Indian hedgehog signaling from endothelial cells is required for sclera and retinal pigment epithelium development in the mouse eye

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Abstract

The development of extraocular orbital structures, in particular the choroid and sclera, is regulated by a complex series of interactions between neuroectoderm, neural crest and mesoderm derivatives, although in many instances the signals that mediate these interactions are not known. In this study we have investigated the function of Indian hedgehog (Ihh) in the developing mammalian eye. We show that Ihh is expressed in a population of non-pigmented cells located in the developing choroid adjacent to the RPE. The analysis of Hh mutant mice demonstrates that the RPE and developing scleral mesenchyme are direct targets of Ihh signaling and that Ihh is required for the normal pigmentation pattern of the RPE and the condensation of mesenchymal cells to form the sclera. Our findings also indicate that Ihh signals indirectly to promote proliferation and photoreceptor specification in the neural retina. This study identifies Ihh as a novel choroid-derived signal that regulates RPE, sclera and neural retina development.

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Introduction

The sclera, choroid, and retinal pigmented epithelium (RPE) are essential for normal eye development and function. The sclera and choroid are derived from the peri-ocular mesenchyme (POM), a mixture of mesoderm and neural-crest derived mesenchymal cells that surround the developing eye beginning at E10.5 in the mouse (Gage et al., 2005). These POM cells proliferate and aggregate around the eyecup to form a distinct cell layer, with the inner-most cells adjacent to the RPE giving rise to the choroid and the outermost layer differentiating as the sclera. The highly vascular choroid nourishes the RPE and photoreceptors while the viscoelastic connective tissue of the sclera provides mechanical support to counteract intra-ocular pressure to maintain the shape of the eye, as well as providing sites of insertion for the extrinsic ocular muscles. Fate mapping experiments in the mouse have revealed a mesodermal origin for choroidal endothelial cells and ocular muscles and a neural-crest origin for the other cell types in the choroid and the sclera (Gage et al., 2005).

The development and maintenance of the RPE, choroid and sclera are highly interdependent. The POM is required for the specification of the RPE and growth of the eyecup (Fuhrmann et al., 2000; Matt et al., 2005) and it subsequently becomes dependent on the RPE for the induction of the choroid (Marneros et al., 2005; Zhao and Overbeek, 2001). Dysgenesis of the choroid is associated with RPE abnormalities (Marneros et al., 2005; Rousseau et al., 2003) and disease or injury to the RPE is often associated with degeneration of the choroid and sclera (Korte et al., 1984; May et al., 1996; Torczynski, 1982), suggesting that interactions between these tissues are required for their maintenance. Several transcription factors, including Foxc1, Foxc2, Pitx2 are required within the POM for the development of the anterior chamber and sclera (Evans and Gage, 2005; Gage et al., 1999; Kume et al., 1998; Lin et al., 1999; Lu et al., 1999; Smith et al., 2000). With the exception of...
 activin-like molecules (Fuhrmann et al., 2000) and retinoic acid (Matt et al., 2005), which mediate the POM effects on the RPE and VEGF, FGf2 and Bmp4, which mediate the RPE effects on the choroid (Marnero et al., 2005; Rousseau et al., 2003; Sakamoto et al., 1995) and anterior segment (Chang et al., 2001), the molecular basis for signaling between these tissues is largely unknown. Here we describe Indian hedgehog (Ihh) as a novel regulator of POM development. Ihh and its mammalian homologues Sonic hedgehog (Shh) and Desert hedgehog (Dhh) belong to the hedgehog (Hh) family of extracellular signaling proteins that control patterning and growth of a number of tissues and cell types in the developing embryo (reviewed in (Ingham and McMahon, 2001)). The Hh signaling pathway is activated in cells by Hh binding to and opposing the activity of its receptor Patched (Ptc1), a 12-transmembrane domain protein (Marigo et al., 1996; Stone et al., 1996), thereby de-repressing the activity of Smothened, a 7-transmembrane domain protein that is required for transduction of the Hh signal (Alcedo et al., 1996; Murone et al., 1999; van den Heuvel and Ingham, 1996). Active Smo signals via cytoplasmic effectors of the Gli transcription factor family to regulate target gene expression (Ingham, 1998). Ptc1 and Gli1 are two universal target genes of the pathway and their expression serves as a convenient readout for the status of Hh pathway activation in tissues (Goodrich and Scott, 1998).

Hh signaling has been shown to play a role in eye development in a number of vertebrate species (reviewed in (Amato et al., 2004; Wallace, 2007)). In the mouse and chick Shh signaling from retinal ganglion cells, the projection neurons of the retina, is required to maintain the progenitor cell pool and also regulates cell fate by inhibiting the production of RGCs and promoting the production of late developing cell types (Moshiri et al., 2005; Wang et al., 2005, 2002; Zhang and Yang, 2001a). In fish and frogs Hh homologues are also expressed in RGCs (Amato et al., 2004) and regulate the onset of retinal neurogenesis and RGC differentiation (Masai et al., 2005; Neumann and Nuesslein-Volhard, 2000; Shkumatava et al., 2004; Stenkamp et al., 2002) in part via effects on cell cycle progression (Locke et al., 2006) and cell cycle exit (Shkumatava and Neumann, 2005). The RPE is also the site of Hh expression with three Hh homologues expressed in this region in Xenopus and two in the zebrafish (Perron et al., 2003; Stenkamp et al., 2000). Pharmacological inhibition of the Hh pathway in frog embryos results in RPE defects (Perron et al., 2003) and direct injection of antisense oligodeoxynucleotides in the zebrafish RPE blocks photoreceptor differentiation (Stenkamp et al., 2000), consistent with a role for RPE-derived Hh signaling in RPE and photoreceptor development.

We showed previously that Ihh is expressed outside the eye in a subset of cells adjacent to the RPE and is required for Hh target gene expression in the POM (Dakubo et al., 2003; Wallace and Raff, 1999). Here we show that cells in the developing choroid express Ihh, which signals to the RPE and POM. Ihh knockout (KO) mice exhibit loss of Hh target gene expression in the POM and extensive defects in the posterior sclera, resulting in deformed ocular shape and increased fragility of the globe. Also evident in Ihh KO mice are abnormalities of the RPE, including abnormal pigment distribution, and a reduction in progenitor proliferation and photoreceptor specification in the neural retina. Gene expression analyses in Shh and Ihh KO mice confirm that Ihh is required for Hh target gene expression in the POM, but not the neural retina. Thus our data show that mammalian eye development is regulated by a second Hh homologue, Ihh, acting directly to promote the differentiation of the RPE and peri-ocular tissues and indirectly to regulate photoreceptor development.

**Materials and methods**

**Mice**

The Ihh KO ([St-Jacques et al., 1999], ShhRGL (Lewis et al., 2001) and Gli1-lacZ (Bai et al., 2002) mice have been described previously and were maintained on a C57Bl/6 and CD1 backgrounds. The GlI3 mutant mice (Hui and Joyner, 1993) were maintained on a mixed C57Bl/6 x C3H background. Embryos were genotyped by the PCR on tail-derived genomic DNA using gene specific primer sets (all genotyping protocols are available on request). Mice were coupled in the late afternoon and the presence of the vaginal plug early the next morning was considered as embryonic day 0 (E0). All mutant embryo phenotypes were compared to littermate controls with the same mixed background. At least three litters of each combination mutant pair were analyzed and compared at each age (E12.5, E13–14 and E17–18). We note that during the course of this study (~2 years) lethality associated with the Ihh mutation manifested at earlier stages in embryonic development such that the latest stage at which we could recover mutant embryos is E13.5. The figures are representative of the phenotypes and gene expression consistently observed at the different embryonic stages.

**In situ hybridization and immunohistochemistry**

Embryos were harvested and tissues fixed in 4% paraformaldehyde and adult mice were perfused with 4% paraformaldehyde prior to dissection of the eye and removal of the lens. Tissues were cryoprotected in 30% sucrose in PBS (Dulbecco’s Phosphate buffered saline, Sigma) before embedding in equal amounts of 30% sucrose and OCT (Tissue Tek). Upper bodies or heads were isolated, depending on the age of the embryo, and tails were collected for genotyping. Tissues were sectioned at 12–14 μm using a Leica CM 1850 cryostat and processed for in situ hybridization (ISH) or β-gal staining. In situ hybridization was performed according to (Wallace and Raff, 1999). Digoxigenin (DIG)-labeled antisense RNA riboprobes were prepared by reverse transcription from linearized plasmids containing complete or partial sequences of the mouse genes of interest; Ihh, Gli1, Mitf, Ptx2, Foxz2, Crx, Shh. Briefly, sections were hybridized overnight with DIG-labeled riboprobes at 65 °C in a moist chamber. Sections were washed at high stringency, incubated with an alkaline phosphatase-conjugated antibody and stained in nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate. X-gal staining in adult Gli1-lacZ retinas was performed as described previously ([Liu et al., 2003]). For IHC the following primary antibodies were used: rabbit polyclonal anti-collagen IV (Biogenesis), mouse monoclonal anti-Ki67 (BD Bioconome), rabbit polyclonal anti-phosphoHistone3 (Upstate Biotechnology), rabbit anti-mouse polyclonal collagen I (Chemicon), mouse monoclonal anti-BrdU (Becton Dickinson). For DAB staining with anti-collagen IV antibodies (rabbit polyclonal, Biogenesis) cytosphere were fixed with 70% ethanol, treated with 0.3% H2O2, and incubated in blocking solution (20% sheep serum in TBS; 50 mM Tris-Cl pH 7.4, 150 mM NaCl; 1% BSA, 50 mM l-lysine, 0.1% Azide) and then incubated with primary antibody (diluted 1:3000 in block) for a minimum of 1 h at room temperature or overnight. After extensive washing, the staining was developed with vectastain® ABC Elite avidin/biotin/ peroxidase kit (Vector laboratories, Burlingame, California) using DAB as a substrate. Sections processed for IHC with other primary antibodies were treated as described above except that the H2O2 pretreatment was omitted. For Ki67 staining the sections were subjected to antigen retrieval by microwaveing slides in a solution of 1X sodium citrate buffer for 6 min. Sections were counterstained with species specific FITC-conjugated secondary antibodies (Jackson Immunoresearch). The proliferation index of cells in the POM in the posterior pole of the retina was quantified by determining the number of nuclei per mm2 in wildtype (E13.5, which is the latest stage at which the phenotype is manifest), with a marker for proliferation, Ki67, a proliferation marker, in two defined areas of the POM on either side of the optic nerve in horizontal sections taken at the level of the optic nerve from wildtype and Ihh KO mice. Data are expressed as mean±SD from 2–4 sections/eye from four mice of each genotype. The thickness of collagen I staining in the sclera was the average of 5 measurements/section taken in the posterior sclera (posterior to the ora serrata) in 3 sections in the horizontal plane in the vicinity of the optic nerve head from 4 wildtype and 4 Ihh KO eyes at E13.5.

**Electron microscopy**

Specimens were fixed in solution of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M Phosphate pH 7.4, postfixed in 1% osmium tetroxide, en bloc stained in 3% aqueous uranyl acetate, dehydrated in ascending grades of ethanol and further processed in Spurr epoxy resin. 0.5 μm sections were cut and stained with Methylene Blue. Thin sections were subsequently cut on a Reichert Ultracut E ultramicrotome and the ultrathin grids counterstained with Reynold’s lead citrate. Sections were examined with a Jeol 1230 TEM equipped with AMT software.

**Explant culture**

Optic cups from E12 C57Bl/6 embryos were dissected in CO2-independent medium ( Gibco) and two explants were placed in a well of a 24 well plate submerged in 0.5 ml of serum free culture medium. (1:1 DMEM/F12 supplemented with insulin (10 μg/ml), transferrin (100 μg/ml), BSA Fraction V (100mg/ml), progesterone (60ng/ml), putrescine (16 μg/ml), sodium selenium (40 μg/ml), N-acetyl cysteine (60 μg/ml), and gentamycin (25 μg/ml). Explants were either untreated (controls) or cultured in the presence of Smoothened agonist (Smo-Ag) at 10 nM (a kind gift of Curis Inc.) for 48 h and serial sections of fixed material were analyzed for ISH and IHC, as described above. Explant sections were examined on a Zeiss Axioplan microscope and digital images captured with the Axio Vision 2.05 (Zeiss) camera and processed with Adobe® Photoshop version 7.
Results

Hh pathway components are expressed in the developing peri-ocular mesenchyme and retinal pigmented epithelium

To clarify the role of Ihh signaling in eye development, we performed a temporal analysis of the expression patterns of Ihh and Hh pathway components by in situ hybridization (ISH) in the developing eye (summarized in Fig. 1A). Ihh expression is first observed at E11 in a discontinuous layer of non-pigmented cells adjacent to the RPE (Fig. 1B). ISH on sagittal sections of the anterior optic cup reveals a lateral gap in this layer of Ihh-expressing cells on the temporal side of the eye (Fig. 1C, arrow). In more posterior regions of the optic cup however, the Ihh-expressing cells surround the entire optic cup and the optic stalk (data not shown). Ihh-expressing cells are detected by ISH in the POM up to E14, after which we are unable to detect expression of this gene by ISH (data not shown). ISH on sections on embryonic eyes from albino mice confirms the absence of Ihh mRNA in the presumptive RPE (Supplementary Fig. 1).

Fig. 1. Ihh and Gli1 expression in extraorbital structures in the developing and adult eye. Diagram of the eye (A) and ISH for Ihh (B–D) and Gli1 (E–H) expression in the eye in the coronal plane at E11 (B, E, F) and sagittal plane at E12 (C, D, G, H). (A) Diagram of the eye in the coronal plane indicating the optic nerve (ON), neural retina (NR), retinal pigment epithelium (RPE) and the lens (L). Expression of Ihh and Gli1 in the peri-ocular mesenchyme (POM) is indicated by the blue and red cells, respectively. The vertical line indicates the plane of section of the eye in panels C, D, G, H. (B–D) Ihh expression is localized to a row of cells adjacent to the RPE. The arrow in C indicates the gap in Ihh expression on the temporal side of the developing eyecup. d, dorsal; v, ventral; n, nasal; t, temporal. Panel D is a higher magnification view of the ventral eyecup in panel C and arrows indicate the position of the Ihh-expressing cells. (E–H) Gli1 is expressed in the RPE, POM and the ventral eyecup at E11 and in the RPE and POM at E12. F is a higher magnification view of the boxed region in E and H is a higher magnification view of the ventral eyecup in panel G. The region between the dashed lines in panels F and H indicates the Gli1-expressing POM. (I) β-galactosidase staining in the RPE and choroid/sclera (area in bracket) in the eye of an adult Gli-LacZ mouse. (J, K) Co-localization of Ihh mRNA (blue) with collagen IV (brown) staining in the choroid at E12. K is a higher magnification view of the posterior pole of the eye in panel J. The arrows indicate examples of apparent co-localization of Ihh and collagen IV staining.
We next examined the expression of genes that are targets and/or mediators of the Hh signaling pathway in the RPE, POM and the neural retina (Figs. 1E–H). At E11 mRNA for Gli1, a Hh target gene (Goodrich and Scott, 1998), is detected in the ventral optic cup, RPE, POM (Figs. 1E, F). By E12, Gli1 expression in the POM forms a ring around the eye cup, but is excluded from a subset of cells adjacent to the RPE, presumably the Ihh-expressing cells, and is downregulated in the optic cup (Figs. 1G, H). From E14.5 onwards the Gli1 expression domain in the sclera is markedly narrowed making it difficult to detect by ISH in pigmented eyes. To circumvent this problem we analyzed reporter gene expression in this region of the eye in adult Gli-lacZ mice (Bai et al., 2002) and observed β-gal+ cells in the RPE and sclera, which indicates that Hh signaling is maintained in these tissues in the adult eye (Fig. 1I).

Since the Ihh-expressing cells are distinct from RPE cells, we investigated whether they were endothelial cells, a cell type that is located in the developing choroid adjacent to the RPE. We performed ISH for Ihh followed by immunohistochemistry for collagen IV, an endothelial cell marker. At E14, the Ihh ISH signal appears to co-localize with collagen IV staining (Figs. 1J, K). Immunohistochemistry with anti-PECAM antibodies confirms the presence of endothelial cells in this region of the developing eye at this stage (Supplementary Fig. 2). It is unlikely that the Ihh-expressing cells in this region are pericytes, as we did not observe staining for smooth muscle actin, a pericyte marker, in this region at this stage of development (data not shown). These results demonstrate that Ihh expression is a property of a subset of endothelial cells of the developing choroidal vasculature.

Ihh signals at a short range in the RPE and POM

Since both Shh and Ihh are expressed in the developing eye and can induce Gli1 expression, it is possible that either or both are responsible for Hh target gene induction in ocular tissues. To identify the relevant target tissues and range of activity within the eye of these two Hh signals, we examined Hh target gene expression in mouse mutants for Shh and Ihh. ISH analysis of retinal sections of Ihh KO mice at E12 reveals a specific loss of Gli1 mRNA in the POM, although Gli1 expression is maintained in the mesenchymal cells that form the orbital bones (Figs. 2A, B). This finding is consistent with our previous report showing the downregulation of Gli1 expression in the scleral condensation at E14 in Ihh KO mice (Dakubo et al., 2003) and demonstrates that Ihh signaling is required for Hh target gene induction in the POM. It is noteworthy that in the Ihh KO mouse this downregulation of Gli1 expression is observed in the anterior and posterior poles of the eye (Fig. 2).

Fig. 2. Gene expression analysis of Ihh and Shh mutant eyes: Ihh expression is required for Hh pathway activation in the POM. (A, B) ISH for Gli1 expression in sagittal sections of the eye in wildtype (A) and Ihh KO (B) mice at E12. Gli1 expression is absent from the POM in the mutant mice. The asterisks indicate the position of the developing tendon insertions of the extrinsic ocular muscles. (C–F) ISH for Shh (C, D) and Gli1 (E, F) in horizontal sections of wildtype (C, E) and Shh−/-Gli3+/− (D, F) eyes at E14.5. Gli1 expression is maintained in the POM in the double mutant mice, indicating that Shh expression is not required for the induction of Gli1 expression in this region of the eye. Note that two lens structures (L) are present in the fused eye of the Shh−/-Gli3−/− mutant shown in panel D.
posterior POM at the level of the optic nerve head, however, in sections taken dorsal to the optic nerve, there is evidence of low levels of \( \text{Gli1} \) expression in the anterior POM (Supp. Fig. 3). \( \text{Dhh} \) expression has also been reported in the RPE (Levine et al., 1997; Takabatake et al., 1997), however, we were unable to detect \( \text{Dhh} \) mRNA by ISH at any stage in the developing mouse eye (data not shown).

To determine whether the range of action of \( \text{Ihh} \) extends to the neural retina, we examined \( \text{Gli1} \) expression in the eyes of mice with a germline deletion of the \( \text{Shh} \) gene (\( \text{Shh}^{−/−} \)). To generate \( \text{Shh}^{−/−} \) embryos that have eye suitable for analysis we crossed the \( \text{Shh} \) mutation onto a \( \text{Gli3} \) mutant background to generate \( \text{Shh}^{−/−}\text{Gli3}^{−/−} \) compound mice, since the \( \text{Gli3} \) mutant background rescues the severe cyclopia that is observed in \( \text{Shh}^{−/−} \) mice (Litingtung and Chiang, 2000). Compared with the bilaterally positioned wildtype eyes, the eyes of \( \text{Shh}^{−/−}\text{Gli3}^{−/−} \) mutants are located at the midline of the brain and appear as fused RPE and retina, but with two lenses (Fig. 2 compare C and D). RGC differentiation is initiated in the retina, based on the presence of \( \text{Shh} \) transcripts, which are transcribed from the mutant allele and detected with a full length antisense \( \text{Shh} \) probe (Wang et al., 2002), in the RGC layer (Figs. 2C,D) but \( \text{Gli1} \) expression is not induced in the neural retina of \( \text{Shh}^{−/−}\text{Gli3}^{−/−} \) embryos (Fig. 2 compare E and F), consistent with previous data that \( \text{Shh} \) expression is required for Hh target gene expression in the retina (Wang et al., 2002). \( \text{Gli1} \) expression is, however, maintained in the POM of \( \text{Shh}^{−/−}\text{Gli3}^{−/−} \) embryos (Fig. 2F), which is consistent with our previous observations that \( \text{Ihh} \), but not \( \text{Shh} \), is required for the maintenance of Hh target gene expression in the POM (Dakubo et al., 2003). Taken together these findings indicate that the range of action of \( \text{Ihh} \) signaling is restricted to the RPE and POM.

**Eyes of \( \text{Ihh} \) KO mice are hypopigmented and misshapen**

A role for \( \text{Ihh} \) signaling in RPE and peri-ocular tissue development is suggested by the restricted signaling of \( \text{Ihh} \) in these tissues. Perinatal (E14–E18) \( \text{Ihh} \) KO mice exhibit two gross ocular abnormalities (Fig. 3). First, the RPE of \( \text{Ihh} \) KO mice has numerous foci of hypopigmented spots giving it a “salt-and-pepper” appearance (Fig. 3, compare A and B). This phenotype is variable and ranged from severe hypopigmentation with irregular papillary boundaries to smaller spots that have eyes suitable for analysis we crossed the \( \text{Shh} \) mutation onto a \( \text{Gli3} \) mutant background to generate \( \text{Shh}^{−/−}\text{Gli3}^{−/−} \) compound mice, since the \( \text{Gli3} \) mutant background rescues the severe cyclopia that is observed in \( \text{Shh}^{−/−} \) mice (Litingtung and Chiang, 2000). Compared with the bilaterally positioned wildtype eyes, the eyes of \( \text{Shh}^{−/−}\text{Gli3}^{−/−} \) mutants are located at the midline of the brain and appear as fused RPE and retina, but with two lenses (Fig. 2 compare C and D). RGC differentiation is initiated in the retina, based on the presence of \( \text{Shh} \) transcripts, which are transcribed from the mutant allele and detected with a full length antisense \( \text{Shh} \) probe (Wang et al., 2002), in the RGC layer (Figs. 2C,D) but \( \text{Gli1} \) expression is not induced in the neural retina of \( \text{Shh}^{−/−}\text{Gli3}^{−/−} \) embryos (Fig. 2F), which is consistent with our previous observations that \( \text{Ihh} \), but not \( \text{Shh} \), is required for the maintenance of Hh target gene expression in the POM (Dakubo et al., 2003). Taken together these findings indicate that the range of action of \( \text{Ihh} \) signaling is restricted to the RPE and POM.

**Abnormalities in the sclera, choroid and RPE of \( \text{Ihh} \) KO mice**

The tough outer coat of the eye, the sclera, maintains the shape and size of the eye against intrinsic intra-ocular pressure changes. Thus, the abnormal eye shape of \( \text{Ihh} \) KO mice suggests the sclera could be defective. We performed histological analysis of the sclera on perinatal \( \text{Ihh} \) KO mice in comparison to their wildtype littermates. Hematoxylin and eosin staining of sections through the eye of E18 mice reveals an extensive defect in the sclera of \( \text{Ihh} \) KO mice (Figs. 3 I–L). Compared with wildtype littermates, \( \text{Ihh} \) KO mice have focal loss of the sclera in the posterior (optic nerve head) region of the eye (Fig. 3 compare I and J). In regions where the sclera is absent, the tissue that replaces it has the appearance of a primitive mesenchyme (Fig. 3J). The extracellular component of this tissue appears to consist of a watery ground substance with no fibers, the cells are dispersed and their nuclei contain multiple nucleoli (data not shown). In more anterior parts of the eye (at the level of the ora serrata), the sclera of the mutants is present but thinner than those of wildtype littermates (data not shown). Careful examination of the POM and sclera in \( \text{Ihh} \) KO mice shows that the regions with defective scleral condensation are also associated with the hypopigmentary RPE defects and reduced collagen IV staining in the choroid (Fig. 3 compare K and L). Thus, \( \text{Ihh} \) KO mice have regions of defective POM condensation, primarily in the posterior pole of the eye, that are closely associated with reduced melanin in RPE cells and abnormalities in the choriocapillaris.

To investigate the basis of the scleral and RPE defects in the \( \text{Ihh} \) KO mice, we analyzed the development of these tissues at E12–E14, a critical period of condensation formation, by examining the expression, of \( \text{Pitx2} \), a homeodomain transcription factor that is expressed by all POM cells and extrinsic ocular muscles (Kitamura et al., 1999), \( \text{Foxc2} \), a forkhead transcription factor that is expressed in the POM (Hiemisch et al., 1998; Winnier et al., 1997) and Mitf, which labels the RPE and a sub-population of neural-crest cells (Hodgkinson et al., 1993). The expression pattern of \( \text{Pitx2} \) in the POM at E12 is similar in \( \text{Ihh} \) KO and wildtype mice, although there is a slight reduction in gene expression in the posterior POM of the mutants (Figs. 4A,B), indicating that \( \text{Ihh} \) expression is not required for the invasion of neural crest and mesoderm-derived mesenchymal cells around the eye. By E13.5, however, the eyes of \( \text{Ihh} \) KO mice exhibit a severe reduction in \( \text{Pitx2} \) and \( \text{Foxc2} \) expression in the POM around the posterior region of the eye (extending from the optic nerve to the ora serrata) (Figs. 4 C–F). \( \text{Foxc2} \) expression is also reduced in the POM anterior to the ora serrata in the \( \text{Ihh} \) KO mice (Figs. 4E, F). The RPE in \( \text{Ihh} \) KO mice is also thinner, based on the pattern of Mitf expression, although the intensity of the Mitf signal is not different (Fig. 4 compare G and H). Hematoxylin and eosin staining at E13.5 confirms the lack of scleral condensate in the posterior pole of the retina, as well as a thinner condensate in the anterior sclera at the level of the ora serrata in the \( \text{Ihh} \) KO compared with wildtype mice (Figs. 5A–F). The choroidal vasculature is intact in the mutants at this stage, as revealed by the presence of red blood cells (Fig. 5F) and Collagen IV* cells adjacent to the RPE (data not shown). Analysis of sagittal sections of wildtype and \( \text{Ihh} \) KO eyes at E13.5, taking care to distinguish between sclera and tendon insertions, confirmed that the scleral defect was more pronounced in the posterior compared with anterior regions of eye (data not shown).

Consistent with a defect in scleral condensation, staining for collagen I, the major extracellular matrix component of the sclera at this stage, is reduced in the posterior pole of the eye in \( \text{Ihh} \) KO mice compared with wildtype littermates (Figs. 5G, H). Direct measurement of the Collagen I stained region in the sclera at E13.5 revealed that there is a two-fold reduction in the thickness of the Collagen I matrix in \( \text{Ihh} \) KO mice compared with wildtype littermates (Collagen I thickness: WT 35.3±2.5 \( \mu \)m \( \mu \)m; \( \text{Ihh} \) KO 15.6 \( \mu \)m ± 1.7 \( \mu \)m; \( n = 5 \) \( p < 0.0001 \)).

**Ultrastructural analysis of the RPE and sclera in \( \text{Ihh} \) KO mice**

To examine RPE and scleral defects at higher resolution, ocular tissues from wildtype and \( \text{Ihh} \) KO mice at E13.5 were examined in thin sections. In the wildtype eye, the RPE and neural retina are closely apposed and junctions between the retinal neuroepithelial cells are visible (Fig. 6A). The RPE-neural retina junction appears to be normal in some regions of the \( \text{Ihh} \) KO eye (Fig. 6B); however, there are also several regions of full retinal detachment (Fig. 6C). The higher incidence of retinal detachment in \( \text{Ihh} \) KO eyes was also noted in many sections examined by light microscopy (data not shown). In addition, abnormalities are also apparent in the mutant RPE, including elongated and misshapen nuclei and basal, microvilli-containing cytoplasmic inclusions, which were larger and more frequent in regions of the \( \text{Ihh} \) KO eye with retinal detachment (Figs. 6C, D).
Fig. 3. Gross morphological and histological analysis of the eyes of Ihh KO mice reveal altered pigmentation and eye shape and defects in the choroid and sclera. (A, B) Macroscopic comparison of wildtype (A) and Ihh KO (B) eyes at E18 reveals uneven pigmentation in the mutant eye. (C, D) Section ISH for Gli1 in wildtype (C) and Ihh KO (D) eyes at E14 shows the reduction in Gli1 expression in the POM and the altered pigmentation pattern in the RPE (compare arrows in panels C and D). (E, F) Dissection of the eye to compare the shape of the wildtype (E) and Ihh KO (F) eye at E18 reveals the uneven contour of the mutant eye (compare the dashed lines in panels E and F). (G, H) ISH for Gli1 in the wildtype (G) and Ihh KO (H) eyes at E18 reveals the presence of abnormal folds in the retina (arrow in H) of the mutant eye. (I, J) Hematoxylin and eosin and (K, L) Collagen IV staining in E18 wildtype (I, K) and Ihh KO (J, L) eyes reveals absence of the choroid and scleral condensates (within the bracket in panel I), which is replaced by undifferentiated spindle-shaped cells (within bracket in J) adjacent to the RPE in the mutant eye. The RPE in the mutant has reduced pigmentation (compare region between red brackets in panels K, L), which is associated with the absence of the choroid, as shown by the reduction in collagen IV staining (compare the area between the black bracket in panels K, L).
Analysis of the sclera at this stage reveals that the wildtype sclera consists of rows of closely packed and elongated cells (Fig. 6E), whereas in the Ihh KO eye the sclera is sparsely populated and the cells have an immature appearance, as shown by the cytoplasmic extensions and failure to elongate (Fig. 6F). Although the initial invasion of mesenchyme around the eye appeared to be normal in the Ihh KO mice, it was not clear to what extent alterations in proliferation or cell survival contribute to the changes in the RPE and the reduction in cell density in the POM at E13.5. There is no significant difference in the proportion of cycling cells, identified by IHC for the Ki67 proliferation marker, in the RPE (data not shown) or in the posterior POM of Ihh KO compared with wildtype littermates (% Ki67+ cells in the POM: Wt 40.7±6.8 n=4; Ihh KO 34.1±6.8 n=4; p>0.05). We also do not observe any difference in cells undergoing apoptotic cell death, as determined by TUNEL staining (data not shown). Thus we conclude that the primary defect in the POM of the Ihh KO mice is a failure of differentiation, although we cannot rule out the possibility that a reduction in mesenchymal cell recruitment to the eye after E12 could also contribute to the phenotype.
Retinal development in Ihh KO mice

We investigated whether the RPE/scleral defects in the Ihh KO mice were associated with altered neural retina development. The RPE plays an important role in maintaining the epithelial polarity in the neural retina (Jensen et al., 2001) and eyecup patterning (Nguyen and Arnheiter, 2000), however, these are normal in the Ihh KO retina, as demonstrated by the apical localization of mitotic cells (Fig. 7) and normal eyecup and optic stalk patterning (Fig. 4 and data not shown). Signaling from the RPE also plays an important role in progenitor proliferation and photoreceptor development in the neural retina (Layer et al., 1998; Pearson et al., 2005; Raymond and Jackson, 1995). Proliferation in the Ihh KO retina is reduced, as indicated by the significant reduction in the proportion of cycling cells (%Ki67+ cells; wildtype 50.0±2.9 n=5; Ihh KO 33.4±7.7 n=6; P<0.001), cells in S-phase of the cell cycle (%BrdU+ cells; wildtype 38.0±4.3 n=6; Ihh KO 25.7±2.6 n=6; P<0.001) and in cell number (total cell number: wildtype 2.5×10⁵±0.1×10⁵ n=5; Ihh KO 1.8×10⁵±0.2×10⁵ n=6; P<0.001). ISH for a panel of transcription factors and markers of cell fate revealed that the density of Crx+ cells is markedly

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**Fig. 5.** Histological analysis of the scleral defect in Ihh KO mice. Hematoxylin and eosin staining on E13.5 wildtype (A, C, E) and Ihh KO (B, D, F) eyes sectioned through the horizontal plane, anterior is to the left. Panels C and D are higher magnification views of the anterior sclera in the green boxed areas in panels A and B, respectively. Panels E and F are higher magnification views of the posterior sclera in the red boxed areas of panels A and B, respectively. Note the reduced mesenchymal condensation in the posterior sclera of Ihh KO mice (compare region between the brackets in panels E, F). Arrows in panels E, F indicate red blood cells in the choroid. (G, H) IHC for collagen I in wildtype (G) and Ihh KO mice (H) at E13.5. The asterisks in panel H indicate the regions of the posterior sclera where collagen I thickness was measured.
reduced in the Ihh KO retina (Fig. 7 compare C and D). The reduction in Crx expression in the Ihh KO retina is not associated with a general defect in neurogenesis, as RGC differentiation and the expression of homeodomain and bHLH transcription factors is unchanged in the mutants (data not shown). Since we were unable to recover a sufficient number of Ihh KO mice after E13.5, presumably because of increased embryonic lethality caused by successive backcrossing of the Ihh mutation onto an inbred C57Bl/6 background, we were not able to investigate the effects of this mutation on photoreceptor development in vivo at later developmental stages.

Effects of Hh agonist treatment in vitro on gene expression in the POM

Previous studies have implicated Hh signaling in the regulation of Foxc2 and Ptx2 expression (Jeong et al., 2004; Logan et al., 1998; St Amand et al., 1998; Yamagishi et al., 2003; Yoshioka et al., 1998) and our analyses support a role for Ihh signaling in the regulation of these genes in the POM. Therefore, we next addressed whether Hh pathway activation is sufficient to induce the expression of these genes in the POM. For these studies, we compared gene expression in whole E12 eyes (with RPE and POM intact) that were cultured for 48 h under control
We showed previously that *Ihh* expression is restricted to non-pigmented cells apposed to the RPE in the developing mouse eye (Wallace and Raff, 1999). Here we extend those findings and show that *Ihh* expression co-localizes with collagen IV staining, indicating that *Ihh*-expressing cells are endothelial cells. The timing of *Ihh* expression around the optic cup, beginning at E11, is also consistent with the first appearance of differentiated endothelial cells of the choroid at E11.5 (Marneros et al., 2005). Although we cannot formally exclude the possibility that *Ihh* is expressed by another cell type(s) that is closely associated with the developing choroidal vasculature, we have excluded the possibility that mature pericytes are a source of *Ihh*. Our data appear to contradict previous reports documenting *Ihh* expression in the RPE of the rat, mouse and newt (Levine et al., 1997; Takabatake et al., 1997). However, because of the difficulty in separating the RPE from the choroid, RT-PCR-based detection of *Ihh* transcripts in the RPE is likely due to signal from contaminating choroidal tissue.

Our findings highlight the endothelial cell as a novel source of Hh. Hh signaling is required for vascularization in a number of tissues, including the yolk sac (Byrd and Grabel, 2004; Pola et al., 2001), and heart (Lavine et al., 2006) and is associated with pathological neovascularization in the retina and choroid (Surace et al., 2006). In several instances, Hh acts indirectly via VEGF and Notch signaling to promote vascular development (reviewed in (Byrd and Grabel, 2004; Pola et al., 2001)), however, it can also signal directly to endothelial cells and pericytes and induce VEGF-independent vessel formation from endothelial cells (Colnot et al., 2005; Kanda et al., 2003; Vokes et al., 2004). We show here that instead of functioning as an Hh target, the vasculature can be a source of Hh that signals at short range to adjacent tissues, in this example from the choroid to the RPE and POM. The maintenance of *Gli1* expression in the RPE and sclera in the adult eye also implicates endothelial-derived Hh signaling in cellular interactions in differentiated ocular tissues and raises the possibility that similar interactions mediated by endothelial cells via Hh occur in other tissues.

**Discussion**

*Ihh* expression in endothelial cells — a novel source of Hh signaling

Previous studies have implicated a role for the choroid in the development of the RPE (Marneros et al., 2005; Rousseau et al., 2000), however, the signals that mediate this interactions have not been
were suggested to be evidence of a polarity defects in the RPE (Defoe
light microscopy
Ultrastructural analysis of the RPE in the
tissues. The RPE in the
pigmentation and is frequently detached from the neural retina.
and sclera and inactivation of Ihh results in abnormalities in these
tissues. The RPE in the Ihh KO mouse is thinner and has reduced
pigmentation and is frequently detached from the neural retina.
Ultrastuctural analysis of the RPE in the Ihh KO mouse confirms the
light microscopy findings and reveals the presence of cytoplasmic
inclusions on the basal side of the RPE cells that contain microvilli.
Similar basilar RPE defects have been reported in the
et al., 2007). However, since the
Ihh
KO mouse is thinner and has reduced
expression and blood vessel morphology in explant cultures of E12 eyes. Section ISH for
Ihh
expression and blood vessel morphology in explant cultures of E12 eyes. Section ISH for
Ihh
and periosteum defects in
KO mice. The scleral
expression to this region of the developing
Ihh
expression in the anterior POM, however, we have not
reductions in Pitx2 and Foxc1 expression in the POM, particularly in
mesenchymal cells in the posterior POM. A reduction in cell density
in the POM is unlikely to be the only explanation for the reduced
expression of these genes and, rather, we suggest that it indicates a
role for these genes downstream of Ihh signaling in the developing
sclera. Foxc2 and Pitx2 are Hh targets in other tissues (Jeong et al.,
2004; Logan et al., 1998; St Amand et al., 1998; Yamagishi et al.,
2003; Yoshioka et al., 1998), and are required for anterior chamber
and sclera development (Evans and Gage, 2005; Lu et al., 1999;
Smith et al., 2000 ). Moreover, reduced expression of Foxc1, a
homologue of Foxc2, in the neural crest is associated with a similar
block in POM cell differentiation as we observe in the Ihh KO sclera
(Ittner et al., 2005). However, Hh pathway activation is not sufficient
to induce the expression of these genes in the POM in vitro. The
discrepancy between our in vivo and in vitro findings suggests that
Ihh-mediated regulation of gene expression in the posterior POM is
complicated, and possibly requires additional signals. In addition,
the expression of Pitx2 and Foxc2 in the anterior POM appear to be less
dependent on Ihh signaling. In this instance there may be
compensation by other signaling pathways, including RA, BMP4 and TgfB, which have all been shown to regulate gene expression in
the POM and the development of neural-crest derivatives in the
anterior chamber (Chang et al., 2001; Ittner et al., 2005; Matt et al.,
2005). It is also possible that Dhh expression could be regulating
gene expression in the anterior POM, however, we have not
been able to localize Dhh expression to this region of the developing
eye.

Fig. 8. Ihh expression and blood vessel morphology in explant cultures of E12 eyes. Section ISH for Ihh (A, C) and IHC for collagen IV (B, D) on eyes of E12 mice at time 0 (A, B) or after
48 h of in vitro culture (C, D). Arrows in panel A indicate the location of Ihh-expressing cells in the choroid at time 0, but these cells are absent by 48 h. Panels B’ and D’ are higher
magnification views of the boxed areas in panels B and D, respectively. Note that at time 0, collagen IV staining outlines cell bodies, whereas by 48 h, it appears as a thin line.

identified. Here we identify Ihh as a novel choroid-derived factor that
directly targets the RPE and the sclera. Ihh expression in the choroid is
required for the maintenance of Hh target gene expression in the RPE
and sclera and inactivation of Ihh results in abnormalities in these
tissues. The RPE in the Ihh KO mouse is thinner and has reduced
pigmentation and is frequently detached from the neural retina.
Ultrastuctural analysis of the RPE in the Ihh KO mouse confirms the
light microscopy findings and reveals the presence of cytoplasmic
inclusions on the basal side of the RPE cells that contain microvilli.
Similar basilar RPE defects have been reported in the p27 KO mice and
were suggested to be evidence of a polarity defects in the RPE (Defoe
et al., 2007). However, since the Ihh KO eyes did not exhibit other
hallmarks of disrupted RPE polarity, such as a loss of apical mitoses in
the neural retina, it is unlikely that loss of Ihh results in a severe
disruption in RPE polarity. Another possibility is that these microvillus-containing basal inclusions represent abnormal differentiation of
the basal infoldings characteristic of RPE cells. Although we did not
find evidence of abnormal RPE proliferation, our results indicate that
Ihh signaling is required for normal RPE cell morphology and
adhessiveness between the RPE and neural retina.

Scleral development in the Ihh KO is severely perturbed, with a
reduction in mesenchymal cell condensation, elongation and
collagen I deposition. This function of Ihh in the eye is analogous
to its role in the development of the periosteum of bones. In Ihh KO
mice, the mesenchymal cells that form the periosteum fail to align
and pack around the cartilage and instead form a loose network of
cells with a reduced production of ECM, in particular collagen I
(Colnot et al., 2005), a phenotype that is remarkably similar to the
scleral abnormalities that we observed in Ihh KO mice. The scleral
and periosteum defects in Ihh KO mice also highlight the role played
by these tissues in maintenance of the vascular supply. Although
present initially, the subsequent development of endothelial cells is
disrupted in the sclera and periosteum of Ihh KO mice (Colnot et al.,
2005).
Indirect effects of Ihh signaling on neural retina development

Ihh inactivation was also associated with a reduction in progenitor proliferation and Crx expression in the neural retina. Since Crx marks post-mitotic cells committed to a photoreceptor fate (Chen et al., 1997; Freund et al., 1997; Furukawa et al., 1997), reduced Crx expression in the Ihh KO retina could indicate a role for Ihh in photoreceptor specification. Unfortunately, the early embryonic lethality of Ihh KO mice precluded a more extensive analysis of the role of this signal in retinal cell type development. Because Ihh does not appear to signal directly to the neural retina, its effect on the neural retina would have to be mediated indirectly via the sclera or RPE. This interpretation is consistent with previous studies implicating the RPE in the regulation of RPC proliferation (Pearson et al., 2005), retinal lamination (Wolburg et al., 1991) and photoreceptor differentiation (Bumsted et al., 2001; Raymond and Jackson, 1995). A role for RPE-derived Hh signaling in photoreceptor development is also supported by studies in zebrafish (Stenkamp et al., 2000). Finally, the evidence that RPC proliferation is disrupted in the Ihh KO retina, together with the evidence for a mitogenic function of Shh on retinal progenitors (Wang et al., 2005, 2002), supports the notion that progenitor proliferation in the neural retina is regulated by two distinct sources of Hh 1) Shh from RGCs, acting directly and 2) Ihh, from the choroid, acting indirectly on progenitor cells.

Peri-ocular Hh function—conservation from frogs and fish?

A role for Hh signaling in RPE specification and differentiation appears to be conserved among several vertebrate species. As in the mouse, the RPE is a Hh target in Xenopus, zebrafish and chick (Perron et al., 2003; Stenkamp et al., 2000; Zhang and Yang, 2001b). Inhibition of Hh signaling in Xenopus and the chick results in RPE defects, including reduced pigmentation and downregulation of RPE markers (Perron et al., 2003; Zhang and Yang, 2001b) and, increased Hh signaling in the chick retina promotes pigment cell development (Zhang and Yang, 2001b). Although inhibition of Hh signaling has not been reported to perturb RPE development in zebrafish, it has been shown to inhibit photoreceptor differentiation (Kay et al., 2005; Masai et al., 2005; Neumann and Nusslein-Volhard, 2000; Stenkamp and Frey, 2003; Stenkamp et al., 2000). However, defects in sclera or choroid in the context of Hh pathway perturbation have not been reported in fish, frog or chick.

Since the same Hh homologues are expressed in both the RPE and RGC layer in Xenopus and zebrafish and because pharmacological inhibition of Smo signaling could be blocking all sources of Hh, it has been difficult to make definitive conclusions about the exact function of the different Hh sources in the eye. Ihh and Shh expression does not overlap in the mouse eye and inactivation of one homologue does not perturb signaling by the other, therefore, the phenotypes of the mouse mutants have helped to clarify the
functions of these different Hh sources in the developing eye. The data reported here provide direct evidence for a role of chordin-derived Ihh signaling in RPE/sclera and retinal development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.05.528.

References


