Proper patterning of the optic fissure requires the sequential activity of BMP7 and SHH

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The optic disc develops at the interface between optic stalk and retina, and enables both the exit of visual fibres and the entrance of mesenchymal cells that will form the hyaloid artery. In spite of the importance of the optic disc for eye function, little is known about the mechanisms that control its development. Here, we show that in mouse embryos, retinal fissure precursors can be recognised by the expression of netrin 1 and the overlapping distribution of both optic stalk (Pax2, Vax1) and ventral neural retina markers (Vax2, RaIdh3). We also show that in the absence of Bmp7, fissure formation is not initiated. This absence is associated with a reduced cell proliferation and apoptosis in the proximoventral quadrant of the optic cup, lack of the hyaloid artery, optic nerve aplasia, and intra-retinal misrouting of RGC axons. BMP7 addition to organotypic cultures of optic vesicles from Bmp7–/– embryos rescues Pax2 expression in the ventral region, while follistatin, a BMP7 antagonist, prevents it in early, but not in late, optic vesicle cultures from wild-type embryos. The presence of Pax2-positive cells in late optic cup is instead abolished by interfering with Shh signalling. Furthermore, SHH addition re-establishes Pax2 expression in late optic cups derived from oculo retardation (or) embryos, where optic disc development is impaired owing to the near absence of SHH-producing RGC. Collectively, these data indicate that BMP7 is required for retinal fissure formation and that its activity is needed, before SHH signalling, for the generation of PAX2-positive cells at the optic disc.

KEY WORDS: Mouse, Optic fissure, BMP, SHH

INTRODUCTION

The optic disc (OD or blind spot of the retina) is the region of the retina where the optic fibres converge to become part of the optic nerve. This structure constitutes the interface between the optic stalk and the retina, and develops from the edges of the optic fissure, a transient groove visible at the ventral pole of the eye rudiment. The optic fissure can be divided into two adjoining parts, the retinal fissure (RF) and the optic groove, which derive from the progressive invagination of the ventral surface the optic vesicle and stalk, respectively. The transition between the RF and the optic groove dictates the position where the OD will form. The optic fissure enables the entrance of surrounding mesenchymal cells into the developing eye chamber, which will form the hyaloid artery, a main blood supply for the eye (Barishak, 1992). Soon after these cells have migrated in, the fissure closes beginning from its central region and proceeding posteriorly and anteriorly. As the RF seals, the first retinal axons egress from the eye, occupying a small crescent just dorsal to the hyaloid artery (Silver and Robb, 1979). As a result of these morphogenetic events, both axons and artery at the nascent OD become encircled by a ring of compact neuroepithelial cells, characterised by the expression of the paired-boxed transcription factor Pax2 (Otteson et al., 1998) (Fig. 2A). These neuroepithelial cells have a long-standing recognised role in retinal ganglion cell (RGC) axon guidance (Ramon y Cajal, 1892) and are the source of different cell surface and secreted axon guidance cues, including laminin, NCAM or netrin 1, which are known to orient the growth of RGC axons towards the disc and then into the optic nerve (Stuermer and Bastmeyer, 2000).

In spite of the importance of the OD, many of the basic questions relating to its development still remain unanswered. For example, it is unclear if the PAX2-positive OD precursors have a unique identity that distinguishes them from the remaining PAX2-positive cells of the optic stalk and/or if they share characteristics with cells of the remaining neural retina. Equally unclear is whether there are specific cellular and molecular determinants that control its formation, as most of our fragmentary knowledge is related to the development of the optic fissure in general.

An increased proliferation rate (Calvente et al., 1988) and an extensive programmed cell death in the ventral optic vesicle (Cuadros and Rios, 1988; Ozeki et al., 2000) are two events that specifically accompany optic fissure formation. The asymmetric expression of several regulatory genes seems also at the basis of ventral optic vesicle specification (Peters, 2002) and hence of optic fissure formation. Among these, the homeodomain-containing transcription factors Pax2 (Torres et al., 1996), Vax1 (Bertuzzi et al., 1999) and the related Vax2 (Barbieri et al., 1999) appear particularly important. Genetic inactivation of either Pax2 or Vax1 strongly interferes with optic stalk development, although the initial formation of the optic fissure is unperturbed (Torres et al., 1996; Bertuzzi et al., 1999). The effects of Vax2 inactivation are instead more closely related to the establishment of dorsoventral polarity of the optic cup (Barbieri et al., 2002; Mui et al., 2002). However, a common feature of the three mouse lines is the presence of an eye coloboma or failure of optic fissure closure (Torres et al., 1996; Bertuzzi et al., 1999; Barbieri et al., 2002), indicating that both ventral retina and optic stalk specific genes contribute to the proper development of the fissure. Furthermore,

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Accepted 14 June 2006
the activity of Pax2, Vax1 and Vax2 contribute to the segregation of the optic stalk from the optic cup domain, at least in part, via repression of the Pax6 gene, an optic cup determinant (Schwarz et al., 2000; Mui et al., 2005).

Besides these cell-autonomous regulators, embryological studies, based on transplantations of 180° rotated optic vesicles in amphibian and chick, postulate the existence of ‘inducers’ of optic fissure formation (Sato, 1933; Silver, 1977; Uemonsa et al., 2002). These ‘inductive’ signals appear to act gradually in the early optic vesicle with a crucial activity at the 10-somite stage, when transplantations of rotated vesicles result in no fissure formation (Uemonsa et al., 2002) and the development of embryos with a microphthalmic eye and no optic nerve (Silver, 1977). It has been proposed that sonic hedgehog (SHH), a member of the hedgehog family of secreted glycoproteins (Marti and Bovolenta, 2002), and bone morphogenetic protein 7 (BMP7), a member of the TGFβ superfamily of signalling factors (Chen et al., 2004), emanating from the prechordal mesoderm, might be likely candidates as ‘fissure inducers’ (Uemonsa et al., 2002), as both factors interact to specify other prosencephalic ventral structures (Dale et al., 1997). Midline-derived SHH is generally involved in the early specification of ventral eye structures by controlling the expression of Pax2 and Vax genes (reviewed by Yang, 2004), but there is no evidence that its signalling is specifically needed for optic fissure formation. Only later in development, when the OD has already formed, is SHH, derived from differentiated RGC, reported to control the development of the OD PAX2-positive cells (Dakubo et al., 2003). Here, we have analysed whether BMP7, the other possible fissure ‘inducer’, might be relevant for early OD development.

BMP genes, particularly Bmp4 and Bmp7, have been implicated at different step of eye development. BMP4, which is expressed in the dorsal retina, has a fundamental role in establishing dorsoventral polarity of the eye cup and controls local proliferation and programmed cell death (Koshiba-Takuechi et al., 2000; Sakuta et al., 2001; Troussse et al., 2001). Different lines of Bmp7-null mice present microphthalmia or a variably penetrant anophthalmic phenotype owing to the requirement of BMP7 at early steps of lens induction (Dudley et al., 1995; Luo et al., 1995; Wawersik et al., 1999), a limiting step in the progression of eye development. Reduction of BMP receptor activity indicates that BMP signalling is also involved in the growth and differentiation of the neural retina (Murrali et al., 2005). Overexpression of the BMP antagonist noggin in the ventral part of the chick optic cup causes coloboma, pecten agenesis and ectopic expression of optic stalk markers in the ventral retina (Adler and Belecky-Adams, 2002), suggesting that BMP signalling does contribute also to ventral eye development.

Consistent with this idea, we show that Bmp7 is necessary at early steps of eye development to initiate RF formation through the activation of cell proliferation and apoptosis in the proximodorsal quadrant of the optic cup. In Bmp7-null mice affected by a microphthalmic phenotype, failure of initiating RF morphogenesis leads to the absence of the hyaloid artery, agenesis of the optic nerve and defects in RGC axon growth. Addition of BMP7 to organotypic cultures of Bmp7−/− optic vesicles rescues the expression of Pax2, a marker for RF precursors. Conversely, the Bmp7 inhibitor follistatin abrogates it in early but not late optic vesicle cultures, when instead SHH is required. On the basis of these results, we propose that BMP7 is necessary for RF (and hence optic nerve) formation and that its activity is needed, before that of SHH, for the generation of PAX2-positive cells at the OD.
Optic cup organotypic cultures
Slices of E9.5-E10 embryonic heads containing the optic primordia were obtained by manual dissection and stored in cold DMEM/F12. The tissue slices were layered on polycarbonate-membrane inserts (Falcon) and cultured in DMEM/F12 supplemented with N2 for 48 hours in the presence or absence of the following reagents used at 1 μg/ml: BMP7 (R&D), Follistatin (R&D), N-SHH (Biogen) and the anti-Shh blocking monoclonal antibody 5E1 (Developmental Studies Hybridoma Bank). Isolated optic cups freed from the RPE were dissected from E12.5 embryos, layered on polycarbonate membranes with the lens upwards and cultured as above. In all conditions, the development of the OD was assessed by whole-mount in situ hybridisation with a Pax2-specific probe.

RESULTS
Absence of Bmp7 impairs optic fissure development
Different observations suggest that Bmp7 might be necessary for optic fissure formation (Uemona et al., 2002). Bmp7 is expressed in the ventral midline and in the proximal region of the developing optic vesicle and then optic cup, particularly associated with the pericocular mesenchyme forming the hyaloid artery (Dudley and Robertson, 1997) (see Fig. S1 in the supplementary material). The expression of different BMP receptors is abundant in RF precursors (Belecky-Adams and Adler, 2001; Trousse et al., 2001; Mishina, 2003), strongly suggesting that Bmp signalling activation may be relevant for RF development. Furthermore, genetic inactivation of Bmp7 leads to eye defects that, in the milder cases, are characterised by microphthalmia (Dudley et al., 1995; Luo et al., 1995), a phenotype also reported in association with the absence of the optic fissure (Silver, 1977). Many of the developmental defects present in Bmp7-null mice have been previously described (Dudley et al., 1995; Luo et al., 1995; Wawersik et al., 1999) but the microphthalmic phenotype has not been analysed in detail, leaving open the possibility of its link with defects in fissure formation.

To explore this possibility, we took advantage of an existing Bmp7-deficient mouse line (Godin et al., 1998) maintained in a C57/B16 genetic background, where the incidence of microphthalmia versus anophthalmia was higher (58%) than that reported in other backgrounds (Dudley et al., 1995; Wawersik et al., 1999). In all Bmp7−/− embryos analysed, eye development was normally initiated (see Fig. S3A,B in the supplementary material) (see also Dudley et al., 1995; Luo et al., 1995), but in 42% of the embryos it did not proceed beyond optic vesicle stage (Fig. 1B,E,H), presumably owing to the failure in lens placode induction and hence lens-vesicle interaction, as already reported (Dudley et al., 1995; Luo et al., 1995; Wawersik et al., 1999). Lens-derived signalling is also crucial for the initial regionalization of the optic vesicle into neural retina and retina pigmented epithelium (RPE) (reviewed by Martínez-Morales et al., 2004). Consistent with this idea, in the optic primordium of these embryos the lens placode was rudimentary (see Fig. S2 in the supplementary material) or absent (Fig. 1E) and the expression of neural retina markers was strongly downregulated, while that of RPE specific genes expanded (see Fig. S2 in the supplementary material), explaining the all-pigmented remnants observed at later stages (Fig. 1E). These features probably account for the anophthalmic phenotype already described in these mice (Dudley et al., 1995; Luo et al., 1995).

In the remaining Bmp7−/− embryos (58%, the only ones considered from now on in this study), these defects were not observed. The lens, neural retina and RPE developed normally although the entire optic cup was smaller (Fig. 1C,F,I; Fig. 4B) when compared with wild-type embryos (Fig. 1A,D,G; Fig. 4A). Similarly, in the mutants Pax6 expression was restricted to its normal domain but the signal appeared more uniformly distributed than in

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**Fig. 1.** Bmp7-null embryos with a microphthalmic phenotype lack a morphologically visible RF. (A-C) Lateral view of intact embryos and Cresyl Violet stained frontal (D-F) or parasagittal (G-I) sections from E12.5 (A-F) or E11.5 (G-I) wild-type (A,D,G) and Bmp7−/− mouse embryos affected by an anophthalmic (B,E,H) or microphthalmic (C,F,I) phenotype. The anophthalmic eye is rudimentary, consisting of a small heavily pigmented neuroepithelium with no evidence of the RF (arrowheads in E,H). Embryos with a microphthalmic phenotype show a well-developed optic cup but a complete absence of the RF (arrowheads in F,I) when compared with wild-type embryos (arrows in D,G). The optic stalk is composed of two juxtaposed neuroepithelial layers (arrow in F). Scale bar: 300 μm in A-C; 100 μm in D,F; 50 μm in E,G-I.
wild type (see Fig. S3B,C in the supplementary material). Notably, this microphthalmic phenotype was always associated with the absence of a morphologically visible RF (compare Fig. 1I, Fig. 2J with Fig. 1G, Fig. 2G) and OD (compare Fig. 2K,L and Fig. 4B with Fig. 2H,I and Fig. 4A). There were no morphological signs of a RF even at earlier stages of gestation (compare Fig. 2D-F with 2A-C; see Fig. S3E,H in the supplementary material), suggesting that its premature closure was an unlikely explanation for this phenotype and, instead, indicating that \textit{Bmp7} activity is required for the initial phases of optic fissure development.

To provide additional evidence that lack of the RF in \textit{Bmp7}–null mice results from a failure in its initial formation, we searched for molecular markers that could help us following the development of RF/OD precursors.

Previous studies have shown that \textit{Pax2} is expressed with a ventral-to-dorsal gradient in the mouse optic vesicle and stalk (see Fig. S3A in the supplementary material) (Nornes et al., 1990; Torres et al., 1990).
Fig. 3. OD precursors, characterised by the combined expression of Pax2, Vax1, Raldh3 and netrin 1 are absent in Bmp7-/- embryos. Frontal sections of the optic cup from wild-type (A,C,E,G) and Bmp7-/- (B,D,F,H) E12.5 embryos were immunostained with antibodies against Pax2 (A,B) or hybridised with specific probes for netrin 1 (C,D), Raldh3 (E,F) and Vax1 (G,H). Arrowheads in A,C,E,G indicate labelled OD precursors in wild-type optic cup. No stained precursors are observed in corresponding sections from mutant embryos. However, staining in the optic stalk (arrows in B,D,H) or ventral retina (arrow in F) precursors is observed in mutants as in wild type (arrows A,C,E,G). The overlap between the different markers demarcates the OD. Scale bar: 50 μm.

Failure in OD formation in microphthalmic Bmp7-/- is associated with aplasia of the optic nerve, intra-retinal misrouting of RGC axons and absence of the hyaloid artery

The RF/OD enables the ingression of mesenchymal cells that form the hyaloid artery and the egression of RGC axons into the optic nerve. Therefore, failure in RF formation should also impair these processes. To test this assumption, we labelled E12.5 optic cup sections with Isolectin B4 that selectively binds to blood vessels. In wild-type embryos, a presumptive IB4-positive hyaloid artery was visible in the initial region of the optic stalk (Fig. 2I, inset; Fig. 3B) as observed in wild-type embryos (Fig. 2L inset; Fig. 3B) but still localised to optic stalk precursors (Fig. 2I inset; Fig. 3B) as observed in wild-type embryos (Fig. 2I inset; Fig. 3B).

These data indicate that Bmp7 activity is required for the generation and invagination of those Pax2-positive cells that form the RF/OD (Fig. 2Q). To corroborate this conclusion, we searched for additional markers that could facilitate the identification of RF/OD cells from the remaining neuroepithelial cells of the optic cup/optic stalk. Among these markers, we analysed Vax1, Vax2, netrin 1 and Raldh3. Vax1 has a distribution similar to that of Pax2 in both optic stalk and RF cells (Fig. 3G). Its close homologue Vax2, and Raldh3, a retinoic acid-synthesising retinaldehyde dehydrogenase (Li et al., 2000), are instead expressed mostly in the ventral neural retina, including the cells of the RF (Fig. 3E; and not shown). In addition, in the eye netrin 1, a recognised guidance cue for RGC axons (Deiner et al., 1997; Hopker et al., 1999), appears mostly confined to RF precursors from E10.5 (see Fig. S3G in the supplementary material; Fig. 3C). Thus, cells of the RF/OD can be distinguished by the expression of netrin 1 and by the overlapping distribution of both optic stalk and ventral neural retina markers, the combination of which gives them a unique identity within the eye cup (Fig. 3I). Supporting our conclusion, Pax2 and netrin 1 expression was clearly diminished in the ventral optic cup of E10.5-Bmp7-null embryos (see Fig. S3E-H in the supplementary material), and by E12.5, Pax2, netrin 1, Raldh3 and Vax1 were not detected in the region where the RF should form (Fig. 3B,D,F,H) but were still present in the initial region of the optic stalk (arrows in Fig. 3B,D,H) or in the cup periphery (Fig. 3F).

As the optic cup forms, PAX2 becomes restricted to the ventral retina (Otteson et al., 1998) (Fig. 2A) in invaginating RF precursors (Fig. 2C; see Fig. S3E in the supplementary material) and then to a small group of neuroepithelial cells that encircle the pioneer RGC axons and hyaloid artery at the OD (Otteson et al., 1998) (Fig. 2G-I; Fig. 3A). Thus, by E12.5, PAX2 expression distinguishes the cells that compose the OD from the remaining cells of the optic cup (Fig. 2G-I; Fig. 3A). In Bmp7-/- optic cup, PAX2 expression was initially undistinguishable from that of wild-type littermates (see Fig. S3B in the supplementary material) but it was reduced in the distoventral region of the optic cup from E10.5, where the RF was undetectable (see Fig. S3F in the supplementary material; Fig. 2D-F). The reduction of invaginating, PAX2-positive ventral precursors was progressively more evident in proximal regions of the cup (Fig. 2E,F) and PAX2-positive cells were observed only in ventral prospective RPE and not in neural retina (Fig. 2E) in the region bordering the optic stalk. Two days later, PAX2 was completely absent from the optic cup of Bmp7-/- embryos (Fig. 2J-L; Fig. 3B) but still localised to optic stalk precursors (Fig. 2L inset; Fig. 3B) as observed in wild-type embryos (Fig. 2I inset; Fig. 3A).

Failure in OD formation in microphthalmic Bmp7-/- is associated with aplasia of the optic nerve, intra-retinal misrouting of RGC axons and absence of the hyaloid artery
In wild-type embryos, many TuJ1-positive RGC axons have already grown through the OD into the optic nerve by E14.5 (Fig. 4C). By contrast, retinal axons of mutant embryos do not leave the eye but instead take aberrant trajectories within the retina, gathering either onto the vitreal surface or in the subretinal space (Fig. 4D,D’). As a consequence of these alterations, in those homozygous mutants that reach birth, the eye globes lack the OD and show several hypo-pigmented areas that may correspond to aberrant accumulations of axons (Fig. 4B,B’ when compared with A,A’). Bmp7-null mice generally die soon after birth. However, in our colony, a few animals (1% of the homozygous) developed to the second and third postnatal weeks. Among these, the vast majority presented complete aplasia and one bilateral hypoplasia of the optic nerves.

The strong alterations in the RGC axon trajectory can be easily explained as a consequence secondary to the physical absence of the OD and of the reduced expression of netrin 1 (Fig. 3C,D). Additional explanations could include the altered expression of other axon guidance cues [such as laminin or NCAM, which are known to participate in RGC axon pathfinding at the optic disk (Stuermer and Bastmeyer, 2000)], an abnormal generation of RGC or a direct effect of BMP7 on the outgrowth or directionality of RGC growth cones. The latter possibility was supported by the observation that BMPs can provide directionality to commissural axons of the spinal cord (Butler and Dodd, 2003) and by Bmp7 expression in the mesenchyme abutting the outgrowing axons (see Fig. S1G,H in the supplementary material). Nevertheless, none of these processes appeared to contribute to these guidance defects: laminin and NCAM appeared expressed at normal levels (data not shown) and RGC differentiated at a normal density (data not shown) and RGC differentiated at a normal density (data not shown) and RGC differentiated at a normal density (data not shown) and RGC differentiated at a normal density (data not shown) and RGC differentiated at a normal density (data not shown) and RGC differentiated at a normal density (data not shown). However, the expression of ventral eye markers such as Vax2 (Fig. 5A,B) or Ephb (Yang, 2004) (data not shown), was initially normal. Similarly, the distribution of components of the Shh signalling pathway (Shh, Ptc1, Gli1 and Gli3, not shown), which are known to control ventral eye development (Lupo et al., 2006), as well as that of the dorsal eye markers Bmp4 (Fig. 5C,D), Tbx5 and ephrin B2 (not shown), was unchanged.

**Absence of the RF in Bmp7+/− embryos is not associated with early changes in the dorsoventral polarity of the optic cup**

We next explored the mechanism by which Bmp7 signalling could affect the development of the RF/OD. After transplantation of rotated optic vesicles, the development of a dorsally located optic fissure is generally accompanied by an inverted dorsoventral polarity of the optic cup (Uemonesa et al., 2002), suggesting a link between the two events. We therefore asked whether absence of Bmp7 could alter dorsoventral polarity of the early optic cup and thus impair RF formation. However, the expression of ventral eye markers such as Vax2 (Fig. 5A,B) or Ephb (Yang, 2004) (data not shown), was initially normal. Similarly, the distribution of components of the Shh signalling pathway (Shh, Ptc1, Gli1 and Gli3, not shown), which are known to control ventral eye development (Lupo et al., 2006), as well as that of the dorsal eye markers Bmp4 (Fig. 5C,D), Tbx5 and ephrin B2 (not shown), was unchanged.
Retinoic acid (RA) signalling has been widely implicated in ventral eye development (Lupo et al., 2006; Matt et al., 2005; Molotkov et al., 2006). Deprivation of RA causes morphological alterations of the ventral retina (Maden, 2002; Matt et al., 2005; Molotkov et al., 2006), while implantation of RA-charged beads within the eye field induces an infolding resembling the optic fissure (Hyatt et al., 1996). The expression of Raldh3 was decreased in Bmp7–/– deficient embryos (Fig. 3F; Fig. 5F), raising the possibility of a RA-mediated Bmp7 activity on RF formation. However, oral administration of RA (Dickman et al., 1997) to pregnant embryos, making this link unlikely.

**BMP7 regulates proliferation and apoptosis during optic fissure development**

Besides dorsoventral patterning, fissure formation has been associated also with an increased cell proliferation (Calvente et al., 1988) and a marked apoptotic cell death (Cuadros and Rios, 1988; Ozeki et al., 2000). BMP signalling is implicated in the regulation of both proliferation and apoptosis in different neural structures, including the eye (Trousse et al., 2001). We, thus, examined whether BMP7 effects on fissure formation could involve alterations in either one of the two processes.

At E10.5, the ventral optic cup of wild-type embryos showed a significantly higher number of BrdU-positive, proliferating cells when compared with the dorsal half (compare 64.91±4.92 with 41.79±7.25; n=5; Fig. 6C). This difference was absent in the Bmp7–/– embryos, where the rate of cell proliferation was almost identical in both the ventral and dorsal aspect of the eye (compare 45.55±5.45 with 48.42±6.07; n=5; Fig. 6A-C). Similarly, apoptotic cell death, detected by TUNEL staining, was markedly decreased in the mutant embryos when compared with wild-type littermates (Fig. 6D-I). This decrease was more evident in the proximal pole of the cup (compare Fig. 6H, I with 6E, F) corresponding to the region where the optic fissure normally forms (Martín-Partido et al., 1988; Ozeki et al., 2000; Silver and Hughes, 1973). Altogether, these data indicate that Bmp7-mediated signalling controls proliferation and programmed cell death of RF precursors.

**Bmp7 requirement for RF formation is independent of Chx10**

The traits we have described for the microphthalmic phenotype of Bmp7–null mice strongly resembled those described for the ocular retardation (or) mouse line. Mice carrying the orJ allele, which causes a premature stop codon in the homeobox of the Chx10 gene, are characterised by microphthalmia, reduced proliferation of retinal progenitors (Burmeister et al., 1996) and optic nerve aplasia associated with decreased programmed cell death and intra-retinal navigation errors (Silver and Robb, 1979). It was therefore possible that Bmp7 and Chx10 would act sequentially in the same genetic cascade. However, Bmp7 is normally expressed in the optic cup of orJ embryos (Fig. 7C, D). Vice versa, the expression of Chx10 did not appear reduced in the optic cup of Bmp7–/– embryos with an evident lens placode (about 50%; Fig. 7A, B). Thus, Bmp7 requirement for early RF formation appeared independent of Chx10 function. Furthermore, careful analysis of orJ early optic cup revealed the presence of a morphologically and molecularly recognisable RF (Fig. 7E, F), although this structure was no longer visible at slightly later stages of development (Fig. 7G-J). A possible explanation for this disappearance is the severely reduced generation of Math5-positive RGC precursors (Fig. 7I, J) (Rowan et al., 2004; Horsford et al., 2005), and thus the reduced availability of RGC-derived SHH, which, in turn, is necessary to maintain the PAX2-positive cells of the OD (Dakubo et al., 2003).

**BMP7 is required, ahead of SHH, for Pax2-positive cells formation at the OD**

Taking our data together with previous studies (Dakubo et al., 2003), we hypothesised that the development of the PAX2-positive RF precursors should initially require the activity of Bmp7 to specify their identity and, thereafter, that of Shh to maintain it. To test this hypothesis, we turned to organotypic cultures of optic vesicle (E10) or of optic cups (E12.5) derived from either wild-type, Bmp7–/– or orJ embryos. If our assumption was correct, we expected that addition of BMP7 to optic vesicles derived from Bmp7–/– embryos would suffice to rescue Pax2-positive cells at the RF, while addition of SHH would do the same in the late optic cup from orJ mutants.

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Fig. 6. Cell proliferation and apoptotic cell death are reduced in Bmp7–/– optic cups. (A–C) Pregnant mice were injected with BrdU and sacrificed 40 minutes later. Embryos were fixed and frontal cryostat sections were immunostained with antibodies against BrdU. The percentage of BrdU-positive cells in the ventral and dorsal wild-type (A) or Bmp7–/– embryos (B) was calculated. The dorsal border (broken lines) of Vax2 expression (insets) was considered as the dividing landmark. (C) The graph represents the percentage of BrdU-positive cells in the ventral and dorsal wild-type (blue) or mutant (purple) optic cups. The decrease of BrdU-positive cells in the ventral optic cup of the mutants is statistically significant (**P<0.0001). (D–I) Parasagittal cryostat sections at different proximodistal levels of wild-type or mutant optic cups were processed for TUNEL staining. In wild type, apoptotic cells are concentrated along the prospective RF (arrowheads in E, F). Apoptotic cells are almost absent in the equivalent sections from the mutants. Abbreviation: lv, lens vesicle; ns, no significance. Scale bar: 60 μm (30 μm in insets).
As shown in Fig. 8, recombinant BMP7 present in the medium of control E10 optic vesicle cultures did not significantly expand the domain of Pax2 expression after 48 hours (Fig. 8A,A’,B,B’; 8/8 treated vesicles), but it rescued Pax2 expression in the ventral region of Bmp7–/– vesicles (Fig. 8E,E’; 4/6 treated vesicles), when compared with equivalent untreated tissue (Fig. 8D,D’; 9/9). Rescued Pax2 expression was observed also when SHH was added to the medium of already formed optic cups from E12.5 or’ embryos (compare Fig. 8 with 8I; 4/5 treated cups). Converse experiments, using the BMP7 antagonist follistatin in cultures of wild-type eye primordia at different stages of differentiation, indicated that inhibition of BMP7 signalling efficiently prevented the formation of Pax2-positive cells in the ventral region of early (Fig. 8C,C’; 78%, 7/9 treated vesicle) but not late (Fig. 8G; 10/10) optic cups. Pax2-positive cells were, however, strongly reduced if the late optic cups were cultured in the presence of SHH-specific blocking antibodies (Fig. 6H; 5/6 treated cups), as previously described (Dakubo et al., 2003). This demonstrates a sequential requirement of BMP7 and SHH for the development of Pax2-positive cells at the RF.

**DISCUSSION**

In this study, we have analysed possible molecular mechanisms underlying OD formation, reaching three main conclusions.

1. PAX2-positive RF progenitors present unique characteristics that distinguish them from the remaining PAX2-positive cells that compose the optic stalk.

2. Development of RF progenitors depends on Bmp7: in mouse embryos deficient for this protein RF but not optic stalk precursors are absent, the hyaloid artery does not form and RGC axons are misrouted.

3. Bmp7 controls RF formation by influencing proliferation and apoptosis of cells in the ventral optic cup. This activity precedes that of RGC-derived SHH, which, later on, sustains the development of PAX2-positive OD cells (this study) (Dakubo et al., 2003).

We thus propose that the OD is composed by a special group of cells, the development of which depends on the sequential activity of BMP7 and SHH.

**Optic disc precursors are a unique group of Bmp7-dependent Pax2-positive cells**

In a developmental study of PAX2-positive cells in the embryonic mouse eye, Otteson and colleagues (Otteson et al., 1998) demonstrated that PAX2-positive distoventral optic cup cells invaginate to demarcate the site of OD formation. Because these cells are in close continuity with those of the optic stalk, they proposed that OD precursors may represent an extension of stalk cells into the optic cup (Otteson et al., 1998). Here, we show that RF precursors have instead an identity of their own, characterised by the combined expression of nefrin 1 and ventral retinal markers (Fig. 3I). In support of this diversity, optic stalk but not RF precursors are normally generated in Bmp7-deficient mouse embryos (Fig. 2 insets), although their development is thereafter impaired, as demonstrated by the aplasia of the optic nerve observed in older mutants. This secondary alteration, however, is a likely consequence of RGC axon failure to leave the eye cup and, thus, of the absence of axon-derived factors, such as Shh (Wallace and Raff, 1999), that are needed to sustain glial cell development (Fields and Stevens-Graham, 2002). By contrast, the absence of different OD markers in Bmp7-null embryos indicates a direct dependence of these cells on BMP signalling, an idea supported also by the highly enriched expression in the RF of the BMP receptor Bmpr1b and of the activin receptors Acvr2a and Acvr1a (Belecky-Adams and Adler, 2001; Trousse et al., 2001; Liu et al., 2003; Mishina, 2003), through which BMP7 can signal (Mishina, 2003).

Bmp signalling appears to regulate the proliferation, survival and morphogenetic behaviour of distal lung epithelial cells (Eblaghie et al., 2006). BMP7 may have a similar pleiotropic activity on RF precursors, specifying their identity, promoting their morphogenetic movements and further controlling their proliferation, as suggested by the reduced mitotic rate observed in the ventral optic cup of Bmp7–/– embryos. In these embryos, a reduced number or absence...
of PAX2-positive cells in the ventral optic cup seems to compromise the correct infolding of its inner layer to initiate fissure formation (Fig. 2Q). A similar defect was observed also in kidney and retinal defect (Krd) haploid embryos, where an insufficient number of PAX2-positive cells could explain the lack of ventral neuroepithelium invagination to form the optic groove (Otteson et al., 1998). In our study, we could not determine whether the reduced apoptosis observed in the mutants is a consequence of the decreased cell proliferation or whether Bmp7 directly controls cell death in the optic fissure. However, optic fissure formation appears to be initiated even in the absence of a significant amount of apoptosis (Silver and Robb, 1979), while preventing optic fissure invagination does not modify programmed cell death in this region (Garcia-Porrero et al., 1987), questioning the real contribution of apoptosis to optic fissure formation.

Previous studies have shown that inhibition of BMP signalling by ectopic expression of dominant-negative receptors or extracellular BMP antagonists (noggin and gremlin) interferes with the development of the chick ventral optic cup, causing microphthalmia, coloboma and ventralisation of the optic cup that is often associated with abnormal growth of RGC axons (Adler and Belecky-Adams, 2002; Huillard et al., 2005). It is not surprising that these defects do not exactly match those observed in the Bmp7-null mice because they result from Bmp signalling interference at relatively late stages of optic cup development, reflecting a contribution of Bmp signalling to late ventral eye cup development (Adler and Belecky-Adams, 2002). Ligands other then BMP7 may contribute to the control of retinal polarity because, in Bmp7-null microphthalmic eye cups, the expression of dorsoventral markers was basically normal with the exception of low levels of Raldh3. However, exogenous administration of RA did not rescue the microphthalmic phenotype of the Bmp7−/− embryos, somehow dissociating RF initiation from the establishment of the ventral identity of the eye cup. Rather, the data presented here provide support to classical embryological studies in amphibian (Beckwith, 1927; Sato, 1933) and the more recent studies in birds (Silver, 1977; Uemonsa et al., 2002) that postulate the existence of an optic fissure initiating factor (or factors). Independently of its precise anatomical source (ventral midline, periorcular mesenchyme or optic stalk, see Fig. S1 in the supplementary material), BMP7 appears to be required as such an initiating factor.

The sequential activity of BMP7 and SHH supports optic disc development

The phenotypic alterations we observed in Bmp7-null microphthalmic embryos strongly resembled those reported for Chx10/orf mice (Silver and Robb, 1979; Burmeister et al., 1996). However, we show that the common OD absence has independent molecular causes. Although Chx10 expression was strongly reduced in the Bmp7-null embryos that presumably develop an anophthalmic phenotype (see Fig. S2 in the supplementary material), it was apparently normal in those affected by microphthalmia. Vice versa, Bmp7 mRNA distribution was unchanged in the eye of orf embryos. Furthermore, in orf− but not in Bmp7-null embryos, RF development was normally initiated indicating that factor(s) other that BMP7 are required at subsequent steps of eye development to sustain OD formation. As demonstrated by conditional inactivation studies, this factor appears to be SHH secreted by differentiated RGCs (Dakubo et al., 2003). Indeed, specific abrogation of RGC-derived SHH causes the loss of

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**Fig. 8. BMP7 and SHH are sequentially required for Pax2 expression in the distoventral optic cup.** Optic vesicle (A-E) or optic cups (F-J) from wild-type (A–C,A′–C′,F–H), Bmp7−/− (D,E,D′,E′) or orf (I,J) embryos were dissected and cultured as described in the Materials and methods in the absence (A,A′,D,D′,F,I) or presence (B,B′,E′,G,J) of BMP7, follistatin (C,C′,G), SHH (I), or the 5E1 anti SHH blocking antibody (H). After 2 days in culture (2DIV) optic cups were fixed and hybridised with a Pax2-specific probe. Images show whole-mount views (A′–E,F–I) or cryostat sections (A–E) of same cultured cups. Addition of BMP7 does not significantly modify the expression of Pax2 in wild-type vesicles (B,B′) but it rescues that of Bmp7-null optic vesicles (arrows in E). By contrast, follistatin completely abrogates Pax2 signal in the optic vesicle (arrow in C) but not optic cups (arrow in G) from wild-type embryos. SHH rescues Pax2 expression in orf− cups (arrow in J), while the 5E1 antibodies prevents expression (arrow in H). Asterisks in H–J indicate RPE remnants; in these vesicles, the lens is visible through the tissue. Scale bar: 50 μm in A–F; 100 μm in G–H; 200 μm in I–J.
OD precursors in already formed optic cups (Dakubo et al., 2003). Supporting this idea, we show that addition of SHH in organotypic cultures rescues Pax2 expression in the optic cups from orl embryos, where RGC are highly reduced in number.

Altogether, these data suggest a model whereby OD formation requires the sequential and independent activity of BMP7 and SHH. BMP7, possibly secreted by the pericellular mesenchyme is sufficient to initiate RF development. BMP7 addition to cultures of Bmp7–/– optic vesicle (well before RGC have begun to differentiate and thus in absence of Shh) is sufficient to induce Pax2 expression in the distalventral optic cup. This requirement is temporal because follistatin abolishes Pax2 expression in early, but not in late, optic cup cultures from wild-type embryos. Once RF precursors are specified, SHH secreted by pioneer RGC axons further maintains OD cells, thereby asuring proper development of axonal connections.

Specification of ventral midline cells (floor plate) in the caudal neural tube requires Shh activity, while differential specification of those in the rostral (diencephalic) region depends on the cooperative interaction between Shh and Bmp7 (Dale et al., 1997). It has been proposed that BMP7 activity may predispose rostral midline cells to acquire diencephalic characteristics in response to SHH, which otherwise would induce cells with floor plate properties (Dale et al., 1997). In a possible speculative analogy, sequential activity of Bmp7 and Shh might be necessary to differentiate retinal fissure precursors from the remaining Pax2-positive cells of the optic stalk.

Intra-retinal axon misrouting, failure of hyaloid artery formation and OD agenesis

In Bmp7–null embryos, optic nerves do not develop because RGC axons do not leave the eye and instead take aberrant trajectories, accumulating predominantly in the subretinal space. Similar alterations in RGC axon navigation have been reported in mice lacking Bmp1b (Liu et al., 2003) or after inhibition of BMP signalling in the ventral optic cup (Adler and Belecky-Adams, 2002; Huilard et al., 2005). Although there is evidence that BMPs can modify growth cone movements (Bovolenta, 2003), we could not detect any direct effect of BMP7 on RGC axon behaviour. Thus, the simpler explanation for the axon guidance phenotype of Bmp7–/– embryos is the absence of a morphological structure through which axons can navigate. An additional likely factor is the reduced expression of netrin1 (Deiner et al., 1997) and possibly of other locally expressed guidance cues, as for example Sfpi1 (Rodríguez et al., 2005).

A similar mechanical explanation may account for the failure of hyaloid artery formation, although a direct dependence on BMP7 activity cannot be excluded in this case. BMP7 is essential for mesenchymal to epithelial transition during kidney development and regeneration (Zeisberg et al., 2003; Zeisberg et al., 2005). It is then possible that a BMP7 autocrine function on pericellular mesenchyme may enable its transition to endothelial cells. The absence of the hyaloid artery in the Bmp7 mutants, together with the expression of this morphogen in the artery forming mesenchyme also raises the interesting possibility that this tissue may actively contribute to the initiation of fissure formation. Organised mesenchymal cells could mechanially promote the invagination of the ventral eye primordium and further provide the morphogenetic signal to control the specification and proliferation of RF precursors. Shaping of the ventral optic cup would then require mesenchymal-neuroepithelial interaction, an event also proposed for the specification of the RPE, a dorsal derivative of the optic vesicle (reviewed by Martinez-Morales et al., 2004).

Independently of the precise relationship between RF and hyaloid artery formation, the Bmp7–/– microphthalmic phenotype strongly resembles a rare congenital pathology known as ‘true aplasia of the optic nerve’ characterised by the absence of the optic nerve, hyaloid vessels and OD (Little et al., 1976; Weiter et al., 1977), opening the possibility that altered expression of BMP7 might also be the molecular cause of these types of malformation in humans.

Another question that deserves attention is whether the mechanisms that may control RF/OD formation in mammals may also apply to other vertebrate as interesting species specific features exist, including possible morphogenetic movements (Holt, 1980), the relative position of the fissure within the eye cup (Schook, 1980; Li et al., 2000b) and the expression of molecular markers. As an example, in Xenopus, cells in the ventral retina where the choroid fissure forms, seem to move into position from the optic stalk (Holt, 1980). Intriguingly, in the chick, where only one Vax gene has been found (Vax) (Schulte et al., 1999), RF is enlarged and the domain of netrin1 expression is wider than in the mouse (F.T. and P.B., unpublished), pointing to possible variations in the molecular mechanisms of OD formation.

We are extremely grateful to Prof. E. Robertson for her generous gift of the Bmp7-null mouse line. We are indebted to Drs I. Conte, P. Esteve and E. Martí for critical reading of the manuscript, and to Dr S. Bertuzzi for discussion and help with Fig. 2Q. We also thank I. Dompablo, Manuel Ángel Escobosa-Gómez and Concha Bailón for excellent technical assistance, help with the mouse colony and preparation of Fig. 2Q, respectively. This study was supported by grants from Spanish Ministerio de Educación y Ciencia (BFU-2004-01585) and the European Union (QLG3-CT-2001-01460) to P.B., and in part by a grant from Fight for Sight. UK to J.C.S. J.M. was supported by a predoctoral fellowship from the Comunidad Autónoma de Madrid (CAM) and an EMBO short-term fellowship.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/16/3179/DC1

References


Bmp7 in retinal fissure development


