a common set of Smad proteins (reviewed in Kawabata et al., 1998), activation of these receptors may elicit different cellular responses as a result of differing availability and/or affinities for ligands. For example, increasing occupancy of a single type of activin receptor elicits different gene expression profiles, which are exquisitely sensitive to small changes in the level of receptor occupancy (Dyson and Gurdon, 1998). Therefore, the ability of BMPs to regulate multiple aspects of chondrogenesis may involve varying levels of activation of a common signaling pathway through multiple BMP receptors rather than activation of distinct pathways. Major challenges for the future will be to understand how multiple type I BMP receptors coordinate aspects of morphogenesis.

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