Distinct developmental programs require different levels of Bmp signaling during mouse retinal development

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Summary

The Bmp family of secreted signaling molecules is implicated in multiple aspects of embryonic development. However, the cell-type-specific requirements for this signaling pathway are often obscure in the context of complex embryonic tissue interactions. To define the cell-autonomous requirements for Bmp signaling, we have used a Cre-loxP strategy to delete Bmp receptor function specifically within the developing mouse retina. Disruption of a Bmp type I receptor gene, Bmpr1a, leads to no detectable eye abnormality. Further reduction of Bmp receptor activity by removing one functional copy of another Bmp type I receptor gene, Bmpr1b, in the retina-specific Bmpr1a mutant background, results in abnormal retinal dorsoventral patterning. Double mutants completely lacking both of these genes exhibit severe eye defects characterized by reduced growth of embryonic retina and failure of retinal neurogenesis. These studies provide direct genetic evidence that Bmpr1a and Bmpr1b play redundant roles during retinal development, and that different threshold levels of Bmp signaling regulate distinct developmental programs such as patterning, growth and differentiation of the retina.

Key words: Bmpr1a, Bmpr1b, Bmp signaling, Mutant mouse, Retinal patterning, Retinal growth, Retinal neurogenesis

Introduction

Vertebrate eye development proceeds through a series of inductive processes involving multiple tissue components and has been studied as a model system to explore the general mechanisms underlying embryonic tissue interactions (Jacobson and Sater, 1988). Several families of secreted signaling molecules including the bone morphogenetic protein (Bmp) family are implicated in the control of inductive processes during normal eye development (Chow and Lang, 2001). Previous studies have implicated in the control of inductive processes normal eye development (Chow and Lang, 2001). Previous studies have implicated at least two members of the Bmp gene family, Bmp4 and Bmp7, in mouse eye development. Both Bmp4+/− and Bmp7+/− null mutants exhibit defects in lens induction (Furuta and Hogan, 1998; Wawersik et al., 1999). In addition, Bmp4+/− heterozygotes show a spectrum of eye abnormalities in the adult (Chang et al., 2001). However, the precise genetic mechanisms by which Bmp signaling regulates these developmental processes are obscured by the embryonic lethality and variably penetrant eye phenotypes in these conventional null mutants.

Within the optic cup, Bmp signaling mediated by the dorsally localized Bmp4 ligand appears to control the patterning of the dorsoventral axis of the developing retina. Ectopic expression of Bmp4 in the embryonic chick retina leads to the upregulation of the dorsal transcription factor, Tbx5, throughout the optic cup, and downregulation of the ventral markers, Vax and Pax2 (Koshiba-Takeuchi et al., 2000). Bmp4 signaling may be subject to negative regulation by the ventrally localized Bmp4 antagonist, ventroptin (Chrdl1 – Mouse Genome Informatics) (Sakuta et al., 2001). Forcing dorsal expression of ventroptin in the early chick retina represses dorsal Bmp4 expression and expands Vax expression. These molecular changes result in severely abnormal projection patterns of retinal ganglion cell axons. Bmp signaling has also been implicated in other aspects of retinal development. Bmp4 can influence proliferation and cell death in the chick retina (Trouse et al., 2001). Inhibition of Bmp signaling in the chick retina by overexpression of the Bmp antagonist, Noggin, leads to disruption of ventral retinal structures (Adler and Belecky-Adams, 2002). At present, however, the possible involvement of Bmp signaling in retinal cell differentiation in vertebrates has not been established,
whereas the Bmp homolog decapentaplegic (Dpp) is a principal regulator of retinal neurogenesis in Drosophila. Several Bmp ligands and cognate receptors are expressed in the developing mouse eye (Dudley and Robertson, 1997; Furuta and Hogan, 1998). One possible model to account for the diverse effects of Bmps in the eye is that the different ligand-receptor combinations can result in qualitative differences in signaling output. Alternatively, different developmental processes may require distinct levels of signaling activity as described in a morphogen model (Freeman and Gurdon, 2002).

The Bmp ligands signal via heteromeric complexes composed of type I and type II transmembrane serine/threonine kinase receptors (Mishina, 2003). In the ligand-activated complex, the type II receptors phosphorylate and activate type I receptors. Activated type I receptors in turn trigger downstream signaling by Smad proteins, which are responsible for transduction of the extracellular signal to the nucleus. In this system, the activity of type I receptors primarily dictates the level and specificity of the intracellular signaling. Among various type I receptors, Acrv1 (Alk2), Bmpr1a (Alk3) and Bmpr1b (Alk6) are capable of mediating Bmp ligand signal, although the affinity for different ligands varies among these receptors (Mishina, 2003). Of these, Bmpr1a and Bmpr1b are expressed within the developing mouse retina (Furuta and Hogan, 1998). Sources of Bmp ligand that can potentially signal to the retina via Bmpr1a and/or Bmpr1b include Bmp7 in the lens and peri-ocular mesenchyme, Bmp4 in the dorsal retina, and Bmp2 and Bmp3 in the retinal pigment epithelium and surrounding mesenchyme, respectively (Dudley and Robertson, 1997; Furuta and Hogan, 1998; Wawersik et al., 1999). In order to resolve this complex signal transduction apparatus, it is crucial to evaluate the cell type specific roles of the signaling pathway in vivo. Moreover, although multiple receptor-ligand pairs have been suggested to exist by biochemical experiments, genetic evidence for redundancy among the various receptors is lacking.

Here, we report the generation of retina-specific Bmp type I receptor mutant mice to investigate the role of Bmp signaling within the developing retina. A graded diminution of Bmp signaling activity, achieved by combining mutations of Bmpr1a and Bmpr1b genes causes defects in multiple aspects of retinal development, including dorsoventral patterning, growth and differentiation. Our studies provide direct genetic evidence that Bmpr1a and Bmpr1b play largely redundant roles during early retinal development, and that different thresholds of Bmp signaling regulate distinct developmental programs in the retina.

Materials and methods

Mice

Mice carrying null and conditional mutant alleles of the Bmpr1a gene [Bmpr1a (Mishina et al., 1995), referred to as Bmpr1a<sup>+/−</sup>], and Bmpr1b<sup>fx</sup> (Mishina et al., 2002), referred to as Bmpr1b<sup>fx</sup> in this paper], a null allele of Bmpr1b [Bmpr1b<sup>−/−</sup> (Li et al., 2000), referred to as Bmpr1b<sup>−/−</sup>], a null allele of Bmp4 (Winnier et al., 1995) and a Six3Cre transgene to drive Cre recombinase expression in the developing retina (Furuta et al., 2000) have been previously described. Among multiple lines of Six3Cre transgenic mice generated, line 69 [designated TgN(Six3Cre)69Frty, according to Standardized Genetic Nomenclature for Mice (www.informatics.jax.org/mgihome/nomen/gene.shtml)] was used to conditionally disrupt the Bmpr1a<sup>−/−</sup> allele, owing to consistency of tissue-specific Cre expression and absence of abnormal phenotypes in homozygotes.

Tissue preparations for marker analyses

Embryonic tissue explant culture using filters and BMP4-soaked beads was performed essentially as described (Furuta and Hogan, 1998). Recombinant human BMP4 protein was kindly provided by the Genetics Institute (Cambridge, MA). Embryos or cultured explants were fixed in 4% paraformaldehyde in PBS, dehydrated through a graded series of methanol, followed by whole-mount in situ hybridization (see below) or histological sectioning. Sections were processed for Hematoxylin and Eosin staining, in situ hybridization or immunohistochemistry (see below). For analyses of retinal cell type markers, fixed retinal tissues were embedded in polyester wax (Electron Microscopy Sciences, PA) for sectioning. For all the experiments described here, three or more animals/embryos of each genotype were examined.

In situ hybridization

In situ hybridization using [35S]UTP-labeled riboprobes on sections or digoxigenin (DIG)-labeled riboprobes on sections or whole-mount embryos was performed essentially as described previously (Nagy, 2003). Templates for Rldh1a, Rldh1a3, Chx10, Brn3b and Math5 (Atoh7 – Mouse Genome Informatics) RNA probes were obtained from a retinal EST library (Mu et al., 2001). Photos for radioactive in situ sections were taken by double exposures, digitally combining dark field images under a red channel and bright-field images under a blue channel. Pictures for DIG in situ sections were photographed under differential interference contrast illumination.

Immunohistochemistry

Antibodies used for immunohistochemical detection were anti-Syntaxin monoclonal antibody (mAB) (HPC-1, Sigma), anti-β-tubulin III mAB (TU-20, Chemicon International), anti-phospho-smad1(ser463/465)/smad5(ser463/465)/smad8(ser426/428) rabbit polyclonal (Cell Signaling Technologies), anti-cyclinD1 mAB (Santa cruz Biotechnology) and rabbit antisera to protein kinase C-α (Sigma). Secondary antibodies were biotin conjugated anti-mouse IgG (goat), Cy2-conjugated anti-rabbit IgG and Cy3-conjugated anti-mouse IgG (Jackson Immuno Research Laboratories) in appropriate combination with primary antibodies. For detection of biotin-conjugated antibodies, specimens were incubated with peroxidase-conjugated streptavidin (Vestacain ABC kit; Vector Laboratories), followed by chromogenic reactions using diaminobenzidine (0.6 mg/ml in 50 mM Tris-Cl, pH=7.6) as a substrate in the presence of 0.03% H2O2.

Analyses for cell proliferation and cell death

Pregnant mice were injected intraperitoneally with 1 ml/100 g body weight of Cell Proliferation Labeling Reagent (Amersham) 2 hours before embryo dissection. Sections of BrdU-labeled embryos were processed for immunohistochemical analysis using an anti-BrdU monoclonal antibody (Chemicon International). A biotin-conjugated anti-mouse secondary antibody, bound by extravidin-FITC was used for fluorescence detection. BrdU-positive cells were counted manually, and expressed as a percentage of total cell number. TUNEL apoptosis detection was performed using Apoptag Cell Death Detection Kit (Chemicon International) according to the manufacturer’s instructions.

Retinotopic mapping analyses

Anesthetized P7-P10 pups were injected with Dil (Molecular Probes) applied focally to dorsal or ventral region of the retina using a Nanoject (Drummond). For each labeling, ~9.2 nl of 10% Dil dissolved in dimethyl formamide was injected. The brain of labeled pups was dissected 2 days after injection. The superior colliculus was
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Results

Bmpr1a function is not essential for retinal development

Bmpr1a is expressed ubiquitously during early embryonic development (Mishina et al., 1995), including the developing eye (Furuta and Hogan, 1998). Mice homozygous for a Bmpr1a-null mutation die prior to gastrulation (Mishina et al., 1995). To directly test a role for this receptor in the developing retina, we specifically disrupted Bmpr1a gene function using the Six3Cre transgene, which drives Cre recombinase expression in the retina and ventral forebrain (Furuta et al., 2000). The conditional Bmpr1a allele results in a null allele upon Cre-mediated recombination (Bartlett et al., 2002). In our mating scheme, we employ a combination of the null allele (Mishina et al., 1995) and conditional allele; mice transheterozygotes for these alleles (referred to as Bmpr1a–/fx below) are thus hemizygous for the Bmpr1a locus prior to Cre-mediated recombination.

Animals lacking retinal Bmpr1a function (Bmpr1a<sup>Δε</sup> in the Six3Cre transgenic background; referred to as Bmpr1a<sup>Δε,Cre</sup> below) develop normally without overt ocular abnormalities. In the adult retina, the characteristic layered structure is retained in the mutant (Fig. 1A), without significant qualitative changes in the distribution of major retinal cell types examined (data not shown). Bmp signaling is implicated in dorsoventral patterning of the retina, which is reflected later in the retinotectal projection maps formed by retinal ganglion cell axons.

We analyzed the trajectory of retinal ganglion cell axons from the retina to the superior colliculus (mid-brain) using focal injection of the lipophilic dye, DiI. In wild-type mice, dorsal retina-derived ganglion cell axons project to the lateralposterior quadrant of the contralateral mid-brain (e.g. Fig. 1B, left) and ventral ganglion cell axons, to the medioanterior domain (not shown). In the mutants, the dorsal axons project to their termination zones in a wild-type pattern, suggesting that retinotectal projections are unaffected (Fig. 1B, right). Genomic PCR analyses confirm extensive Cre-mediated recombination of the Bmpr1a<sup>Δε</sup> allele throughout the differentiated retina in the adult (Fig. 1C,D), suggesting that the retina of Bmpr1a conditional mutants is entirely composed of Bmpr1a-deficient cells. As discussed below (Fig. 3), loss of Bmpr1a function appears to take place early during retinal development. Therefore, we conclude that Bmpr1a function is not essential for gross patterning, morphogenesis and differentiation of the developing retina.

Both Bmpr1a and Bmpr1b function to maintain dorsoventral patterning of the retina

Transcripts of another Bmp type 1 receptor, Bmpr1b, are present in a ventral-high-to-dorsal-low gradient in the developing retina (Furuta and Hogan, 1998). Bmpr1b null mutant mice are viable, although they exhibit defects in chondrogenesis and female fertility (Baur et al., 2000; Yi et al., 2000). These animals also exhibit abnormal ventral retinal ganglion cell axon behavior (Liu et al., 2003). To test whether Bmpr1a and Bmpr1b function redundantly in the retina, a null allele of Bmpr1b was introduced into the conditional Bmpr1a background. Animals lacking Bmpr1a and one copy of Bmpr1b (Bmpr1a<sup>Δε</sup>;Bmpr1b<sup>+/−</sup>;Cre) have normal eye size (Fig. 2A) and layered retinal morphology (Fig. 2B), without obvious changes in the distribution of major retinal cell types (Fig. 2C-E). However, analyses of retinotectal mapping revealed that in all mutant animals examined, many dorsal retinal ganglion cell axons form ectopic termination zones (Fig. 2F, bottom, arrows). The transmembrane receptor tyrosine kinases EphBs and the ephrin B (Efnb – Mouse Genome Informatics) ligands are expressed in countergradients along the retinal dorsoventral axis, and are implicated in ganglion cell axonal guidance during retinotectal map formation (McLaughlin et al., 2003). At embryonic day 17.5 (E17.5), dorsal Efnb2 expression is completely abolished in the Bmpr1a<sup>Δε</sup>;Bmpr1b<sup>+/−</sup>;Cre embryos (Fig. 2G). Conversely, the ventral-high-to-dorsal-low gradient of Ephb2 is no longer seen, with uniform transcript

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**Fig. 1. Bmpr1a function is not required for embryonic retinal development.** Control samples are from Bmpr1a<sup>Δε</sup>;Cre mice and mutants are from Bmpr1a<sup>Δε</sup>;Cre mice. (A) Hematoxylin and Eosin stained sections of the adult retina from 3-month-old mice. Retinal lamination pattern and various mutant alleles. (B) Genomic PCR to detect Bmpr1a alleles using primers indicated in C. The genotypes of the sample DNAs are indicated at the top. In the retina of adult mice carrying the Cre transgene, the amplicon corresponding to the unrecombined conditional allele is readily detected. Consistent with recombination of the floxed allele occurring only in the retina, the dE2 amplicon, which represents the recombined allele with exon 2 removed, is present only in the retina of mice carrying the Cre transgene. gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer; rpe, retinal pigment epithelium.
levels throughout the retina (Fig. 2H). Earlier during development (E12.5), expression of a T-box transcription factor, Tbx5, implicated in dorsal specification of the retina (Koshiba-Takeuchi et al., 2000), is abolished (Fig. 2I). Furthermore, the expression domain of a ventral marker Vax2 (Barbieri et al., 1999) is expanded throughout the entire retina (Fig. 2J). These observations are consistent with the notion that dorsal retinal character is lost in these mutants.

Bmp4 expression in the optic vesicle initiates at around the 12-somite stage in the mouse and concomitant with lens formation, becomes progressively localized to the dorsal quadrant of the optic cup (Furuta and Hogan, 1998). Initiation of Tbx5 expression at around the 16-somite stage (Bruneau et al., 1999) is thus preceded by that of Bmp4. In Bmp4<sup>−/−</sup> mutants, Tbx5 expression in the optic vesicle is absent from the 16-somite to >25-somite stages (Fig. 3A). This indicates that among multiple Bmps expressed in the developing mouse eye, Bmp4 is the primary mediator of dorsal-specific gene expression. Although dorsoventral patterning is disrupted in Bmpr1a<sup>−/−</sup>;Bmpr1b<sup>−/−</sup>;Cre embryos by E12.5, Tbx5 expression appears normal until E10.5 (Fig. 3B). However, its expression is rapidly downregulated, and completely lost by E11.5 (Fig. 3C). These observations strongly indicate that a certain level of Bmp signaling is required not only for the induction, but also for the maintenance of Tbx5 expression. Moreover, this loss of Tbx5 expression between E10.5 and E11.5 probably reflects the timing of loss of Bmpr1a function as a result of Cre-mediated recombination of the Bmpr1a<sup>−/−</sup> allele. This is further supported by the distribution of phosphorylated Smad1/5/8 protein (P-Smad) in the retina, which is a direct read-out of active Bmp signaling (Massague, 1998). High levels of P-Smad are detected in the dorsal retina of control animals but not in the mutant retina at E11.5 (Fig. 3D).

The retinoic acid signaling pathway is also implicated in the regulation of retinal dorsoventral patterning (Hyatt et al., 1996; Marsh-Armstrong et al., 1994; McCaffery et al., 1999). Genes coding for enzymes regulating retinoic acid synthesis, including Rldh1a1 for Raldh1 and Rldh1a3 for Raldh3, are differentially expressed along the dorsoventral axis of the retina (Fig. 4A,C). It has been suggested that the differential expression of these genes along this axis may be regulated by a Bmp4-Tbx5 pathway (Mic et al., 2002). However, the expression of these genes appears unaffected in Bmpr1a<sup>−/−</sup>;Bmpr1b<sup>−/−</sup>;Cre mutants (Fig. 4B,D).

Bmp signaling is essential for retinal growth and neurogenesis

To address the absolute requirement of Bmp signaling in the retina, we also generated mutants that completely lack function of both Bmpr1a and Bmpr1b in the retina. The Bmpr1a<sup>−/−</sup>;Bmpr1b<sup>−/−</sup>;Cre double mutants appear indistinguishable from the normal littermates until E10.5 (not shown). At birth, however, these double null mutants exhibit anophthalmia (Fig. 5A). Gross morphological abnormalities are apparent by E12.5, when double mutant embryos show smaller eyes with a rough margin of the retinal pigment epithelium and discontinuity of the pigment ventrally (Fig. 5B,C). Histological examination of double mutant retinas revealed that the abnormal retinal morphology first manifests as reduced retinal neuroectoderm thickness beginning at E11.25-11.50 (not shown), associated with excess apoptosis (Fig. 5D).
During mouse embryonic development, retinal neurogenesis initiates at around E11.5, the time at which the abnormal phenotypes of the double mutant retina become first apparent. Initiation of neurogenesis is associated with the expression of Math5, a mouse homologue of the Drosophila pro-neural gene, atonal. Math5 expression gradually expands from center to periphery to represent a wave of retinal ganglion cell differentiation (Brown et al., 1998; Brown et al., 2001). In Bmpr1α−/−;Bmpr1β−/−;Cre double mutants, Math5 expression is not initiated in the retina at E11.5 (Fig. 6E). A potential target gene of Math5 is Brn3b, which encodes a POU-homeodomain-containing transcription factor required for normal retinal ganglion cell differentiation (Liu et al., 2001; Wang et al., 2001). Consistent with the loss of Math5 expression in the double null mutants, expression of Brn3b is not initiated in the mutant retina (Fig. 6F). By contrast, Pax6, which is required for the expression of a number of neurogenic bHLH genes (including Math5) and in the regulation of retinal progenitor population (Marquardt et al., 2001), is expressed in the double mutant retina at E11.5 (Fig. 6G) and E12.5 (not shown). We next examined general neuronal markers to determine if these cells had any neural
character. Some βIII-tubulin-positive cells were detected in the mutant retina (Fig. 6H), although they were not restricted to a central domain.

Bmp signaling is required for the expression of Fgf15 in the developing retina

Fibroblast growth factors constitute another class of secreted signaling molecules that have potential functions in retinal development (see Discussion). These include the regulation of cell survival and proliferation, as well as retinal neurogenesis. As these processes are affected in the Bmp receptor mutants, it is possible that Fgfs act as mediators of Bmp signaling in this regard. One of the Fgf family members expressed specifically within the optic vesicle is Fgf15 (McWhirter et al., 2003).

Fig. 5. Defects in retinal growth in Bmpr1a<sup>αβ</sup>;Bmpr1b<sup>λ+</sup>;Cre double mutant mice. (A) Side views of neonates. The double null mutant has an anophthalmic phenotype (bottom). (B) Side views of the eyes of E12.5 embryos. Reduced eye size and incomplete closure of the retinal pigmented epithelium ventrally are seen in the double mutant (bottom). (C) Coronal sections of embryos shown in B. (D) Cell-death analyses at E11.5 indicate a significant increase in TUNEL-positive cells (green) in the mutant retina (counterstained with propidium iodide). Although there is no significant change in the percentage of BrdU-positive cells up to E11.5 (green/yellow cells) (E-G), there is a rapid reduction of cell proliferation by E12.5 (F,G; broken line in F outlines the degenerating mutant retina; a relatively large error bar in the E11.5–12.5 mutant group reflects a spectrum of phenotypic severity during these stages). le, lens; nr, neuroretina. Scale bar: 500 µm in B; 200 µm in C; 100 µm in D-F.

Fig. 6. Bmpr1a<sup>αβ</sup>;Bmpr1b<sup>λ+</sup>;Cre double null mutants display a range of defects in the expression of retinal marker genes. Analyses of gene and protein expression in coronal sections of embryonic retina at the indicated stages by radioactive in situ hybridization (A,C,D), DIG in situ hybridization (E,F,G) or immunostaining (B,H). (A) At E11.5, expression of Chx10 is attenuated in the mutant retina (right) compared with control. (B) CyclinD1 is specifically absent in the mutant retina (right), whereas protein expression in the adjacent lens tissue is maintained. (C,D) Lhx2 and Rx, two early pan-retinal markers, are expressed in the mutant retina at comparable levels with the control, despite the apparent degenerative phenotypes at this stage. (E) The mutant retina fails to initiate retinal neurogenesis at E11.5 as indicated by the loss of Math5 expression. Arrows indicate comparable Math5 expression both in the diencephalons of both control and mutant. This is not simply a developmental delay, as Math5 is absent even at E12.5 (not shown). (F) Brn3b, a downstream target of Math5 expressed in the newly born ganglion cells in the central retina, fails to be induced in the mutants. (G) Expression of Pax6 is maintained in the mutant. (H) Neuronal tubulin expression is detected in the mutant retina. le, lens; nr, neuroretina; rpe, retinal pigment epithelium. Scale bar: 100 µm.
Fig. 7. Fgf15 is a potential downstream target of Bmp signaling in the retina. (A) Fgf15 is strongly expressed in the distal optic vesicle at E9.0-9.5 in a wild-type embryo. (B) The expression of Fgf15 is absent in the eye (arrowhead) of an advanced Bmp4+/− mutant embryo with equivalent number of somites to the wild-type embryo shown in A. (C,D) Application of BMP4-soaked beads (asterisks) restores the expression of Fgf15 in the distal optic vesicle of Bmp4+/− by 18 hours in culture (C, arrowhead). Under the same condition, BSA fails to restore the expression of Fgf15 (D, arrowhead). Arrows indicate the future retinal pigmented epithelium slightly displaced anteriorly during explant culture. (E) Fgf15 expression persists through later stages as shown for an E11.5 normal retina (Bmpr1a+/−;Bmpr1b+/−;Cre control). (F) In Bmpr1a−/−;Bmpr1b−/−;Cre double mutant, the expression of Fgf15 is absent (arrows). le, lens; ov, optic vesicle. Scale bar: 50 μm in A-D; 100 μm in E,F.

In wild-type embryos, the expression of Fgf15 initiates at around the 15-somite stage (not shown), a few hours following the onset of Bmp4 expression in the prospective retinal neuroectoderm. Fgf15 expression is upregulated subsequently, and persists throughout embryonic retinal development (not shown). First, to determine whether the Fgf15 expression is dependent on Bmp signaling, we examined Bmp4−/− mutant embryos for Fgf15 transcripts. In the absence of Bmp4, Fgf15 expression is drastically reduced or absent in the optic vesicle (Fig. 7B). Furthermore, in explant cultures, the expression of Fgf15 can be restored by exposure of the optic vesicle of Bmp4−/− mutants to Bmp4 protein carried by beads (Fig. 7C). Consistent with these observations, the expression of Fgf15 is diminished in the retina of Bmpr1a+/−;Bmpr1b−/−;Cre double mutants at E11.5 (Fig. 7E,F). Thus, Bmp signaling largely mediated by Bmp4 is essential to induce the expression of Fgf15 in the early developing retina. Bmp4 cannot induce Fgf15 expression in the retinal pigmented epithelium, suggesting that this regulation may be permissive rather than instructive.

Discussion

Using conditional inactivation of Bmp type I receptor function to disrupt Bmp signaling in a cell-autonomous manner, our analyses have revealed the in vivo role for Bmp signaling in mouse retinal patterning, thus validating previous gain-of-function studies in other vertebrate systems. In addition, they have uncovered a unique role of Bmp signaling in maintaining growth and differentiation of the developing retina in the mouse.

Bmpr1a and Bmpr1b play redundant roles during retinal development

Our studies provide the first direct genetic evidence for functional redundancy between the two Bmp type I receptors, Bmpr1a and Bmpr1b. These two receptors share a high degree of sequence similarity and are both capable of binding to Bmp2, Bmp4 and Bmp7 ligands, albeit with varying affinities (Mishina, 2003). However, previous studies indicate that they may possess distinct biological functions. A study using forced expression of constitutively active Bmpr1a and Bmpr1b receptors, both in transgenic mice and in neural stem cells, suggested a role for Bmpr1a in regulating proliferation in the developing central nervous system and for Bmpr1b in regulating apoptosis and terminal differentiation (Panchision et al., 2001). Similarly, in chick limb bud development, expression of constitutively active and dominant-negative forms of the two receptors revealed a role for Bmpr1b in mediating formation of the initial cartilaginous skeleton, and for Bmpr1a in controlling the later differentiation process (Zou et al., 1997). However, during chick neural tube patterning, expression of constitutively active forms of the two receptors revealed no significant qualitative differences in the cellular responses (Timmer et al., 2002). The apparent discrepancies in these studies may be attributable, in part, to differences in the experimental models and tissue or cell types examined. With respect to the phenotypes described here, the lack of retinal abnormalities in the conditional Bmpr1a null mutants or Bmpr1b mutants in comparison with the Bmpr1a/Bmpr1b double null mutant retina indicates the functional redundancy between the two receptors in vivo. One broad implication of the current study is that Bmpr1a and Bmpr1b may also play redundant roles in other tissues. For example, analysis of Bmpr1a function in the embryonic telencephalon revealed its requirement only in the dorsal midline, a domain where Bmpr1b is not expressed, unlike the more lateral telencephalic regions where the expression of Bmpr1a and Bmpr1b overlap (Hebert et al., 2002). Thus, it is likely that disruption of both receptors in the telencephalon may reveal a more extensive requirement for Bmp signaling. Furthermore, this general principle may be extended to receptors for other subgroups of the TGFβ superfamily ligands. For example, canonical TGFβ ligands can bind to both Tgfbr1 (Alk5) and Acrvl1 (Alk1), and activins can bind to Acrv1b (Alk4) and Acrv1 (Alk2). We do note that the degree of functional overlap between Bmpr1a and Bmpr1b may vary depending on the developmental context. For example, within the eye itself, optic nerve head axonal guidance may require only Bmpr1b function (Liu et al., 2003). Dorsoventral patterning defects are present in Bmpr1a−/−;Bmpr1b+/−;Cre, but not in Bmpr1a−/−;Bmpr1b−/−;Cre mutants, suggesting that the two receptors are not completely equivalent. Such differences could arise from varying temporal/spatial expression domains, levels of expression and/or biochemical properties.

Bmp signaling is necessary for the patterning of the retinal dorsoventral axis

The requirement for Bmp signaling in dorsal retina specification is analogous to its role in dorsal neural tube patterning and dorsal forebrain differentiation in vertebrates.
(Furuta et al., 1997; Hebert et al., 2002; Timmer et al., 2002), suggesting that this function is conserved in different parts of the developing nervous system. One potential implication is a conservation of the underlying genetic mechanisms, such as interactions with the Shh-mediated patterning activity that occurs in the developing neural tube. In the chick optic vesicle, reducing endogenous Shh activity results in an expansion of Bmp4 expression (Zhang and Yang, 2001), suggesting opposing roles of Bmp and Shh signaling in retinal dorsoventral patterning.

Our study has identified the bona fide receptors that mediate the Bmp signaling in retinal dorsoventral patterning. The genetic evidence presented in this study indicates that the dorsal Bmp signal intersects with the ventral pathway at the level of or upstream of Vax2 expression (Fig. 8D). In Vax2 mutants, Tbx5 expression, a downstream readout of Bmp signaling, is unaffected (Barbieri et al., 2002; Mui et al., 2002), whereas loss of Tbx5 expression in Bmpr1a<sup>−/−</sup>;Bmpr1b<sup>+/−</sup>;Cre mutant animals results in expansion of the Vax2-positive domain. In the Bmp receptor mutant animals, asymmetric expression patterns of Bmp4 and Bmpr1b appear unaffected (data not shown), suggesting that Bmp signaling is one of the most upstream events in dorsal retinal cell fate specification.

Taken together, one possible model is that the ventral program is a default state, and Bmp signaling provides the instruction to initiate the dorsal program. Although the mechanisms underlying the gradient expression patterns of Eph-Ephrin molecules remain unclear, the Bmpr1a<sup>−/−</sup>;Bmpr1b<sup>+/−</sup>;Cre mutant animals, as well as Vax2-null mice (Barbieri et al., 2002; Mui et al., 2002), will provide useful genetic tools for future investigations of topographic map formation.

Some aspects of dorsoventral asymmetry are maintained in the Bmpr1a<sup>−/−</sup>;Bmpr1b<sup>+/−</sup>;Cre. Although retinoic acid activity in the Bmpr1a<sup>−/−</sup>;Bmpr1b<sup>+/−</sup>;Cre retina has not been directly analyzed, the differential expression of Raldh1 and Raldh3 along the dorsoventral axis at least, is independent of Bmp-mediated patterning within the retina. Our results thus favor a model wherein the asymmetric retinoic acid activity in the early optic vesicle acts upstream of dorsal Bmp4 expression in the retina. Alternatively, these two pathways may function independent of each other. It is not currently clear whether the potential retinoic acid activity gradient is translated into any aspect of dorsoventral patterning in the retina (McLaughlin et al., 2003).

**Bmp signaling regulates cell survival during retinal growth**

Bmps have been implicated in the regulation of cell proliferation and cell death in various developmental contexts, including the developing retina (Trousse et al., 2001). The current genetic studies have uncovered potential downstream effectors of Bmp signaling in these processes. In the Bmpr1a<sup>−/−</sup>;Bmpr1b<sup>+/−</sup>;Cre double homozygous mutants retinal structures are specified initially, but cell proliferation and cell death are severely affected during mid-gestation. A previous study has shown that Chx10 and cyclin D1, two genes whose expression is affected in the double mutants, may have a regulatory relationship in the retina (Chang et al., 2003). The phenotypes of individual homozygous mutants for Chx10 and cyclin D1 include microphthalmia and photoreceptor degeneration, respectively (Burmeister et al., 1996; Sicinski et al., 1995), however, most retinal neurons are present in the eye of these mutants. The Bmpr1a<sup>−/−</sup>;Bmpr1b<sup>+/−</sup>;Cre double null mutant phenotype is more severe possibly due to the combinatorial loss of Chx10 and cyclin D1.

The Bmpr1a<sup>−/−</sup>;Bmpr1b<sup>+/−</sup>;Cre double mutants show
increased levels of apoptosis from E11.25, possibly owing to a lack of survival signals to the retinal progenitor cells. Although Bmps themselves may act as survival factors in the eye, fibroblast growth factors are also candidate mediators of Bmp signaling in this regard. Within the eye, they are expressed in the surface ectoderm (Fgf1, Fgf2), the neuroretina (Fgf15, Fgf9) (Nguyen and Arnheiter, 2000; Zhao et al., 2001) and the central retina-optic stalk region (Fgf8) (Chow and Lang, 2001). Several studies have implicated Fgfs in the specification and survival of the neuroretina (Lillien and Cepko, 1992; Park and Hollenberg, 1989; Pittack et al., 1997). Fgf signal transduction per se may operate through the Ras/MAPK or the PI-3 kinase/Akt pathway, which, in turn, are known to regulate cell survival by transcription-dependent and -independent inhibition of apoptosis (Brunet et al., 2001; Powers et al., 2000). Thus, the loss of Fgf15 signaling may, in part, contribute to retinal degeneration resulting from apoptosis in the Bmpr1a<sup>b+/c</sup>;Bmpr1b<sup>b+/c</sup>;Cre double mutants.

A novel role for Bmp signaling in retinal neurogenesis

Despite well-characterized roles of signaling molecules, including hedgehog (Hh) and Dpp, in Drosophila retina neurogenesis, the signaling events upstream of mouse retinal neurogenesis remain elusive. In particular, the requirement of Hh in retinal ganglion cell specification (i.e. induction of atonal homologs) is unclear (Stenkamp and Frey, 2003). Analysis of Bmpr1a<sup>b+/c</sup>;Bmpr1b<sup>b+/c</sup>;Cre double mutants has revealed a novel function for Bmp signaling in retinal neurogenesis. Although we observe abnormal neuronal tubulin expression in the E12.5 double mutant retina, these labeled cells are not positive for early pro-neural genes such as Math5 (Fig. 6) and Neurod1 (data not shown), or for neurofilament (data not shown). The rapid degeneration of the double mutant retina subsequent to E12.5 precludes analysis of later-born neurons. We therefore conclude that Bmp signaling is required for early neurogenesis in the mouse retina. This is in contrast to the observation that initiation of neurogenesis and expression of atonal in the Drosophila eye can occur in the absence of Dpp signaling (Fu and Baker, 2003). Given that Pax6 expression is maintained in the absence of neurogenesis, it is likely that Bmp signaling intersects with the Pax6-mediated pathway to initiate differentiation of retinal progenitor cells. In this context too, the effects of Bmp signaling on pro-neural gene expression may involve an Fgf receptor-Ras/MAPK signal. Exogenous FGFS are reported to be capable of inducing ganglion cell markers in retinal explants (Guillemot and Cepko, 1992). This model is also analogous to that in Drosophila where the Egfr-Raf-MAPK pathway has been implicated in the induction of atonal (Greenwood and Struhl, 1999; Kumar and Moses, 2001). There also remains the possibility that this abnormality in retinal differentiation is tightly coupled to the decreased growth in the mutant retina (Fig. 8E). The possible causative regulatory relationships between these multiple developmental processes are the subject for future studies.

Different threshold requirements for Bmp signaling in various aspects of retinal development

Our in vivo genetic analysis has generated novel insights regarding the mechanisms by which Bmp signaling regulates various aspects of retinal development. Given the range of Bmp ligands and receptors expressed in the developing retina (Fig. 8A,B), in principle, potentially distinct signaling properties of individual ligands and receptors may underlie different cellular responses. Our data are more consistent with an alternative model wherein different aspects of retinal development, such as patterning and morphogenesis, are regulated at distinct threshold levels of Bmp signaling input (Fig. 8D,E). Our results strongly indicate the existence of at least two threshold levels of Bmp signaling. First, specification of the dorsal retina requires relatively high levels of Bmp signaling that cannot be maintained by only one functional allele of Bmpr1b (Fig. 8D). The observations that the wild-type dorsal retina expresses high levels of both Bmp4 ligand (Furuta and Hogan, 1998) and P-smad protein (Fig. 3D) corroborate this idea. Second, complete elimination of the function of both Bmpr1a and Bmpr1b receptors in double null mutants leads to catastrophic effects in the developing retina, including defects in retinal proliferation and retinal neurogenesis. These data suggest the presence of a lower threshold of signaling throughout the retina that can maintain overtly normal retinal morphogenesis and differentiation in the Bmpr1a<sup>b+/c</sup>;Bmpr1b<sup>b+/c</sup>;Cre or Bmpr1a<sup>b+/c</sup>;Bmpr1b<sup>b+/c</sup>;Cre mutants. A recent study using Noggin overexpression in chick has shown a requirement for Bmp signaling in the development of the ventral optic cup (Adler and Belecky-Adams, 2002). In these experiments, inhibition of Bmp signaling resulted in a range of abnormalities, including microphthalmia, ventral retinal colobomas, altered expression of dorsoventral markers and optic nerve pathfinding defects similar to those reported for the Bmpr1b<sup>b+/c</sup> mutants (Liu et al., 2003). These ventral retina defects may represent a phenotype intermediate between the dorsoventral patterning defects and the retinal degeneration described in our system, indicating possible existence of a third threshold of Bmp signaling during retinal development. The mechanisms by which individual cells divide the overall receptor-mediated signaling into graded units of activities and assign them to elicit qualitatively distinct genetic programs may have implications for other growth factor signaling systems.

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Note added in proof

During the review of our revised manuscript, a study revealing redundant roles of Bmpr1a and Bmpr1b in the developing mouse neural tube was published (Wine-Lee et al., 2004).

References


factors are necessary for neural retina but not pigmented epithelium differentiation in chick embryos. Development 124, 805-816.


