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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.

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

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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 (Marneros 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 (Ptch1), a 12-transmembrane domain protein (Marigo et al., 1996; Stone et al., 1996), thereby de-repressing the activity of Smoothened, 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 (Locker 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 oligonucleotides 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), Shh^{null} (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 G*li3* mutant mice (Hui and Joyner, 1993) were maintained on a mixed C67Bl/6×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 pairing 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*, M*itf*, *Pitx2*, *Foxc2*, *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-indoylphosphate. X-gal staining in adult *Gli-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 Bioscience), 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) cryosection were fixed with 70% ethanol, treated with 0.3% H_2O_2, and incubated in blocking solution (20% sheep serum in TBLS; 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 H_2O_2 pretreatment was omitted. For Ki67 staining the sections were subjected to antigen retrieval by microwaving 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 eye at E13.5 was quantified by determining the proportion of cells staining with 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 is the average±SD 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 resulting 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 CO_2-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 mg/ml), BSA Fraction V (100mg/ml), progesterone (60ng/ml), putrescine (16 μg/ml), sodium selenite (40 ng/ml), N-acetyl cysteine (60 μg/m), 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.