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Extraocular mesenchyme patterns the optic vesicle during early eye development in the embryonic chick

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SUMMARY

The vertebrate eye develops from the neuroepithelium of the ventral forebrain by the evagination and formation of the optic vesicle. Classical embryological studies have shown that the surrounding extraocular tissues – the surface ectoderm and extraocular mesenchyme – are necessary for normal eye growth and differentiation. We have used explant cultures of chick optic vesicles to study the regulation of retinal pigmented epithelium (RPE) patterning and differentiation during early eye development. Our results show that extraocular mesenchyme is required for the induction and maintenance of expression of the RPE-specific genes *Mitf* and *Wnt13* and the melanosomal matrix protein MMP115. In the absence of extraocular tissues, RPE development did not occur. Replacement of the extraocular mesenchyme with cranial mesenchyme, but not lateral plate mesoderm, could rescue expression of the RPE-marker Mitf. In addition to activating expression of RPE-specific genes, the extraocular mesenchyme inhibits the expression of the neural retina-specific transcription factor Chx10 and downregulates the eye-specific transcription factors Pax6 and Optx2. The TGFβ family member activin can substitute for the extraocular mesenchyme by promoting expression of the RPE-specific genes and downregulating expression of the neural retina-specific markers. These data indicate that extraocular mesenchyme, and possibly an activin-like signal, pattern the domains of the optic vesicle into RPE and neural retina.

Key words: Retinal pigmented epithelium, Neural retina, *Mitf*, *Chx10*, Explant culture, Surface ectoderm, Activin

INTRODUCTION

The vertebrate eye has been one of the most intensively studied organs due to its accessibility and role as a classical model for inductive mechanisms. During early eye development, the neuroepithelium of the ventral diencephalon evaginates laterally to form the optic vesicles. Subsequent invagination of the distal portion of the optic vesicle leads to the formation of a two-layered optic cup. The distal layer differentiates into the multilayered neural retina, the proximal layer develops into the retinal pigmented epithelium (RPE), consisting of a single layer of non-neuronal, cuboidal pigment cells. The presumptive neural retina of the optic vesicle is in close contact with the overlying surface ectoderm which invaginates to form the lens. The presumptive RPE is surrounded by extraocular mesenchyme originating from mesodermal cells and neural crest migrating from diencephalic and mesencephalic brain regions (Johnston et al., 1979).

Classical experiments suggest that signals from surrounding tissues are essential during early eye development. Holtfreter (1939) observed in explant cultures derived from amphibian anterior neural plate that eye development arrests at the optic vesicle stage in the absence of contact with the epidermis and neural crest-derived mesenchyme. Dragomirov (1937; for review see Lopashov, 1963) suggested that the overlying surface ectoderm polarizes the distal optic vesicle to become neural retina. This polarizing action is not specific for the lens ectoderm since the otic placode exhibits the same capacity to induce a secondary retina from adjacent RPE tissue (Ikeda, 1937; for review see Lopashov, 1963). Accordingly, ablation of the lens ectoderm in vivo interferes with eye development in chick embryos (Hyer et al., 1998). Recent evidence indicates that fibroblast growth factor (FGF) is one candidate factor that induces neural retina differentiation in the distal optic vesicle (see Discussion; Pittack et al., 1997; Desire et al., 1998).

While these studies have demonstrated the importance of the lens ectoderm in retinal development, the regulation of the other major derivative of the optic vesicle, the RPE, is much less understood. When eye rudiments of amphibian embryos were cultured alone or transplanted into host eyes with various amounts of extraocular mesenchyme, a correlation between the amount of surrounding mesenchyme and differentiation of the RPE was observed (Lopashov and Stroeva, 1961; Lopashov, 1963). Rodent optic vesicles without extraocular tissues undergo complete transformation into retina (Stroeva, 1960; Buse and de Groot, 1991). However, these studies were based on morphological observations and it remains unclear whether extraocular mesenchyme is necessary for induction,

differentiation, and/or maintenance of RPE identity at optic vesicle and optic cup stages.

The present study was performed to determine the specific role of the extraocular mesenchyme in the regulation of RPE formation in the early developing eye. Using microdissection of optic vesicles of chick embryos in combination with molecular markers, we studied the contribution of the extraocular mesenchyme to RPE and neural retina development. We examined Mitf and Chx10, key transcription factors that are the earliest known markers for the domains of the RPE and neural retina, respectively. The basic-helix-loop-helix-zipper protein Mitf is crucial for the activation of expression of pigment-specific genes such as tyrosinase, TRP1 and QNR71 (Bentley et al., 1994; Yasumoto et al., 1994, 1997; Turque et al., 1996). Mice with severe mutations in the *Mitf* gene are non-pigmented and microphthalmic because of an abnormal development of the RPE (Boissy et al., 1993; Nakayama et al., 1998, Bumsted and Barnstable, 2000). *Chx10* is a retinal expressed paired-like cvc homeobox gene, originally cloned in goldfish (Levine and Schechter, 1993, Levine et al., 1994) and mouse (Liu et al., 1994), and more recently in chick (Belecky-Adams et al., 1997). The expression of *Chx10* is highly specific to retinal progenitor cells (Liu et al., 1994; Passini et al., 1997) and correlates with developmental defects in or mice which exhibit a mutation in the *Chx10* gene (Burmeister et al., 1996).

Our data indicate that removal of extraocular mesenchyme interferes with the expression of *Mitf* and additional RPE-specific markers. In contrast, the expression of the neural retina-specific transcription factor *Chx10* is upregulated in the explant cultures under these conditions. We could identify the TGFβ family member activin as a possible candidate that mimics exactly the effects of extraocular mesenchyme on RPE and neural retina. These results indicate an important regulatory role of the extraocular mesenchyme during RPE and neural retina development, possibly acting antagonistically to signals from the surface ectoderm in patterning the optic vesicle.

MATERIALS AND METHODS

Culture experiments

White Leghorn chick eggs were incubated at 37°C and staged according to Hamburger and Hamilton (HH; 1951). For explant cultures of optic vesicles, embryonic chick heads of HH 11-15 were briefly treated with collagenase to facilitate the removal of the surface ectoderm. After inactivation of the enzyme by rinsing with culture media (DMEM/F12, Gibco) containing 5% fetal bovine serum, 5 mM Hepes, 0.6% glucose, 0.11% $NaHCO_3$, penicillin (1 unit/ml) and streptomycin (1 mg/ml), optic vesicles were carefully dissected with or without extraocular tissues in Hank's balanced salt solution (Gibco). To keep extraocular mesenchyme in place after removal of the surface ectoderm, approximately one third of the adjacent diencephalon was left with the optic vesicles. Optic vesicles were cultured for approximately 2 days in 24- or 48-well plates at 5% CO_2 in a 37°C humidified incubator. The explant cultures were maintained on a nutator to avoid attachment of the tissue to the bottom of the well and subsequent flattening. Factors (Activin A, R&D systems; and BMP5, BMP7 and GDF5, Creative Biomolecules, Hopkinton, Massachusettes) were prepared according to the manufacturer's instructions and added to the media at the beginning of the culture period at the indicated concentrations (see Results). For control experiments, explants were cultured in media without factors and/or with the appropriate diluent. Following the culture period, optic vesicle explants were fixed overnight at 4°C or 1-2 hours at room temperature with 4% paraformaldehyde for immunostaining. For in situ hybridization, cultures were fixed with 4% formaldehyde in phosphate-buffered saline solution (PBS) containing 2 mM EGTA, rinsed in PBS with 0.1% Tween-20, dehydrated and stored in methanol at −80°C for at least 12 hours.

For co-culture experiments, cranial mesenchyme or lateral plate mesoderm of chick embryos (HH 13-14) was carefully dissected and transferred to drops of 30 μl placed on a lid of a petri dish (60 × 15 mm). Each drop received cranial mesenchyme dissected from the region between the diencephalon and the hindbrain anteriorly to the otic placode of one or both sides of the chick head. Lateral plate mesoderm of one side or half of one side of the chick embryo was used for one drop. The cells were incubated in hanging drop cultures and, after one day, the optic vesicles (without extraocular tissues) were added. We were not able to orient the mesenchymal/mesodermal cells precisely with the proximal or distal optic vesicle. In addition, the explants were not always completely surrounded by co-cultured cells but always in contact. After two days in culture, the explants were fixed and processed for in situ hybridization.

Immunohistochemistry

Immunohistochemical analysis of the explants was carried out in the following way. Optic vesicles explant cultures were cryoprotected in 20% and 30% sucrose with PBS and embedded in OCT compound. As RPE differentiation marker, the monoclonal antibody MC/1 was used (generously provided by Dr M. Mochii), which was raised against the melanosomal matrix protein MMP115 (Mochii et al., 1988, 1991). Extraocular mesenchyme was labeled using a monoclonal antibody against Pax7 protein (Developmental Studies Hybridoma Bank, University of Iowa) which detects a population of migrating neural crest cells in the cranial mesenchyme (Kawakami et al., 1997). Cryostat sections (10-12 μm) were incubated in PBS containing MC/1 antibody (1:200) with 0.1% Triton X-100 and 5% milk powder overnight at 4°C. For immunostaining with Pax7 antibody, cryostat sections adjacent to MMP115-labeled ones were incubated in primary antibody (1:50) with 0.3% Triton X-100 and 3% normal goat serum at room temperature overnight. The antigens were visualized using rhodamine- (Cappel, 1:400) or Cy3-conjugated secondary antibody (Amersham, 1:1000).

In situ hybridization

Analysis of mRNA expression was performed on whole embryos or optic vesicle explant cultures according to Henrique et al. (1995) with the modification that prehybridization occurred for 3-6 hours to reduce nonspecific background labeling. The chick *Chx10* was cloned from E6 chick retina performing RT-PCR with the following primer sequences: forward, 5′ TTCGGCATCCAGGAGATCCTG 3′; and reverse 5′ TTCTGTGATGCACTGGACTTC 3′. The DNA fragment (978 bp) was subcloned into a pCRII vector using the TA Cloning Kit (Invitrogen, Carlsbad, CA). Digoxigenin-labeled antisense or sense probes specific for chick *Chx10*, *Mitf* (Mochii et al., 1998), *Wnt13* (Jasoni et al., 1999), *Wnt5A* (Hollyday et al., 1995), *Optx2* (Toy et al., 1998), *Pax6* (Li et al., 1994), and activin receptor type IIA and IIB (Stern et al., 1995) were used at a concentration of 0.5-1.0 μg/ml. Statistical analysis was done using a method based on arcsine transformation (Sokal and Rohlf, 1969).

RESULTS

Early patterning of the optic vesicle into the domains of RPE and neural retina

While eye formation is visible during the formation of the optic

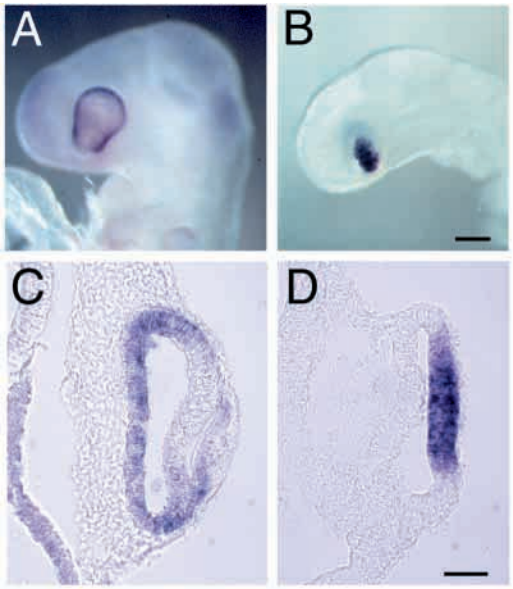

Fig. 1. Expression of *Mitf* and *Chx10* during chick eye development analyzed by whole-mount in situ hybridization. (A) Chick embryo (HH15) showing expression of *Mitf* exclusively in the presumptive RPE. (B) *Chx10* is expressed in the presumptive neural retina (HH12). (C,D) Cryostat sections indicate the complementary expression pattern of *Mitf* (C, HH14) and *Chx10* (D, HH11) in RPE and neural retina, respectively. Scale bars, 200 μm in A,B; 50 μm in C,D.

vesicle, only later, at the optic cup stage, are both RPE and neural retina morphologically distinguishable. However, using specific molecular markers, it is possible to see that both of these domains are already patterned at the optic vesicle stage (Fig. 1). Microphthalmia associated transcription factor (Mitf), a key transcription factor for RPE and melanocyte development, is expressed in the presumptive RPE of the optic vesicle of the chick embryo starting at HH12 (Mochii et al., 1998). Chx10, a paired homeobox transcription factor, (Levine et al., 1994, Burmeister et al., 1996; Belecky-Adams et al., 1997) is expressed at HH10 and remains restricted to the distal part of the optic vesicle (Fig. 1D, compare with Fig. 6D). Therefore, both Mitf and Chx10 represent good markers for a clear distinction between RPE and neural retina domains in the embryonic chick eye.

Extraocular mesenchyme is essential for RPE development in explant culture

Ablation of anterior cranial neural crest in vivo does not lead necessarily to a complete removal of cranial mesenchyme since replacement by late migrating neural crest cells derived from posterior brain regions can occur (Etchevers et al., 1999). Therefore, to examine the effects of extraocular tissues on early eye development in a system that prevents the regeneration of extraocular tissues, we used an explant culture system. Explant cultures of optic vesicles with surrounding extraocular tissues have been shown to develop the normal expression of differentiation markers for the neural retina, RPE and lens (Pittack et al., 1997). We performed microdissection of optic vesicles and cultivated explants in the presence or absence of surrounding extraocular tissues (the overlying lens or surface

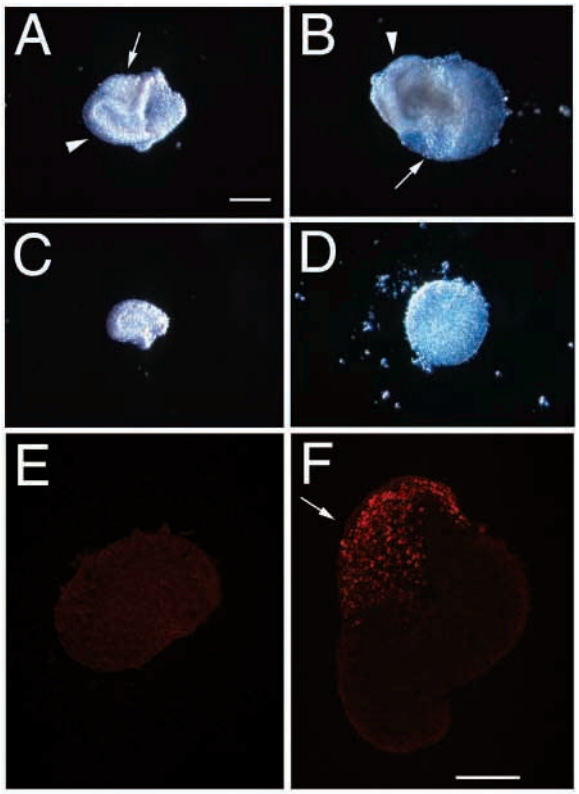

Fig. 2. Explant cultures of optic vesicles during cultivation in the presence or absence of extraocular mesenchyme. In all cases the overlying surface ectoderm was removed. (A) Optic vesicle shown after removal of the overlying surface ectoderm (0 hours of incubation). The extraocular mesenchyme (arrow) is attached to the neuroepithelium of the optic vesicle (arrowhead). (B) The same explant grown for 46 hours in nutating culture exhibits significant growth during this time period. The extraocular mesenchyme (arrow) is closely attached to the optic vesicle (arrowhead).
(C) Neuroepithelial tissue of the optic vesicle shown after removal of both surface ectoderm and extraocular mesenchyme (0 hours). Note the complete removal of the extraocular tissues. (D) The same optic vesicle culture shown after incubation for 46 hours. Note that even in the absence of extraocular tissues the explant grew considerably.
(E) Optic vesicle explant grown without extraocular tissues was immunolabeled for the cranial neural crest marker Pax7. No expression of Pax7 is detectable confirming the absence of mesenchymal cells. (F) Pax7-positive neural crest cells are present in the optic vesicle explant cultured with extraocular mesenchyme (arrow). Scale bars, 200 μm in A-D; 100 μm in E,F.

ectoderm and extraocular mesenchyme). In Fig. 2, optic vesicle explants are shown that were cultured in the presence (Fig. 2A,B) or absence (Fig. 2C,D) of extraocular tissues. Under all conditions, explants grown for 2 days exhibited extensive growth through the cultivation period. To ensure that we removed the extraocular mesenchyme completely, we labeled cryostat sections of optic vesicle explant cultures using an antibody against Pax7, which labels the migrating neural crest cells in the extraocular mesenchyme (Kawakami et al., 1997; see Materials and Methods). Cultures grown in the absence of extraocular tissues showed no expression of Pax7 (Fig. 2E, 0% of labeled cultures, n=11). In contrast, strong expression of Pax7 was detectable in most of the explants cultured with

extraocular mesenchyme (Fig. 2F, 75% of labeled cultures, *n*=8).

The effect of the extraocular mesenchyme on RPE development was determined in explant cultures from chick embryos HH11-15. In all cases the surface ectoderm had also been removed. To identify the extent of RPE differentiation we assayed the genes for several pigment-specific markers, including *Mitf*, *Wnt13* and MMP115. In optic vesicles cultured with extraocular mesenchyme, *Mitf* was expressed in a small discrete band in the explant similar to the expression in the RPE layer in vivo (compare Fig. 3A with 3B). In contrast, when the extraocular mesenchyme is removed, most explants did not express *Mitf* (Fig. 3C). Since the removal of extraocular mesenchyme was complete (Fig. 2E), residual *Mitf* expression in a few explants could be due to a stimulating signal within the neuroepithelium itself. The effect of the extraocular mesenchyme on RPE development was confirmed by examining changes in the expression of additional RPE markers *Wnt13* and MMP115 (Fig. 3D-I). In the embryonic chick eye, *Wnt13* expression was detectable in the dorsal RPE of the optic cup (Fig. 3D, Jasoni et al., 1999). When the optic vesicles are cultured with extraocular mesenchyme, *Wnt13* was expressed in most explants in a pattern similar to that of *Mitf* (compare Fig. 3E with 3B). However, none of the explants expressed *Wnt13* in the absence of extraocular mesenchyme (Fig. 3F). We also used a monoclonal antibody against the melanosomal matrix protein MMP115 which starts to be expressed in the RPE around HH18 (Fig. 3G, Mochii et al., 1988, 1991). MMP115 expression was detectable in every optic vesicle explant in the presence of extraocular mesenchyme (Fig. 3H), but only a small number of the optic vesicles cultured without extraocular mesenchyme showed MMP115-expressing cells (Fig. 3I). In Fig. 4A, quantitative results of the effect of extraocular mesenchyme on *Wnt13* and *Mitf* expression are shown. *Mitf* expression was observed in 96% of the explants with extraocular mesenchyme, but in only 18% of the explants cultured without mesenchyme (Fig. 4A). Similarly, the number of *Wnt13*-expressing explants was decreased from 50% to 0% after removal of the extraocular mesenchyme (Fig. 4A). These results indicate that expression of RPE markers occurs normally in explant cultures in the presence of extraocular mesenchyme, whereas removal of this tissue precludes proper RPE development. Interestingly, expression of an additional RPE-specific gene, *Wnt5a*, which starts at optic cup stage in the RPE, is not expressed in these cultures under any conditions (not shown).

To determine whether extraocular mesenchyme was necessary at later stages of RPE development we dissected optic vesicles from embryos at several stages around the time of onset of *Mitf* expression at HH12. In optic vesicles from chick embryos at HH11, cultured without extraocular mesenchyme, *Mitf* and MMP115 protein were detectable in only approx. 28% (*n*=21) of the explants. Similarly, explants from chick embryos HH13-15 grown under the same condition exhibited expression of both RPE-specific markers in only 12.5% (*n*=24). Thus, the extraocular mesenchyme is necessary not only for the induction of RPE development at HH12, but also for the maintenance of the RPE-specific gene expression at HH13-15. The downregulation of pigment-specific markers after extraocular mesenchyme removal is not likely caused by cell death of presumptive RPE cells because other eye-specific markers are still expressed under this condition.

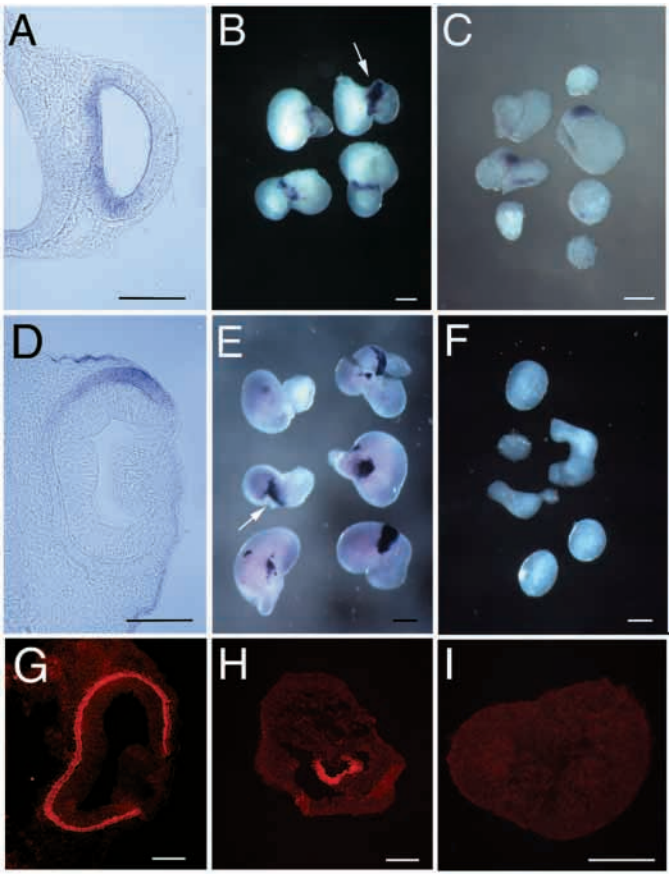

Fig. 3. Expression of RPE-specific markers during chick eye development and in optic vesicle explant cultures in the presence or absence of extraocular tissues. Embryos or cultures were analyzed by whole-mount in situ hybridization or immunostaining for *Mitf* mRNA (A-C), *Wnt13* mRNA (D-F) and melanosomal matrix protein MMP115 (G-I). (A) Section through the optic vesicle after in situ hybridization for *Mitf*, revealing the onset of expression in the RPE at HH12. (B) Examples of optic vesicle explants grown for 2 days in the absence of surface ectoderm express *Mitf*, typically as a stripe across the middle (arrow). (C) Explants grown in the absence of extraocular mesenchyme and surface ectoderm do not express *Mitf*, although the occasional explant will show a small patch of expression. (D) Section through the optic vesicle after in situ hybridization for *Wnt13* in chick embryonic eye at HH17, showing *Wnt13* expression in the dorsal RPE. (E) Many explants cultured with extraocular mesenchyme show *Wnt13* expression (arrow). (F) Explants cultured without extraocular mesenchyme show no *Wnt13* expression. (G) E3-chick embryo showing immunolabeling for MMP115 protein in the RPE. (H) Section through explant cultured for 2 days in the presence of extraocular mesenchyme shows MMP115 expressed in a stripe of cells similar to that observed in vivo. (I) Section through explant cultured in the absence of extraocular tissues; no MMP115 expression is detectable. Scale bars, 100 μm in A,D,G-I; 200 μm in B,C,E,F.

To investigate the role of the overlying surface ectoderm in RPE development, we cultured optic vesicle explants with different combinations of extraocular mesenchyme and surface ectoderm (Fig. 4B). MMP115 expression was observed in 95% of the explants grown with both tissues and in 100% of the

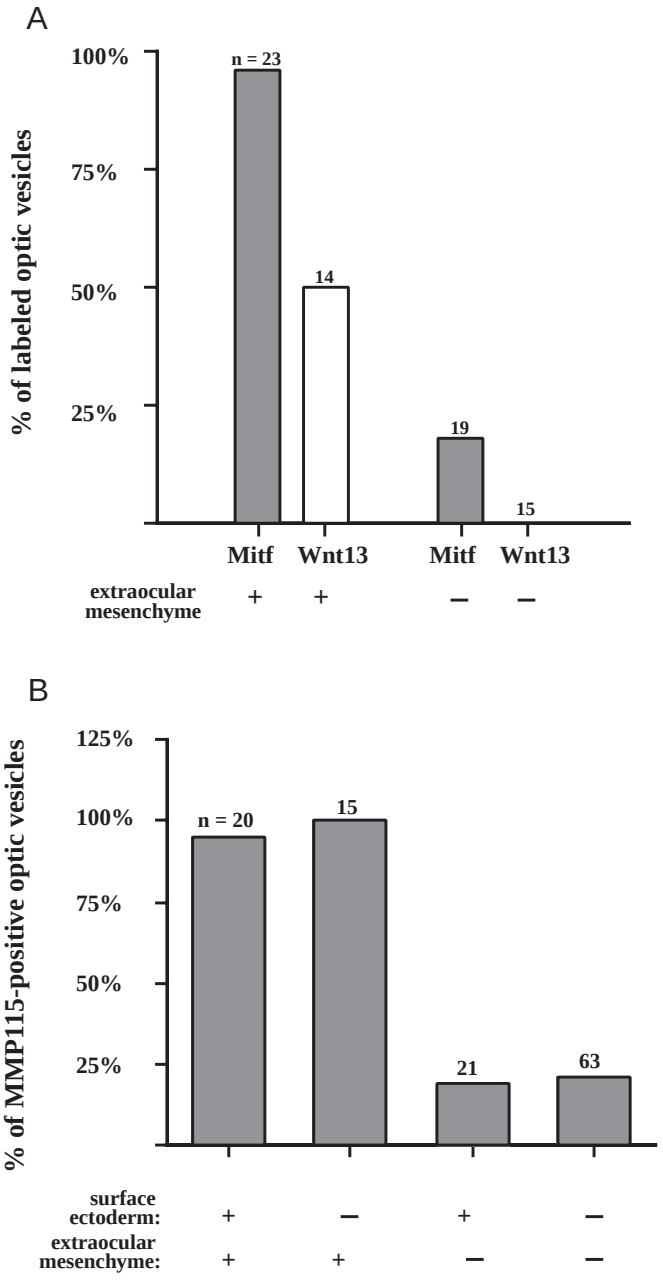

Fig. 4. Quantification of the effect of extraocular tissues on RPE marker expression. (A) After removal of surface ectoderm, optic vesicle explants were grown in the presence or absence of extraocular mesenchyme for 2 days in culture and processed for whole-mount in situ hybridization for *Mitf* (gray bars) or *Wnt13* (white bars). Expression of both *Mitf* and *Wnt13* is greatly reduced in the absence of extraocular mesenchyme. The number of explants showing expression of one of these markers is shown as percentage of the total number of labeled cultures, which is indicated on the top of the bars for each condition. (B) MMP115 protein expression was assayed in optic vesicle explants after 2 days of culture under the following conditions: (1) in the presence of both surface ectoderm and extraocular mesenchyme; (2) after removal of surface ectoderm; (3) after removal of extraocular mesenchyme; and (4) in the absence of both extraocular tissues. Extraocular mesenchyme was necessary for MMP115 expression, while the surface ectoderm was not. For each condition, the absolute numbers are shown on top of the bars.

explants in which only the surface ectoderm had been removed. Removal of extraocular mesenchyme caused a decrease in the number of MMP115-expressing explants to 21%. A similar percentage of explants cultured without either surface ectoderm and extraocular mesenchyme expressed MMP115 (Fig. 4B). These data suggest that the surface ectoderm has no RPE-promoting signal in the developing eye.

We further asked if the reintroduction of mesenchyme to optic vesicles could rescue RPE development in vitro. Cranial mesenchyme was dissected from the region between the diencephalon and the hindbrain, anterior to the otic placode of chick embryos at HH13-14 and incubated in hanging drop cultures. Similar cultures were established with lateral plate mesoderm. After one day, optic vesicles (without surrounding extraocular tissues) were added and the co-cultures incubated for another 2 days (Fig. 5A). In the presence of cranial mesenchyme, 70% of the optic vesicle explants expressed *Mitf* mRNA in (Fig. 5B,D). The expression of *Mitf* was induced in patches in presumably proximal regions of most of the explants rather than throughout the vesicle (Fig. 5B), possibly caused by an incomplete attachment of the mesenchymal cells. In contrast, the number of *Mitf*-expressing explants was decreased to 26% when co-cultured with lateral plate mesoderm (Fig. 5C,D). This percentage shows no significant difference to optic vesicles grown in the presence of extraocular mesenchyme (18%, Fig. 4A). Thus, cranial mesenchyme can substitute for the extraocular mesenchyme to induce the expression of a RPE marker in vitro. However, trunk mesoderm is not able to provide this signal.

Extraocular mesenchyme downregulates expression of the gene for neural retina-specific transcription factor *Chx10*

The above data show that the extraocular mesenchyme is necessary for RPE development in the optic vesicle. If the mesenchyme is acting to pattern the domains of the optic vesicle, we predict that by promoting RPE development, the extraocular mesenchyme will also act to restrict the neural retina domain. Therefore, we examined the effect of the extraocular mesenchyme on the expression of the genes for the eye-specific transcription factors *Pax6*, *Chx10* and *Optx2* to test whether the neural retina domain is expanded in the absence of extraocular mesenchyme (Fig. 6). Pax6 is a key transcription factor for eye and lens development in vertebrates and is strongly expressed in these tissues (Fig. 6A). Expression of *Pax6* starts in the whole optic vesicle and becomes downregulated in the developing RPE during late optic cup stages (not shown; Li et al., 1994; Belecky-Adams et al., 1997). Pax6 was present in explants with extraocular mesenchyme after removal of surface ectoderm (Fig. 6B, n=12) suggesting that the surface ectoderm is not essential for *Pax6* expression in the neuroepithelium of the optic vesicle. In the absence of both extraocular tissues, *Pax6* expression seemed to be expanded (Fig. 6C, n=14). We then looked at the expression of the gene for the homeodomain transcription factor *Chx10*, which is specifically expressed in the presumptive neural retina. Expression of *Chx10* started at HH10 and was restricted to the distal optic vesicle (Fig. 6D). *Chx10* expression in explant cultures with extraocular mesenchyme was similarly restricted distally when compared with *Mitf* (compare Fig. 3B with 6E) and, thus, closely correlates with the in vivo

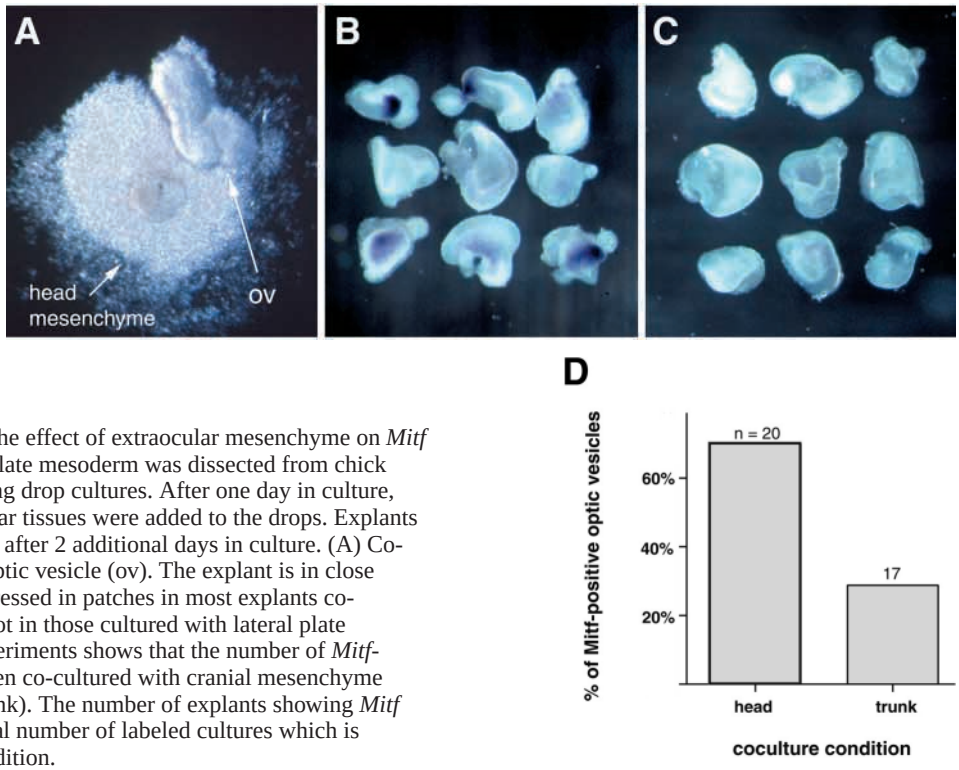

Fig. 5. Cranial mesenchyme substitutes for the effect of extraocular mesenchyme on *Mitf* expression. Cranial mesenchyme or lateral plate mesoderm was dissected from chick embryos (HH13-14) and incubated in hanging drop cultures. After one day in culture, optic vesicles without surrounding extraocular tissues were added to the drops. Explants were processed for *Mitf* in situ hybridization after 2 additional days in culture. (A) Co-culture of cranial (head) mesenchyme and optic vesicle (ov). The explant is in close contact with mesenchymal cells. *Mitf* is expressed in patches in most explants co-cultured with cranial mesenchyme (B) but not in those cultured with lateral plate mesoderm (C). (D) Quantification of all experiments shows that the number of *Mitf*-expressing optic vesicles is much higher when co-cultured with cranial mesenchyme (head) than with lateral plate mesoderm (trunk). The number of explants showing *Mitf* expression is shown as percentage of the total number of labeled cultures which is indicated on the top of the bars for each condition.

expression (*n*=13). Interestingly, removal of extraocular mesenchyme caused an increase of *Chx10* expression that was observed in 100% of labeled explants (*n*=13) and extended throughout the explant in most cases (Fig. 6F). In some explants, very small portions did not express *Chx10*, which may be caused by residual *Mitf* expression under these conditions (compare Figs 3C and 6F). This suggests that expression of *Chx10* and *Mitf* do not overlap in these explants, similar to their expression patterns in vivo (Fig. 2). The expansion of the *Chx10* expression domain in the explant cultures occurred even in the absence of surface ectoderm (Fig. 6F). Similar results were obtained using *Optx2*, a member of the six family of homeobox transcription factors that is expressed in the distal and ventral optic vesicle but not in the presumptive RPE in the chick embryo (Toy et al., 1998; Fig. 6G). In explants grown with extraocular mesenchyme, *Optx2* was strongly expressed in those parts of the optic vesicle that are not surrounded by extraocular mesenchyme (Fig. 6H, *n*=12). In the absence of extraocular mesenchyme, *Optx2* was expressed throughout the explants (Fig. 6I, *n*=10). Therefore, extraocular mesenchyme downregulates expression of *Chx10* and of the more widely distributed *Pax6* and *Optx2*.

Activin substitutes for the extraocular mesenchyme in optic vesicle explant cultures

Our results so far strongly suggest that the extraocular mesenchyme produces a signal that regulates the expression of genes specific for the neural retina and RPE domains in the optic vesicle. The TGFβ family member activin represents a good candidate since expression of activin βA and βB subunits was detected in the extraocular mesenchyme of frog and mouse (Dohrmann et al., 1993; Feijen et al., 1994). To investigate the effect of activin on the expression of eye-specific genes. we incubated optic vesicles without extraocular tissues in media containing 100 ng/ml activin A for 2 days and processed at least 10 explants for each marker for whole-mount in situ hybridization or immunostaining (Fig. 7). Addition of activin to the culture media caused an upregulation of all of the RPE-specific markers we examined: *Mitf*, *Wnt13* and MMP115. This effect is shown in Fig. 7 and can be seen by comparing Fig. 7A,B,C with Fig. 3C,F,I, respectively. Although the expression domains of these genes appeared extended by activin treatment, it is not possible to determine whether expansion into the neural retina domain occurred. In contrast, expression of the neural retina-specific genes *Pax6*, *Chx10* and *Optx2* was downregulated or strongly repressed. This effect can be seen by comparing Fig. 6C,F,I with Fig. 7D-F, respectively. In addition, the size of activin-treated explants appeared slightly smaller than untreated explants (compare Figs 3, 6 and 7) possibly caused by inhibition of proliferation as previously shown in human RPE cells in vitro (Jaffe et al., 1994). These data indicate that activin exactly mimics the effect of extraocular mesenchyme on RPE and neural retina development in explant culture.

We quantified these effects of activin and other TGFβ family members and the data are shown in Fig. 8. The effect of activin on *Mitf* expression in vitro is specific since BMP5, BMP7, and GDF5 did not show a comparable effect (Fig. 8A). In addition, activin promotes the expression of MMP115 in a dose-dependent manner, with a maximal effect between 100 and 130 ng/ml (Fig. 8B). A possible role for activin a vivo is supported by the expression pattern of the ligand-binding activin receptors type IIA and IIB in the chick embryo at HH11 (Fig. 9; Stern et al., 1995). Activin receptor type IIB and IIA are expressed in the central nervous system in the head (Fig. 9A,B). Cryostat sections of optic vesicles show strong

expression of both activin receptors in the presumptive RPE and neural retina (Fig. 9C,D). In addition, activin receptor type IIA is present in the extraocular mesenchyme (Fig. 9D).

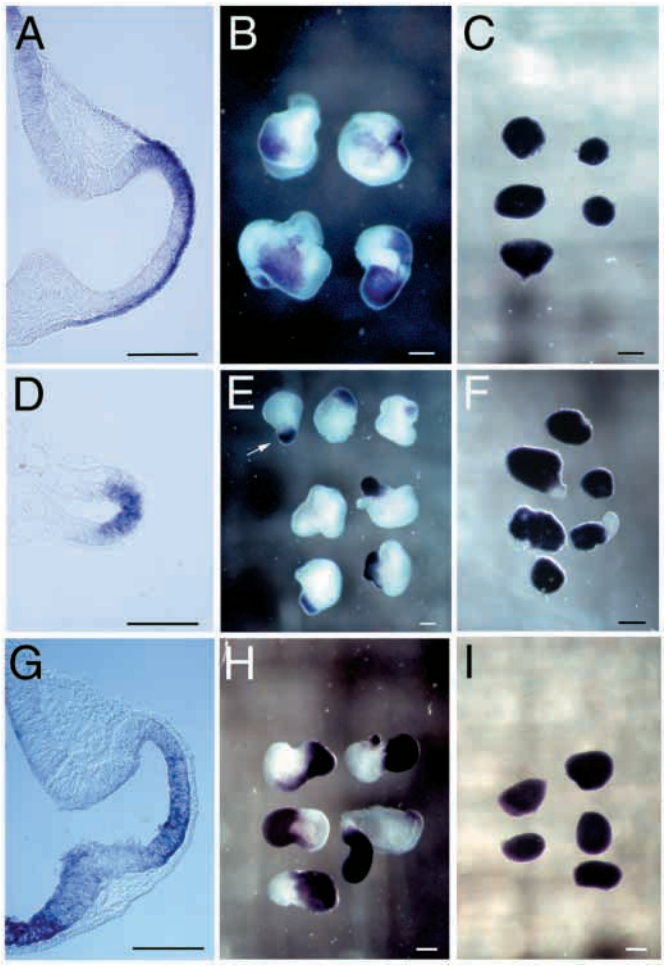

Fig. 6. Effect of extraocular mesenchyme on the expression of *Pax6, Chx10* and *Optx2*. Embryos or cultures were analyzed by whole-mount in situ hybridization for *Pax6* mRNA (A-C), *Chx10* mRNA (D-F) and *Optx2* mRNA (G-I). (A) Section through the optic vesicle after in situ hybridization showing *Pax6* expression at HH11. *Pax6* is expressed in the dorsal optic vesicle, overlying surface ectoderm, and dorsal diencephalon. (B) Explants grown for 2 days in the absence of surface ectoderm, also express *Pax6* through much of the optic vesicle. (C) Explants grown in the absence of extraocular tissues express *Pax6* throughout the explant. (D) Section through the optic vesicle after in situ hybridization for *Chx10* at HH10. *Chx10* is expressed in the presumptive retina adjacent to the overlying surface ectoderm, but excluded from the proximal optic vesicle that contacts the extraocular mesenchyme. (E) In optic vesicle explant cultures with extraocular mesenchyme, *Chx10* is restricted to a small region of the optic vesicle (arrow). (F) Removal of extraocular mesenchyme causes an upregulation of *Chx10* throughout the whole optic vesicle. Occasionally, very small patches of the explant do not express *Chx10*. (G) Section of an embryo HH12 showing expression of *Optx2* in the presumptive neural retina in the distal optic vesicle and ventral diencephalon. (H) Optic vesicle explants cultured with extraocular mesenchyme express *Optx2* in the presumptive neural retina. (I) In the absence of extraocular tissues, *Optx2* is expressed throughout the explant. Scale bars, 100 μm in A,D,G; 200 μm in B,C,E,F,H,I.

DISCUSSION

Extraocular mesenchyme regulates patterning of the domains of RPE and neural retina in the chick optic vesicle

Using molecular markers expressed at different stages of RPE development, the present study shows that the extraocular mesenchyme is crucial for patterning/induction and subsequent differentiation of the RPE in chick optic vesicle explants. The extraocular mesenchyme activates the expression of a critical key transcription factor Mitf as well as the expression of the later RPE-specific markers Wnt13 (Jasoni et al., 1999) and the melanosomal protein MMP115 (Mochii et al., 1988). Furthermore, our data reveal that the extraocular mesenchyme regulates expression of the neural retina-specific transcription factor Chx10 in the opposite manner. In optic vesicle explants with extraocular mesenchyme, *Chx10* expression is restricted to the distal domain that is not surrounded by mesenchymal cells. Removal of the extraocular mesenchyme causes an expansion of the *Chx10* domain in the explants. In addition, the homoeobox transcription factors Pax6 and Optx2 are also downregulated by extraocular mesenchyme. In optic vesicle explant culture, removal of extraocular mesenchyme causes the expansion of *Pax6* and *Optx2* expression throughout the whole optic vesicle. Thus, our data suggest that the extraocular mesenchyme patterns the optic vesicle by activating *Mitf* and

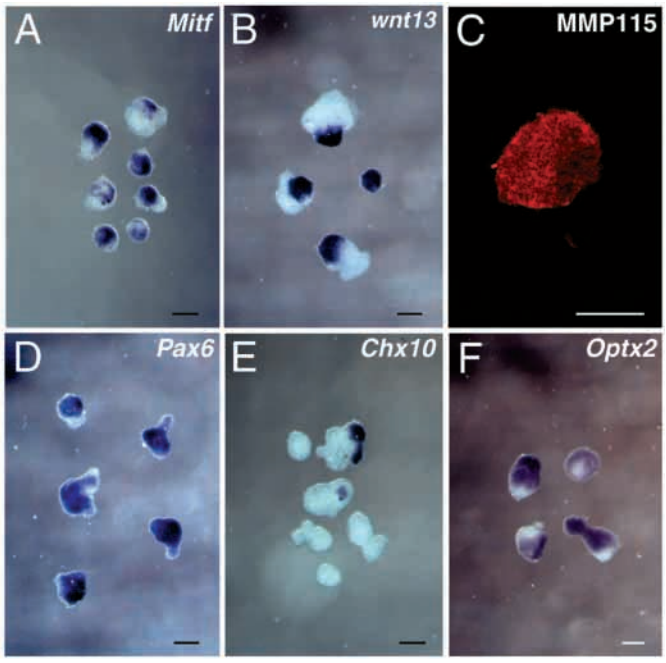

Fig. 7. Activin can substitute for the effect of extraocular mesenchyme on the expression of RPE-markers and the neural retina-specific genes. Optic vesicles without extraocular tissues were cultured with 100 ng/ml activin A for 2 days and analyzed by whole-mount in situ hybridization immunolabeling for *Mitf* (A), *Wnt13* (B), MMP115 (C), *Pax6* (D), *Chx10* (E) and *Optx2* (F). (A-C) The expression of the RPE-specific markers *Mitf*, *Wnt13* and MMP115 is upregulated in activin-treated explants. (D-F) In contrast, the expression of the neural retina-specific genes *Pax6* (D) and *Optx2* (F) is downregulated or strongly inhibited (*Chx10* in E) by activin. Scale bars, 200 μm in A,B,E,F; 100 μm in C.

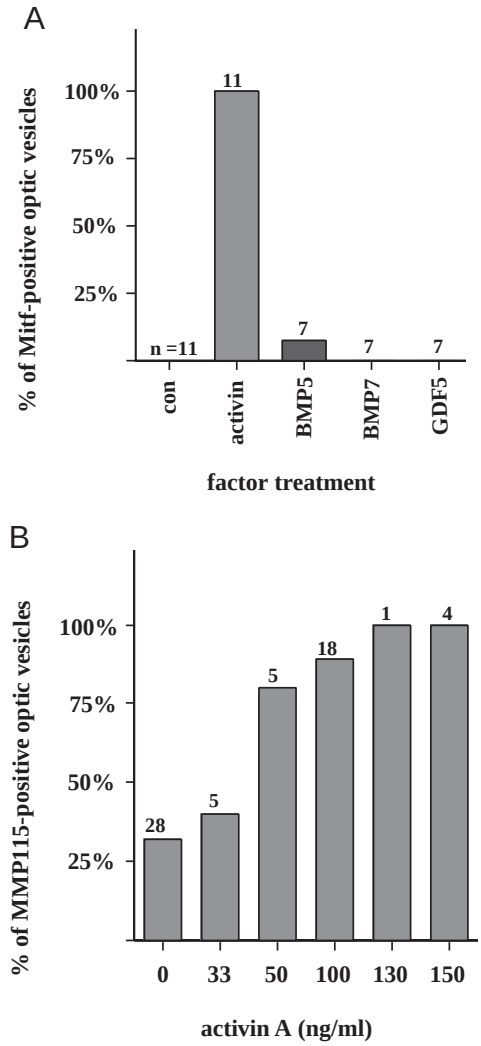

Fig. 8. Activin specifically promotes expression of *Mitf* and MMP115 in optic vesicle explant cultures. Factors were added at the beginning of the culture period and, after 2 days, explants were hybridized with the *Mitf* probe (A) or immunolabeled for MMP115 (B). (A) Optic vesicles were grown with no factor added (con) or in media containing 100 ng/ml activin A, BMP5, BMP7 or GDF5. Activin A upregulates expression of *Mitf* in all of the explants. In contrast, treatment of the explants with BMP5, BMP7 or GDF5 did not promote *Mitf* expression. (B) Explants were cultured in the presence of different activin A concentrations as indicated. The effect of activin A on MMP115 expression in the optic vesicle explants is dose dependent. Expression of MMP115 in all of the explants is detectable when between 100 and 130 ng/ml activin was added to the cultures.

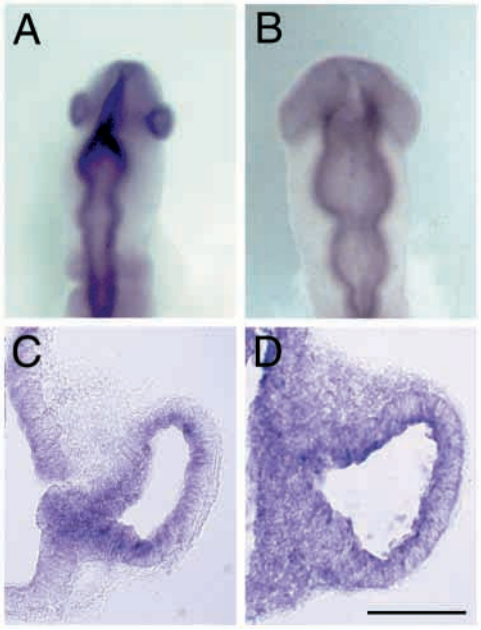

Fig. 9. Expression of the activin receptors type II in the neuroepithelium of the optic vesicles in the chick embryo. Whole-mount in situ hybridization for activin receptor type IIB (A,C) and IIA (B,D) was performed using chick embryos HH11. (A) Head of a chick embryo showing expression of activin receptor type IIB in the central nervous system. (B) Similarly, activin receptor type IIA is expressed in the neuroepithelium of the forebrain. (C,D) Cryostat sections indicate the expression of both activin receptors in the optic vesicle. Scale bar, 100 μm in C,D.

inhibiting *Chx10, Pax6* and *Optx2*. This suggests that signal(s) in the extraocular mesenchyme are necessary to induce and maintain the RPE domain during further eye development. We could identify activin as a potential candidate molecule that induces expression of the RPE-specific markers and downregulates expression of the neural retina-specific genes.

These observations are summarized in the following model (Fig. 10), which proposes a crucial role for the extraocular mesenchyme in RPE development. Our results indicate that extraocular mesenchyme is essential for the expression of RPE-specific genes but downregulates expression of neural retina-specific markers. We could identify an activin-like signal as a candidate molecule. Previous studies strongly suggest that FGF localized in the overlying surface ectoderm is important for the differentiation of the neural retina (Pittack et al., 1997; Hyer et al., 1998). FGF also has been shown to inhibit the development of the RPE and induce its transdifferentiation into neural retina (Park and Hollenberg, 1989; Pittack et al., 1991; Guillemot and Cepko, 1992; Zhao et al., 1995). Therefore, we propose that extraocular mesenchyme (possibly an activin-like signal) and surface ectoderm (FGF) act antagonistically in patterning the optic vesicle into the domains of RPE and neural retina.

Our results indicate that the extraocular mesenchyme produces a signal that activates development of the RPE domain in the optic vesicle. Co-culture experiments revealed that this activator is also present in the cranial mesenchyme that originates from the region between diencephalon and otic placode. Interestingly, a signal promoting RPE development is not present in trunk mesoderm, as shown by co-culture experiments with lateral plate mesoderm. It is also possible that trunk mesoderm expresses an inhibitor of RPE development. FGF family members are known to inhibit differentiation of the RPE and endogenous FGF3 expression in the lateral plate mesoderm was recently demonstrated (Mahmood et al., 1995). However, in co-culture with lateral plate mesoderm, *Mitf* is still expressed in a small percentage of explants, suggesting that inhibition does not occur.

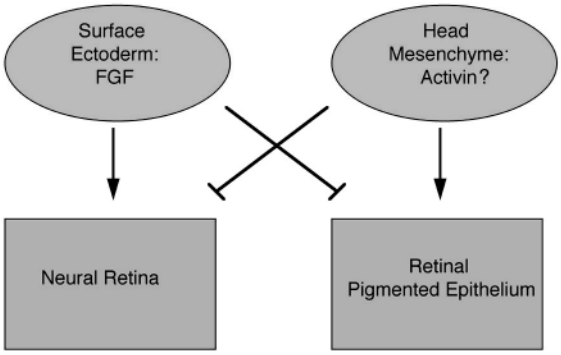

Fig. 10. Proposed model for the regulation of patterning of the neural retina and retinal pigmented epithelium (RPE) domains in the developing eye. Previous studies strongly suggest that FGF localized in the overlying surface ectoderm is important for the differentiation of the neural retina. FGF also has been shown to inhibit the development of the RPE and induce transdifferentiation of the RPE into neural retina. Our results show that extraocular mesenchyme is essential for the expression of RPE-specific markers but downregulates expression of neural retina-specific genes. We could identify an activin-like signal as a candidate molecule. Therefore, we propose that extraocular mesenchyme (possibly an activin-like signal) and surface ectoderm (FGF) act antagonistically in patterning the optic vesicle into the domains of RPE and neural retina.

The role of extraocular mesenchyme and surface ectoderm in vertebrate eye development

Evidence from other studies are consistent with our data. The transcription factor AP2 is a retinoic acid-responsive gene that is strongly expressed in the neural crest-derived cranial mesenchyme (Shen et al., 1997, West-Mays et al., 1999). Mice with a null mutation in the AP2-α gene exhibit defects in ocular development and formation of an ectopic retina in the dorsocentral region of the presumptive RPE (Nottoli et al., 1998; West-Mays et al., 1999). Since AP2-α is not expressed in the developing RPE, this defect is most likely secondary, caused by an inappropriate differentiation of the extraocular mesenchyme leading to decreased or no production of a RPE-promoting signal in the extraocular mesenchyme (West-Mays et al., 1999).

The differential expression pattern of *Mitf* in the optic vesicle of chick and mouse supports the presence of a RPE-promoting signal in the extraocular mesenchyme. In contrast to chick, *Mitf* expression in mouse starts throughout the optic vesicle and later becomes restricted to the presumptive RPE (Bora et al., 1998; Mochii et al., 1998). In rodents, mesenchymal cells are often observed between surface ectoderm and the distal part of the optic vesicle prior to the induction of lens placode (Kaufman, 1979; de Iongh and McAvoy, 1993; Bora et al., 1998; Furuta and Hogan, 1998). In chick, the distal part of the outgrowing optic vesicle is always in close contact with the overlying surface ectoderm (Hilfer, 1983) and, therefore, does not receive a RPE-promoting signal.

While the extraocular mesenchyme has an important role in patterning the optic vesicle, several previous studies have proposed that the overlying surface (lens) ectoderm also plays a critical role during early eye development. Dragomirov (1937) first proposed that contact with the lens ectoderm of the distal part of the optic vesicle promotes neural retina development in this domain. He tested this idea by transplanting the optic vesicle to other locations in the embryo and found that the presumptive RPE developed as neural retina when placed near the lens ectoderm. Some other epithelia such as the otic and olfactory placode also possess this capability (Dragomirov, 1937). Recent studies have implicated FGF as a candidate factor released from the lens ectoderm that patterns the distal optic vesicle. At least two members of the FGF family of signalling molecules have been shown to be expressed in the lens ectoderm (de Iongh et al., 1993; Pittack et al., 1997). In addition, treatment of the optic vesicles with FGFs causes the presumptive RPE to develop as neural retina (Zhao et al., 1995; Pittack et al., 1997; Hyer et al., 1998) and inhibition of FGF signaling with either antibodies or antisense oligonucleotides inhibits neural retinal development (Pittack et al., 1997; Desire et al., 1998). Part of the mechanism of this response to FGF may be due to the fact that it downregulates *Mitf* expression (Mochii et al., 1988). However, while removal of the ectoderm interferes with neural retinal development from the optic vesicle (Holtfreter, 1939; Hyer et al., 1998), it does not appear to be required for the maintenance of *Chx10* expression (this study). *Chx10* is expressed very early indicating that the domain of the presumptive neural retina is already determined at the time of manipulation. Since both tissues are in close contact before induction of lens formation in chick embryos (Hilfer, 1983), the distal part of the optic vesicle could have already been exposed to a neural-retina inducing signal before the surface ectoderm was removed. Interestingly, Winkler et al. (2000) found that the temperature-sensitive mutation *eyeless* (presumably unrelated to *Pax6*) in medaka fish exhibits no evagination of optic vesicles and a failure of subsequent neural retina and lens differentiation consistent with the hypothesis that the overlying surface ectoderm is required to induce retinal differentiation.

An activin-related molecule may represent the RPE-promoting signal in the extraocular mesenchyme

We tested the TGFβ family member activin for its ability to regulate expression of eye-specific genes in optic vesicle explant cultures. Our data show that activin specifically induces expression of the RPE-specific markers *Mitf*, *Wnt13* and MMP115, and downregulates expression of the neural retina genes *Pax6*, *Chx10* and *Optx2*. This indicates that activin can substitute for the extraocular mesenchyme in promoting RPE development and downregulating expression of neural retina-specific genes. In addition, we confirmed that the activin receptors type IIA and IIB are present in the neuroepithelium of the optic vesicle suggesting a possible role for activin or a related molecule during early eye development in vivo. Our data are consistent with previous studies. Activin inhibits neuronal differentiation in murine P19 carcinoma cells (Ameerun et al., 1996) and expression of activin βA and βB subunits is detectable in the extraocular mesenchyme in frog and mouse (Dohrmann et al., 1993; Feijen et al., 1994). However, we were not able to detect expression of activin βA or βB subunits in the extraocular mesenchyme in the chick embryo at optic vesicle stages (not shown; Connolly et al., 1995). It is therefore possible that a related molecule is produced by the extraocular mesenchyme that binds to the activin receptors and has not yet been identified. For example, other activin β-subunits have been identified in frog and

rodents (Oda et al., 1995; Fang et al., 1996, 1997; O'Bryan et al., 2000) suggesting that additional activins may also exist in the chick. Mice heterozygous for Smad2, a transcription factor mediating intracellular signaling by activin, lack eyes, suggesting that the activin signaling pathway could be involved in eye formation (Nomura and Li, 1998). Animals with an inactivation of both activin receptor type IIA and IIB do not develop beyond gastrulation making it impossible to determine the effect on eye development (Song et al., 1999). However, none of the already identified activin subunits may be important for early eye development, since mice deficient in activin βB subunits or type IIA receptor exhibit defects in eyelid development but no obvious disturbances during early formation of the RPE and neural retina (Vassalli et al., 1994; Matzuk et al., 1995). Our next step will be to clarify the role of activin or a related signal during early eye development.

In summary, our study provides evidence for the presence of molecules in the extraocular mesenchyme that are involved in the early regulation of both neural retina and RPE development and act antagonistically to FGF localized in the overlying surface ectoderm. The effect of activin on expression of RPE- and neural retina-specific markers suggests a possible mechanism underlying the regulation of early eye development by the surrounding extraocular mesenchyme.

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