Bone morphogenetic proteins specify the retinal pigment epithelium in the chick embryo

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In vertebrates, the neuroepithelium of the optic vesicle is initially multipotential, co-expressing a number of transcription factors that are involved in retinal pigment epithelium (RPE) and neural retina (NR) development. Subsequently, extrinsic signals emanating from the surrounding tissues induce the separation of the optic vesicle into three domains: the optic stalk/nerve, the NR and the RPE. Here, we show that bone morphogenetic proteins (BMPs) are sufficient and essential for RPE development in vivo. BMP4 and BMP7 are expressed in the surface ectoderm overlying the optic vesicle, the surrounding mesenchyme and/or presumptive RPE during the initial stages of eye development. During the initial stages of chick eye development the microphthalmia-associated transcription factor (Mitf), important for RPE development, is expressed in the optic primordium that is covered by the BMP-expressing surface ectoderm. Following BMP application, the optic neuroepithelium, including the presumptive optic stalk/nerve and NR domain, develop into RPE as assessed by the expression of Otx2, Mitf, Wnt2b and the pigmented cell marker MMP115. By contrast, interfering with BMP signalling prevents RPE development in the outer layer of the optic cup and induces NR-specific gene expression (e.g. Chx10). Our results show that BMPs are sufficient and essential for RPE development during optic vesicle stages. We propose a model in which the BMP-expressing surface ectoderm initiates RPE specification by inducing Mitf expression in the underlying neuroepithelium of the optic vesicle.

KEY WORDS: BMP, Eye development, Retinal pigment epithelium, RPE specification

INTRODUCTION
The vertebrate eye primordia are first visible as an outgrowth of the prosencephalic neuroepithelium (reviewed by Chow and Lang, 2001; Martinez-Morales et al., 2004). Enlargement of the distal portion of the optic vesicle and dorsal expansion divides the optic vesicle into three territories (Hilfer, 1983): the narrow optic stalk (proximal), the neural retina (NR) and the retinal pigment epithelium (RPE). Formation of the lens vesicle from the surface ectoderm induces the distal region of the optic vesicle to invaginate and this process results in the development of the bilayered optic cup. The inner layer develops into the multilayered NR, whereas the outer layer develops into the single-layered, pigmented RPE (reviewed by Chow and Lang, 2001).

In vertebrates, optic vesicle cells initially co-express a number of transcription factors (TFs) that become restricted to NR, RPE and optic nerve later on, implicating that these cells are competent to develop into these tissues (reviewed by Martinez-Morales et al., 2004). Extrinsic signals emanating from the surface ectoderm and ocular mesenchyme appear to induce and repress specific TFs, which subsequently pattern the optic vesicle into NR and RPE domains (for reviews, see Chow and Lang, 2001; Martinez-Morales et al., 2004). For example, fibroblast growth factors (FGFs) expressed in the surface ectoderm and/or distal optic vesicle appear to be involved in NR induction and differentiation (Pittack et al., 1997; Hyer et al., 1998; Nguyen and Arnheiter, 2000; Vogel-Höpker et al., 2000; Martinez-Morales et al., 2005). Embryonic transplantations and in ovo explant cultures of the chick optic vesicle have shown that the dorsoventral polarity of the eye is already specified by stage 10 (Uemonsa et al., 2002; Kagiyama et al., 2005). At this time point, the dorsal half of the optic vesicle is fated to develop mainly into RPE, whereas the ventral portion develops mainly into NR (Kagiyama et al., 2005).

Little is known about the molecular mechanisms that specify the RPE (reviewed by Martinez-Morales et al., 2004). The mesenchyme adjacent to the optic vesicle appears to be crucial for RPE development, but the molecular nature of the signal(s) is still unclear (reviewed by Chow and Lang, 2001; Martinez-Morales et al., 2004). Activin, a member of the transforming growth factor-β (TGF-β) superfamily, or a related growth factor appears to be released from the mesenchyme to induce RPE development (Fuhrmann et al., 2000). Cell-intrinsic TFs mediate the effect of mesenchymal signalling molecules on RPE development (reviewed by Chow and Lang, 2001). The best-studied example is the microphthalmia-associated transcription factor (MITF), a basic helix-loop-helix leucine zipper TF that is crucial for the acquisition and maintenance of RPE cell identity (reviewed by Martinez-Morales et al., 2004). Ectopic Mitf expression in cultured avian neural retina cells results in the induction of pigmentation by initiating the expression of two markers of differentiated pigment cells: melanosomal matrix protein 115 (MMP115) and tyrosinase (Mochii et al., 1998; Planque et al., 1999). By contrast, inhibition of Mitf by small interfering RNAs (siRNA) decreases MMP115 expression and promotes de-differentiation of the RPE (Iwakiri et al., 2005). In Mitf mutants, the RPE remains unpigmented and displays areas developing into a second NR (Burnsted and Barnstable, 2000; Nguyen and Arnheiter, 2000). Members of the orthodenticle-related family of TFs, Otx1 and Otx2, are also required for RPE specification during vertebrate eye development (Martinez-Morales et al., 2001; Martinez-Morales et al., 2003). In Otx1/Otx2 mutants, RPE development is disturbed and instead the outer layer of the optic cup develops NR-like features. Similar to Mitf, Otx2 overexpression induces a pigmented phenotype in cultured NR cells. Otx1 and Otx2 are initially
expressed in the entire optic vesicle. Subsequently, Otx2 expression is maintained in the presumptive RPE and expression persists in the adult RPE (reviewed by Martinez-Morales et al., 2004).

There appear to be differences in the Mitf expression pattern between chick and mouse (Mochii et al., 1998; Fuhrmann et al., 2000; Nguyen and Arnheiter, 2000). In chick, Mitf expression seems to be restricted to the dorsal region of the optic vesicle, the presumptive RPE, and this region is covered by the surrounding mesenchyme. By contrast, the entire mouse optic vesicle is initially covered by a small amount of mesenchyme and here Mitf expression is observed throughout the optic vesicle. Once the mesenchyme is displaced at the distal part of the optic vesicle at the time this region contacts the FGF-expressing surface ectoderm, Mitf expression is inhibited and instead NR induction occurs in the mouse (Bora et al., 1998; Nakayama et al., 1998; Nguyen and Arnheiter, 2000). The paired-like homeobox gene Chx10 is a specific marker of retinal progenitor cells and functions to repress Mitf expression in the distal optic vesicle (Rowan et al., 2004; Horsford et al., 2005). Moreover, overexpression of Chx10 in the chick RPE causes downregulation of Mitf expression and other pigment markers, leading to a nonpigmented RPE (Rowan et al., 2004). Thus, the current model is that the ocular mesenchyme is necessary to induce the RPE domain during vertebrate eye development, whereas FGFs released from the surface ectoderm ensure that the NR develops at the distal part of the optic vesicle (reviewed by Chow and Lang, 2001).

Like activin, BMPs belong to the TGF-β superfamily and several BMP ligands and their receptors are expressed in the developing chick and mouse eye and surrounding tissues (reviewed by Chow and Lang, 2001; Martinez-Morales et al., 2004). BMPs are involved in several aspects of vertebrate eye development. For example, BMP signalling is required for patterning the eye primordia during blastula and gastrula stages in zebrafish (Hammerschmidt et al., 2003), whereas later on BMPs function in both dorsal and ventral patterning of the vertebrate eye (Koshiba-Takeuchi et al., 2000; Sakuta et al., 2001; Adler and Belecky-Adams, 2002; Sasagawa et al., 2002; Murali et al., 2005). In addition, the generation of retina-specific BMP type 1 receptor mutant mice has shown that different threshold levels of BMP signalling regulate distinct developmental processes such as dorsoventral patterning of the NR, as well as NR growth and differentiation (Murali et al., 2005). At present, however, the possible involvement of BMP signalling in RPE development during optic vesicle stages has not been established (for a review, see Martinez-Morales et al., 2004).

In this study, we show that BMP family members are expressed at the right time and place to be involved in inducing Mitf expression in the chick optic vesicle. Mitf expression is first observed at optic vesicle stages, being strongest in the distal optic vesicle that is covered by the BMP-expressing surface ectoderm. Gain-of-function experiments show that BMPs are sufficient to elicit RPE development in vivo. BMP treatment converts cells of the presumptive optic stalk and NR region into RPE. By contrast, interfering with BMP signalling at optic vesicle stages inhibits RPE formation and induces NR-specific gene expression in the outer optic cup. Thus, we provide evidence that during optic vesicle stages, BMPs are necessary and sufficient for RPE development in vivo.

MATERIALS AND METHODS
Assaying gene expression in chick embryos by in situ hybridisation (ISH)
ISH was performed on whole embryos according to Wilkinson (Wilkinson, 1993) and Henrique et al. (Henrique et al., 1995) and on cryostat sections using the technique described by Reissmann et al. (Reissmann et al., 1996). In some cases, we enhanced the signal by staining whole-mount embryos two to three times or by leaving the colour reaction on sections overnight. Antisense RNA probes specific for chicken Bmp2, Bmp4 and Bmp7 (Reissmann et al., 1996; Vogel-Höpker and Rohrer, 2002), Bmp5 (Oh et al., 1996), Bmpr1b (L. Niswander, Sloan-Kettering Institute, NY), Rx and Fgf8 (T. Ogura, Tohoku University, Aoba, Japan), Mitf (Mochii et al., 1998), Mmp115 (Rowan et al., 2004), Chx10 (D. Schulte, MPI Brain Research, Frankfurt/M, Germany), Wnt2b (H. Roelink, University of Washington, Seattle, WA) and Sox10 (M. Wegner, University of Erlangen, Germany) were used.

In vivo manipulations of the developing chick embryo
Gain-of-function experiments
A 2 μl drop of recombinant mouse BMPs or BMP4 (0.7 mg/ml or 1 mg/ml; R&D Systems) was placed in a Petri dish and about eight drops (10 μl each) of distilled water were placed around it to keep it from evaporating. Ten to fifteen agarose beads (Affi-Gel blue beads, Biorad) were added to the BMP solution, taking care to avoid transferring any fluid with the beads. These beads were incubated in the BMP4 or BMP5 solution for a minimum of 1 hour at room temperature.

Fertile white leghorn chicken eggs were incubated at 37.8°C until they reached the desired stages (stages 8-12) according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). The embryonic membranes were removed and a small incision was made either temporal (posterior) to the optic vesicle/cup or into the midline of the forebrain. One BMP-soaked bead was transferred to the egg, inserted through the slit in the membranes and placed either temporal to the optic vesicle/cup into the mesenchyme or placed into the forebrain/optic vesicle region. The embryos were left to develop at 37.8°C until they reached the desired stages (stages 13-26). At this point, the embryos were fixed in 4% paraformaldehyde in PBS (PFA) at 4°C for 24–48 hours. Embryos to be used for whole-mount ISH were dehydrated and stored in 100% methanol. Those intended for ISH on sections were cryoprotected overnight in 15% sucrose in PBS at 4°C; consecutive 12-16 μm sections were then cut and analysed by ISH. For control experiments, beads were soaked in PBS and implanted according to the same protocol.

Loss-of-function experiments
Noggin-expressing Chinese hamster ovary (CHO B3A4) cells were cultured and implanted as described (Vogel-Höpker and Rohrer, 2002). Briefly, for implantation, a 90% confluent culture was harvested and centrifuged to form a pellet for implantation. The embryonic membranes of stage 8-12 chick embryos were removed and noggin-expressing CHO cells implanted/injected into the mesenchyme temporal to the optic vesicle or into the optic vesicle using fine glass micropipettes. For control experiments, CHO cells were cultured, harvested and implanted according to the same protocol. After incubation for a further 1-6 days, the embryos were fixed and sectioned as described above.

Replication-competent RCAS (B) retroviruses engineered to express the dominant-negative BMPR1B (referred to here as dnBmpr1B) were kindly provided by L. Niswander. Retroviral stocks were prepared as described previously (Vogel et al., 1995; Vogel et al., 1996). For the infection of embryos with dnBmpr1B-RCAS (B), or with RCAS (B) as control, retroviral stock was injected either into the optic vesicle or into the mesenchyme temporal to the optic vesicle at stages 6-11, using fine glass micropipettes. The embryos were incubated for a further 3-8 days and analysed as described above.

RESULTS
Gene expression in the neuroepithelium of the optic vesicle during the initial stages of chick eye development
At the beginning of eye development, the entire optic vesicle co-expresses several genes known to be involved in RPE and NR development. To determine the time point when RPE and NR development is initiated in the chick, we analysed and compared the distribution of transcripts of genes known to be involved in RPE and NR development in vertebrates.
In the chick, the separation of the optic vesicle into NR and RPE domains is initiated in the distal region of the optic vesicle at stage 10 (see below). Initially, Otx2 transcripts are detected throughout the optic vesicle (data not shown) (Bovolenta et al., 1997). At stage 10, Otx2 expression weakens in the distal portion of the optic vesicle (Fig. 1A) and, by stage 13, Otx2 transcripts are abundant in the dorsal part of the optic vesicle, the cells that will give rise to the RPE (Fig. 1B). Otx2 expression is maintained in the RPE thereafter and, from about stage 23 onwards, Otx2 expression is also detected in NR cells (unoperated eye in Fig. 5B) (Bovolenta et al., 1997).

No Mitf transcripts were observed in the eye primordia of the chick at stage 8 (Fig. 2F). However, Mitf expression was observed in the optic vesicle at stage 9, where expression is strongest in the distal region (Fig. 2G) that is covered by the overlying surface ectoderm (Fig. 2, compare G with J). In the temporal part of the optic vesicle, downregulation of Mitf expression was observed in the distal portion at stage 10 (Fig. 1C; Fig. 2S,T), while expression is still observed in the distal optic vesicle more nasally (Fig. 2R). Subsequently, at around stage 12/13, Mitf transcripts were restricted to the presumptive RPE (Fig. 1D). A marker of differentiated pigment cells is melanosomal matrix glycoprotein 115 (MMP115), which is involved in melanin production. Unlike Otx2 and Mitf, we did not observe MMP115 expression at the initial stages of chick eye development (Fig. 1E). The first MMP115 transcripts were detected in the presumptive RPE from stage 13 onwards (Fig. 1F; Fig. 4C). This is about five stages earlier than previously reported (Mochii et al., 1988; Mochii et al., 1998). At stages 13-18, Wnt2b expression is detected in the presumptive RPE and no transcripts are detected within the NR (Fig. 3B) (Jasoni et al., 1999).

Next, we investigated the time point at which the NR domain is established during chick eye development. The retinal homeobox-containing gene Rx is initially expressed throughout the optic vesicle (data not shown) (Mathers et al., 1997). At stage 10, Rx expression was seen to be downregulated in the presumptive RPE (Fig. 1G) and, by stage 13, expression was restricted to cells in the distal portion of the optic vesicle (Fig. 1H). Chx10 is a NR-specific gene expressed in progenitor cells of the NR. At stage 10, Chx10 expression is detected distally in the temporal region of the optic vesicle (Fig. 1I) (Fuhrmann et al., 2000), the region where Mitf transcripts are first downregulated (compare Fig. 1C or Fig. 2T with Fig. 1I).

A second NR-specific marker is FGF8, which appears to be involved in NR induction and differentiation (Vogel-Höpker et al., 2000; Martinez-Morales et al., 2005). At stage 10, Fgf8 transcripts were not detected in the distal neuroepithelium of the chick optic vesicle (Fig. 1K). Fgf8 transcripts in the presumptive NR are first observed at stage 11/12 (13-16 somites) (Vogel-Höpker et al., 2000; Crossley et al., 2001) and expression persists in the central region of the chick NR at optic cup stages (Fig. 1L) (Vogel-Höpker et al., 2000).

Thus, in the chick, the subdivision of the optic vesicle into NR and RPE is observed at stage 10.

**BMP expression during the initial stages of chick eye development**

A signal released from the mesenchyme is thought to be the primary inducer of Mitf expression in the chick and mouse optic vesicle (Fuhrmann et al., 2000; Kagiyama et al., 2005). At stage 9, Mitf expression was seen to be strongest in the distal part of the optic vesicle that is covered by the surface ectoderm (Fig. 2G). The first mesenchymal cells that surround the dorsal region of the optic vesicle are of neural crest origin (Johnston et al., 1979; Hilfer, 1983), suggesting that initially a signal released from the surface ectoderm induces Mitf expression within the optic vesicle, rather than a signal released from the adjacent mesenchyme. To document the presence of neural crest-derived mesenchyme in more detail, we next compared the expression of the neural crest marker gene Sox10 with the Mitf expression pattern during the initial stages of chick eye development. At stage 8+, the optic primordia are first visible. At this stage, Sox10 expression was detected in migrating neural crest cells in the dorsal neural folds (Fig. 2C). At stages 9 and 10, Sox10
expression was restricted to migrating neural crest cells that overlie the dorso-temporal part of the optic vesicle and no transcripts were observed in the distal region (Fig. 2D,E) where Mitf expression is strongest (Fig. 2G,H,R).

Two candidate genes that are detected in the presumptive lens ectoderm in the mouse are Bmp4 and Bmp7 (Furuta et al., 1997; Furuta and Hogan, 1998). In the chick, Trousse et al. (Trousse et al., 2001) did not detect Bmp7 transcripts in the neuroepithelium of the optic vesicle or overlying ectoderm until stage 13, whereas we previously observed Bmp7 transcripts in the presumptive RPE at stages 11-16 (Vogel-Höpker et al., 2000). Therefore, we investigated the expression pattern of BMP family members and their receptors in comparison with the Mitf expression pattern during the initial stages of chick eye development (stages 8-14). By stage 8+ (6 somites) the neural folds have closed and the neuroepithelium of the optic primordia are first visible. At this time point, the neuroepithelium appeared to be close to the surface ectoderm (Fig. 2) and transcripts of Bmp4, Bmp5 and Bmp7 were detected in the dorsal neural folds. Within the neural folds, Bmp7 expression was diffuse (Fig. 2I), whereas there appeared to be a regional restriction of Bmp4 and Bmp5 transcripts (Fig. 2L,O). Both Bmp4 and Bmp7 transcripts, but not Bmp5 transcripts, were observed in the overlying ectoderm between stages 8 and 11 (Fig. 2B,I-Q). Both Bmp4 and Bmp7 transcripts were also detected in the ectoderm overlying the ocular mesenchyme at stage 10 (Fig. 2K,N). At optic cup stages (stages 13-16), Bmp4 transcripts are present in the dorsal NR, whereas Bmp5 and Bmp7 transcripts are detected in the presumptive RPE and surrounding mesenchyme (data not shown) (Vogel-Höpker et al., 2000; Trousse et al., 2001).

BMP receptor type 1a and type 1b are expressed in the eye field during the initial stages of vertebrate eye development (Furuta and Hogan, 1998; Trousse et al., 2001; Hyer et al., 2003). Consistent

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**Fig. 2. Comparison of the Sox10, Mitf and Bmp expression patterns during the initial stages of chick eye development.**

(A) Schematic illustrating the location of the section in H, showing the nasal part of the optic vesicle at stage 10/11. (B) Bmp4 expression in the surface ectoderm overlying the optic vesicle following whole-mount in situ hybridisation (13 somites). The optic primordium is shown at stage 8 (6 somites; C,F,I,L,O), at stage 9 (7-9 somites; D,G,J,M,P) and stage 10 (10-12 somites; E,H,K,N,Q). (C) At stage 8, Sox10-positive neural crest cells are observed in the dorsal neural folds of the prosencephalon (arrows). (D) Sox10-expressing neural crest cells are detected in the dorsal-most region of the prosencephalon (arrows) and no transcripts are detected distally. (E) Sox10 expression is detected in neural crest cells overlying the neural fold of the optic vesicle (arrows). (F) At stage 8, Mitf expression is not observed in the optic primordium (arrow). (G) Mitf expression is strongest in the distal part of the optic vesicle at stage 9 (arrows). The arrowheads indicate neural crest cells dorsally. (H) At stage 10, Mitf expression is observed in the presumptive RPE (dorsal optic vesicle) and in the distal part (arrows). See A for the location of this section; see also R,S,T. (I) Bmp7 expression is observed in the ectoderm overlying the optic primordium at stage 8 (arrows), and diffuse expression is detected in the neural folds (arrowheads). (J) Parallel section of the embryo shown in G. At stage 9, strong Bmp7 expression is observed in the overlying ectoderm (arrows). Bmp7 transcripts are also detected in the dorsal ectoderm that covers the neural crest cells (arrowheads). (K) Bmp7 expression in the ectoderm overlying the distal region of the optic vesicle at stage 10 (arrows). Transcripts are still observed in the ectoderm overlying the mesenchyme (arrowhead). (L) At stage 8, Bmp4 transcripts are detected in the overlying ectoderm (arrow) and in the neural folds (arrowheads). (M) Strong Bmp4 expression is detected in the ectoderm overlying the distal portion of the optic vesicle at stage 9 (arrows). Weak or no expression is observed in the dorsal-most ectoderm overlying the mesenchymal cells (arrowheads). (N) At stage 10, Bmp4 expression is still strong in the ectoderm overlying the distal portion of the optic vesicle (arrows), whereas weak expression is observed in the ectoderm overlying the surrounding mesenchyme (arrowhead). Note that Bmp4 expression appears to be stronger in the ectoderm overlying the dorsal portion of the optic vesicle. (O) At stage 8, Bmp5 expression is strong in the dorsal midline, the neural folds (arrowheads). Transcripts appear to be absent from the following ectoderm. (P) Bmp5 expression weakens in the dorsal midline at stage 9 (arrowhead). (Q) No Bmp5 transcripts are detected in the neuroepithelium of the chick optic vesicle and surrounding tissues (arrow) at stage 10. (R) Nasal region of the optic vesicle at stage 10. Mitf expression in the distal and dorsal part of the optic vesicle (arrowheads). (S) Higher magnification of the Mitf expression pattern in the more-temporal region of the optic vesicle shown in H. Mitf expression is detected in both the dorsal and distal region of the optic vesicle (arrowheads), although expression weakens ventrally (arrow). (T) In the most-temporal region of the optic vesicle, Mitf expression is downregulated in the disto-ventral region (arrow).
with these observations, we detected Bmpr1b transcripts in the neuroepithelium of the optic vesicle, the overlying ectoderm and surrounding mesenchyme at stages 8-10 (data not shown).

In summary, BMP family members are expressed at the right time (stage 8/9) and place (surface ectoderm) to be involved in RPE specification by inducing Mitf expression in the neuroepithelium of the chick optic vesicle.

**BMP application induces RPE development in the presumptive optic stalk and NR**

If BMP levels determine whether the cells of the neuroepithelium of the optic vesicle acquire a RPE instead of a NR phenotype, overexpression of BMPs should result in ectopic generation of RPE from cells of the optic vesicle (presumptive NR/optic stalk region). We implanted BMP-soaked beads into the head mesenchyme or optic vesicle at stages 8-12 and analysed these embryos for changes in NR and RPE gene expression patterns. Vertebrate BMPs have been divided into two subgroups, suggesting that different ligands might have different functions during embryogenesis. In our experiments, we implanted BMP4 and BMP5, which belong to different BMP subclasses. BMP4 and BMP2 belong to the Dpp family, whereas both BMP5 and BMP7 belong to the 60A family (Zhao, 2002).

As described above, Otx2 and Mitf expression is downregulated in cells that develop into NR, but maintained in cells that will develop into RPE during vertebrate eye development. By contrast, the RPE-specific marker MMP115, which is involved in melanin
pigment production, is first detected in the presumptive RPE at stage 13 (Fig. 1F). Similarly, Wnt2b expression is detected in the presumptive RPE at early optic cup stages (Fig. 3B) (Jasoni et al., 1999; Fuhrmann et al., 2000). Application of BMP4 or BMP5 at stages 8-12 induced (MMP115, Wnt2b) and maintained (Otx2, Mitf) RPE genes in both the distal and proximal region of the optic vesicle in 43% of the embryos (n=19/44). In some cases in which the bead had been placed close to the eye region, optic cup and nerve formation was not observed, so that the BMP-treated eyes had still optic vesicle-like morphology (Fig. 3E; Fig. 4E-G). The distal and proximal regions of these BMP-treated eyes had lost the characteristic morphology of the multilayered NR and optic stalk/nerve, respectively. Instead, these regions developed RPE-like features, including the appearance of pigment granules (Fig. 5J). In these embryos, the expression of Mitf, Otx2, MMP115 and Wnt2b was maintained or induced in the proximal region that normally develops into the optic nerve (Fig. 4F; Fig. 5C,F), and/or in the distal region that normally gives rise to the NR (Fig. 3E; Fig. 4E,F,I; Fig. 5D,G,N,O). In BMP-treated embryos that developed RPE-like features, expression of NR-specific genes such as Rx, Chx10 and Fgf8 was downregulated or absent in the distal optic vesicle (Fig. 4G,H; Fig. 5I,K). Three BMP-treated embryos that were left to develop until stage 25/26 developed a single-layered pigmented region within the neuroepithelium of the forebrain, expressing both Otx2 and MMP115 (data not shown).

BMP beads placed into the mesenchyme lying more temporal to the optic vesicle did not prevent optic cup formation, and eye morphogenesis, including lens development, appeared normal (Fig. 4H,I; Fig. 5L-O). In 44% of these cases, the RPE-specific marker MMP115 was expressed in single cells within the NR (n=12/27; Fig. 4I; Fig. 5N,O). Application of PBS-soaked beads into the optic vesicle or into the mesenchyme temporal to the optic vesicle did not
convert NR into RPE (n=12; data not shown) (Vogel-Höpker et al., 2000). These results show that BMPs are sufficient to induce RPE development in vivo.

**BMP signalling is required for RPE development**

To address the functional relevance of BMPs by loss-of-function experiments during the initial stages of eye development, we interfered with BMP signalling using the protein noggin. Noggin specifically inhibits BMP signalling by binding to BMP dimers, thereby preventing their interaction with cell surface receptors (reviewed by Balemans and Van Hul, 2002).

Noggin-expressing CHO cells were injected either into the head mesenchyme or into the ventricle of developing chick embryos at stages 8-11. At several time points after the injection, the embryos were analysed for the expression of RPE- and NR-specific markers. In general, the noggin-treated eyes were smaller than the contralateral unoperated eye and displayed aberrant development of the optic stalk/nerve (coloboma), NR, RPE and lens as previously reported by Adler and Belecky-Adams (Adler and Belecky-Adams, 2002). We therefore implanted the cells slightly further away, at the level of the midbrain. In 32% of the embryos, parts of the outer optic cup no longer had a single-layered morphology and instead a region developed with NR-like morphology (Fig. 3F, arrowheads; Fig. 6B,D, arrowheads; Fig. 6E-J, arrows). In these regions, the pigment marker MMP115, Wnt2b and Mitf expression was downregulated (n=7/22; Fig. 3F; Fig. 6B,D,J) and pigment granules were not observed (Fig. 6E,F,J, arrow). Instead, we observed expression of the retinal markers Rx and Chx10 in these regions (Fig. 6G; data not shown). Pax6 is initially expressed throughout the optic vesicle, but expression is lost from the proximal RPE at late optic cup stages (Fig. 6I, arrowhead) (reviewed by Martinez-Morales et al., 2004).

At stage 25, Pax6 expression is strong within the chick NR and no Pax6 transcripts are observed within the single-layered RPE (Fig. 6H,J). Overexpression of Pax6 in the chick RPE induces differentiation of the RPE into NR (Azuma et al., 2005). Following noggin treatment, we observed strong Pax6 expression in a small, multilayered region of the RPE (Fig. 6H,I) whereas expression of the RPE-specific gene MMP115 was downregulated and pigment granules were absent (Fig. 6J, arrow). However, weak induction of Pax6 expression within the RPE (Fig. 6H, arrowhead) did not result in the downregulation of MMP115 (data not shown).

In control experiments, CHO cells were grafted into the mesenchyme temporal to the optic vesicle of stage 10-11 chick embryos. In these cases, eye morphology was normal and MMP115 expression and pigment granules were restricted to the RPE (n=12; data not shown).

In a second set of experiments, we blocked BMP signalling within the RPE by viral overexpression of a dnBmpr1b construct. Injection of dnBmpR1b-RCAS (B) into the eye field at stages 6-11 resulted in partial loss of RPE development in 21% (7/33) of cases. The most dramatic effects were observed when the operation was carried out at stage 6/7. The outer layer of the optic cup was no longer single-layered and instead developed a NR-like morphology (3/4 cases). Expression of both Otx2 and MMP115 was downregulated in the outer optic cup (Fig. 7G,J). By contrast, the NR marker Chx10 was now detected in the outer layer of the optic cup (Fig. 7H). Thickening of the outer layer was not as prominent when the operation was carried out at stages 8-11 (observed in 4/29 cases; Fig. 7K,L). Injection of control RCAS (B) retrovirus at the same stages of development did not result in any alterations in gene expression, and pigment granules were still observed in the outer layer of the optic cup (n=8; Fig. 7A-D).
Fig. 7. Effects of interfering with BMP signalling on the distribution of transcripts known to be involved in NR and RPE development. (A–D) Control experiments overexpressing a viral RCAS (B) (labelled RCAS-B) construct only (parallel sections). (A) Eye morphology is normal following viral infection with RCAS (B). Expression of the viral reverse transcriptase gene (RT) indicates infected areas of the RPE (arrows). (B) In the eyes infected with RCAS (B), Otx2 expression is still restricted to the RPE (arrowheads) and no transcripts are observed in the NR at stage 20. (C) Following viral infection of RCAS (B), MMP115 expression is unchanged and is restricted to the RPE (arrowheads). (D) Expression of the NR-specific marker Chx10 is not observed in the outer layer of the optic cup following infection of RCAS (B) (arrowheads). (E–H) The effects following injection of the viral dnBmpR1b–RCAS (B) construct at stage 6. (E) Following injection of dnBmpR1b, a large region of the outer layer of the optic cup is thickened (arrowheads). The infection of the RPE is shown by the expression of RT. (F) Otx2 expression weakens in the outer layer of the optic cup following injection of dnBmpR1b (arrowheads). (G) MMP115 expression is downregulated following inhibition of BMP signalling (arrowheads). (H) Expression of the NR-specific gene Chx10 is induced in the outer layer of the optic cup following viral overexpression of the dnBmpR1b construct (arrowheads). (I) RT expression in the outer layer of the optic cup (arrowheads) following viral infection of the dnBmpR1b construct at stage 7. (J) MMP115 expression is downregulated (arrowheads) in the infected region of the outer layer of the optic cup. Strong MMP115 expression is still observed in the single-layered portion (arrow). (K) RT expression within the proximal RPE (arrowheads) following infection at stage 10. (L) The arrowheads indicate the expression of the NR marker gene Chx10 in the RPE in the parallel section. L, lens; NR, neural retina.

Taken together, the data suggest that BMP signalling is required during the initial stages of chick eye development for proper development of the RPE.

DISCUSSION

In this study, we have determined the time point at which the optic vesicle is subdivided into NR and RPE domains. We present evidence from gain- and loss-of-function studies that BMPs are necessary and sufficient for RPE development during optic vesicle stages in the chick. In addition, the BMP expression pattern in comparison to the expression of RPE and/or NR marker genes suggests that the BMP-expressing surface ectoderm, rather than the mesenchyme, is involved in RPE specification by inducing Mitf expression in the underlying optic vesicle.

In vertebrates, the neuroepithelium of the optic vesicle initially co-expresses several TFs that are involved in RPE and NR development. For example, Mitf and Otx2 are initially expressed in the entire optic vesicle, but expression is subsequently maintained only in the presumptive RPE. MITF and OTX2 are key signals involved in initiating and maintaining pigmentation in the RPE of vertebrates (for reviews, see Chow and Lang, 2001; Martinez-Morales et al., 2004). The retinal homeobox-containing gene Rx, which is also initially expressed throughout the optic vesicle, becomes downregulated in the presumptive RPE, whereas expression is maintained in the presumptive NR (Mathers et al., 1997). In this study, we show that in the chick optic vesicle, RPE development is initiated first and that induction of NR development, marked by Chx10 expression, leads to the separation of the chick optic vesicle into NR and RPE. Expression of Chx10, a marker of retinal progenitor cells, is detected at stage 10 in the distal region of the chick optic vesicle (this study) (Fuhrmann et al., 2000) (for a review, see Chow and Lang, 2001) at the time when Mitf expression is downregulated in this region (this study). Members of the FGF family – Fgf1, Fgf2 and Fgf19 – are expressed in the surface ectoderm overlying the distal portion of the chick optic vesicle (reviewed by Chow and Lang, 2001; Martinez-Morales et al., 2004; Kurose et al., 2004). The separation of the optic vesicle into NR and RPE domains is initiated through FGF-mediated induction of Chx10, which subsequently leads to the repression of Mitf (Rowan et al., 2004; Horsford et al., 2005) and possibly also of Otx2 in the presumptive NR. An antagonistic interaction between Chx10 and Mitf regulates retinal cell identity. CHX10 negatively regulates Mitf expression by binding to its promoter, thereby ensuring NR development in the distal portion of the optic vesicle (Rowan et al., 2004; Horsford et al., 2005). Thus, it appears that, similar to the situation in mouse, RPE development is the fate of the neuroepithelium of the optic vesicle in the absence of NR-inducing signals. Removal of the ectoderm after BMP-mediated RPE induction and before FGF production should thus lead to RPE development. Indeed, surface ectoderm removal at stage 10 prevents the separation of the optic vesicle into NR and RPE, and instead a pigmented vesicle develops (Hyer et al., 1998; Nguyen and Arnheiter, 2000). At stage 10, Mitf expression is mainly observed in the distal optic vesicle, whereas at this time only a few cells express Chx10 (this study). Thus, in the absence of FGF-induced Chx10 expression, the neuroepithelial cells will mainly develop into RPE and only a few neuronal cells are observed (Hyer et al., 1998). FGF application to the distal optic vesicle restores proper separation of the NR and RPE domains in the absence of the surface ectoderm (reviewed by Martinez-Morales et al., 2004). FGF family members are also expressed in the presumptive NR (reviewed by Chow and Lang, 2001; Martinez-Morales et al., 2004; Kurose et al., 2004). For example, in the chick, Fgf8 and Fgf19 transcripts are observed in the distal optic vesicle at about the time when Chx10 expression is first detected in this region (Vogel-Höpker et al., 2000; Crossley et al., 2001; Kurose et al., 2004). Indeed, Fgf8 application into the chick ocular mesenchyme inhibits Mitf, Otx2 and Bmp7 expression in the presumptive RPE and Bmp7 expression in the surrounding mesenchyme, and this allows NR development to occur in the outer optic cup (Vogel-Höpker et al., 2000; Martinez-Morales et al., 2005). On the other
hand, BMP application leads to a downregulation of Fgfg expression within the NR, and this allows RPE development to occur in the distal region of the neuroepithelium (this study, see below).

It has been suggested that RPE development is initiated by signals released from the ocular mesenchyme (for a review, see Martinez-Morales et al., 2004; Kagiyama et al., 2005). Previous studies considered the mesenchyme as a source of RPE-inducing signals for three reasons. First, Mitf expression was first detected in the presumptive chick RPE at stage 12/13 (Mochii et al., 1998; Fuhrmann et al., 2000), at the time the presumptive RPE is surrounded by mesenchyme. Second, in the mouse, the initial expression of Mitf throughout the optic vesicle coincides with the time when it is entirely covered by a small amount of mesenchyme (Bora et al., 1998; Nguyen and Arnheiter, 2000). Third, embryonic transplantations and explant studies supported the idea that the mesenchyme induces RPE-specific gene expression within the neuroepithelium of the optic vesicle (Fuhrmann et al., 2000; Kagiyama et al., 2005). In the chick, Mitf expression is induced in the distal optic vesicle before mesenchymal cells are present (this study) (Hilfer, 1983; Johnston et al., 1979; Sullivan et al., 2004; Kagiyama et al., 2005), and here expression is strongest in the distal optic vesicle that is covered by the Bmp4- and Bmp7-expressing surface ectoderm. Our gain-of function experiments show that ectopic BMP application at optic vesicle stages can induce the development of a single-layered RPE, including the appearance of pigment granules, by inducing the pigment cell marker Mmp115 and/or maintaining the expression of Otx2 and Mitf in cells that would normally have developed into the optic nerve or NR. In vitro studies have shown that optic vesicles isolated at stages 11-15 and cultured in the presence of mesenchyme express Mitf, Wnt2b and Mmp115 (Fuhrmann et al., 2000). The results of these co-culture experiments may be explained by BMP-producing/-containing mesenchymes that has a maintenance function at this later stage (see below). Fuhrmann et al. (Fuhrmann et al., 2000) reported that activin, but not BMPs, can substitute for the mesenchyme to induce RPE development in optic vesicle explants. The discrepancy with the present in vivo data might be best explained by the fact that BMPs specify different cell fates in a concentration-dependent manner and that the BMP concentrations used were either too low or too high to elicit RPE induction (Wilson et al., 1997; Simeoni and Gurdon, 2007). BMP beads applied close to the optic vesicle induce the development of a single-layered RPE in cells that would have normally developed into NR or optic stalk. By contrast, following exposure to a lower BMP concentration owing to a different position of the BMP-soaked bead, only single cells within the NR itself express the RPE-specific marker Mmp115.

What is the cellular mechanism that is responsible for the generation of RPE instead of a two-layered optic cup with NR? BMP treatment does not lead to increased apoptosis, excluding the possibility of selective death of presumptive NR (Ohkubo et al., 2002). The significant defects in eye vesicle morphogenesis upon BMP overexpression raised the question of whether the effect of BMPs is direct or, alternatively, is secondary to an invagination defect. Optic vesicle invagination fails when the NR domain has not been correctly specified (Uemensa et al., 2002). The finding that lower BMP levels do not interfere with optic cup formation and lead to RPE-specific gene expression in single cells within the NR argues in favour of a direct BMP-induced differentiation process (e.g. Fig. 5D).

In the chick and mouse, several BMP family members and relevant receptors are expressed at the right time and place to play a role in inducing and maintaining RPE development (Lyons et al., 1995; Dudley and Robertson, 1997; Furuta et al., 1997; Furuta and Hogan, 1998; Wawersik et al., 1999; Fuhrmann et al., 2000; Vogel-Hopker et al., 2000; Crossley et al., 2001; Trouse et al., 2001; Belecky-Adams et al., 2002; Müller and Rohrer, 2002; Hyer et al., 2003; Liu et al., 2003). BMPs mainly signal via complexes composed of type 1 and type 2 transmembrane serine/threonine kinase receptors, which are both required for signal transduction (Mishina, 2003). Activated type 1 receptor kinases subsequently phosphorylate intracellular mediators known as Smad proteins. The type 1 receptors, also known as activin receptor-like kinases (ALKs), ALK1 (ACVR1L1), ALK2 (ACVR1), ALK3 (BMPR1A) and ALK6 (BMPR1B) phosphorylate SMAD1, SMAD5 and SMAD9 (also known as SMAD9 in mouse), which transduce the extracellular signal to the nucleus. Activin

Fig. 8. Proposed model for the regulation of the RPE and NR domain in the developing chick eye. (A) The RPE is specified first at stage 9. BMP genes expressed in the surface ectoderm at stage 8 (e.g. Bmp4 and Bmp7) induce Mitf expression in the underlying optic vesicle. Mitf expression is strongest in the region of the optic vesicle that directly contacts the overlying ectoderm. (B) At stage 10, NR specification is initiated by signals released from the FGF-expressing surface ectoderm. FGF-mediated (e.g. FGF1, 2 and 19) induction of expression is strongest in the region of the optic vesicle that directly contacts the overlying ectoderm. (C) At early optic cup stages, Mitf expression is still maintained in the presumptive RPE by BMPs expressed within the RPE itself and in the surrounding mesenchyme (e.g. BMP5, BMP7). At these stages, BMP family members expressed in the dorsal surface ectoderm and adjacent diencephalon could also be emanating into the underlying mesenchyme to maintain Mitf expression and hence RPE development. On the other hand, FGFs present in the NR itself (e.g. Fgf3, 8, 15 and 19) maintain Chx10 expression, which allows NR development in the adjacent inner layer of the optic cup. The antagonistic interaction between BMPs/Mitf within the RPE and FGFs/CHX10 within the NR ensures the development of the vertebrate eye at early optic cup stages.
receptor type 2 mediates BMP signalling when bound to BMPR1A or BMPR1B (for reviews, see Balemans and Van Hul, 2002; Larsson and Karlsson, 2005). In the chick, Bmpr1a, Bmpr1b and activin type 2a and type 2b receptors are expressed in the neuroepithelium of the optic vesicle and/or surrounding tissues at optic vesicle stages (data not shown) (Stem et al., 1995; Fuhrmann et al., 2000; Hyer et al., 2003) and ACTR1 is present in the optic primordia of the developing mouse embryo (Yoshikawa et al., 2000). Interestingly, neither the βA nor βB activin subunit has been detected at optic vesicle stages in the developing chick embryo (Fuhrmann et al., 2000), whereas phosphorylated SMAD1 was observed in both the neuroepithelium of the optic vesicle and in the surface ectoderm (Belecke-Adams et al., 2002; Faure et al., 2002; Sakai et al., 2005). We finally demonstrate the physiological importance of BMPs in RPE development by interfering with BMP signalling at optic vesicle stages. Application of the BMP-inhibitor noggin or of the dnBmpR1b construct downregulated Mmp115, Mitf and Otx2 expression in the RPE and instead induced the expression of the NR marker genes (e.g. Chx10, Rx). FGF8 application into the mesenchyme near to the optic vesicle/cup induces the development of a second NR in the outer optic cup (Vogel-Höpker et al., 2000; Martinez-Morales et al., 2005). However, during chick eye development, Fgfr8 and Fgfr19 are expressed within the NR, but NR induction in the outer optic cup does not occur. If BMPs within the RPE and FGFs within the NR act antagonistically, the absence of BMPs within the RPE should allow the development of NR-like features in the outer optic cup (see Figs 6 and 7). BMP inhibition at optic cup stages results in the upregulation of Fgfr8 expression within the NR itself (Adler and Belecke-Adams, 2002).

BMPs have multiple functions during early and late stages of vertebrate eye development (Koshiba-Takeuchi et al., 2000; Sakuta et al., 2001; Adler and Belecke-Adams, 2002; Sasagawa et al., 2002; Hammerschmidt et al., 2003; Murali et al., 2005). For example, deletion of the BMPR1A/B function specifically within the mouse retina leads to reduced growth of the NR and failure of retinal neurogenesis (Murali et al., 2005). We show that at optic vesicle stages, BMPs are involved in patterning the vertebrate eye by regulating RPE gene expression within the neuroepithelium of the optic vesicle. On the basis of our results, we propose the following model (Fig. 8). Within a short period of time, both RPE and NR specification are induced by signals released from the overlying ectoderm. Initially, the BMP-expressing surface ectoderm is involved in inducing and maintaining Mitf expression in the neuroepithelium of the chick optic vesicle. At this time, the optic vesicle is in direct contact with the surface ectoderm (this study) (Johnston et al., 1979; Hilfer, 1983; Sullivan et al., 2004; Kagiymaya et al., 2005). The subdivision of the optic vesicle into NR and RPE domains is initiated by FGFs (e.g. FGF1, 2 and/or 19) released from the surface ectoderm a few hours later at stage 9/10. FGF-mediated induction of Chx10 expression in the distal portion of the optic vesicle downregulates genes involved in RPE development (e.g. Mitf). Subsequently, during early optic cup stages, BMPs (e.g. BMP5 and BMP7) in the presumptive RPE itself, the mesenchyme and/or released from the surrounding tissues (dorsal surface ectoderm, diencephalon) into the mesenchyme, are involved in stabilising the RPE domain in the outer optic cup. FGF family members (e.g. FGF3, 8, 15 and 19), being now expressed in the NR itself, maintain Chx10 expression and allow NR development to occur adjacent to the RPE. Thus, at the early optic cup stages when the NR and RPE are in close contact, BMPs/MITF within the RPE and FGFs/CHX10 within the NR, act antagonistically to ensure vertebrate eye development.

BMP ligands are expressed in overlapping domains and genetic studies strongly argue that BMP family members are functionally redundant in vivo (Solloway et al., 1998; Solloway and Robertson, 1999; Kim et al., 2001). It is possible that cooperative signalling of different BMP family members, which may also involve BMP heterodimers (Butler and Dodd, 2003), might be involved in regulating RPE development at optic vesicle stages. However, which specific BMP family members are involved in RPE specification, differentiation and maintenance remains to be elucidated.

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