Activation of the Hedgehog Signaling Pathway in the Developing Lens Stimulates Ectopic *FoxE3* Expression and Disruption in Fiber Cell Differentiation

Christine L. Kerr,¹ Jian Huang,² Trevor Williams,^{*,2} and Judith A. West-Mays^{*,1}

PURPOSE. The signaling pathways and transcriptional effectors responsible for directing mammalian lens development provide key regulatory molecules that can inform our understanding of human eye defects. The hedgehog genes encode extracellular signaling proteins responsible for patterning and tissue formation during embryogenesis. Signal transduction of this pathway is mediated through activation of the transmembrane proteins smoothened and patched, stimulating downstream signaling resulting in the activation or repression of hedgehog target genes. Hedgehog signaling is implicated in eye development, and defects in hedgehog signaling components have been shown to result in defects of the retina, iris, and lens.

METHODS. We assessed the consequences of constitutive hedgehog signaling in the developing mouse lens using Cre-LoxP technology to express the conditional M2 smoothened allele in the embryonic head and lens ectoderm.

RESULTS. Although initial lens development appeared normal, morphological defects were apparent by E12.5 and became more significant at later stages of embryogenesis. Altered lens morphology correlated with ectopic expression of *FoxE3*, which encodes a critical gene required for human and mouse lens development. Later, inappropriate expression of the epithelial marker *Pax6*, and as well as fiber cell markers *c-maf* and *Prox1* also occurred, indicating a failure of appropriate lens fiber cell differentiation accompanied by altered lens cell proliferation and cell death.

CONCLUSIONS. Our findings demonstrate that the ectopic activation of downstream effectors of the hedgehog signaling pathway in the mouse lens disrupts normal fiber cell

differentiation by a mechanism consistent with a sustained epithelial cellular developmental program driven by *FoxE3*. (*Invest Ophthalmol Vis Sci.* 2012;53:3316–3330) DOI: 10.1167/iovs.12-9595

In vertebrates, the Hedgehog (Hh) family comprises three developmentally important homologues, Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh).^{1,2} In a receiving cell, signal transduction driven by the Hh ligand relies on the Patched (Ptch) and Smoothened (Smo) transmembrane proteins. In the absence of Hh, Smo is repressed by Ptch and internalized, leading to an inhibitory transcriptional output. When Hh is present, it binds to Ptch and relieves the repression of Smo, generating a transcriptional cascade via Gli proteins and resulting in the activation or repression of Hh target genes.¹ Hh signaling is critical for many developmental processes in multiple vertebrate and invertebrate species. With respect to human eye formation, disruptions in Hh signaling components, including *SHH* and *PTCH1*, can result in cyclopia, due to an underlying defect in brain and facial development. Furthermore, *PTCH1* mutations, which lead to activation of the Hh pathway even in the absence of Hh,³ results in Gorlin syndrome (BCNS), which often can present with defects in the retina, iris, or lens, including cataracts.^{4–6}

In a broader evolutionary context, an expanded domain of Shh expression in the developing central nervous system (CNS) is responsible for the inhibition of eye development in the blind cavefish via its effects on the optic cup.⁷ A number of studies in fish and amphibians have provided further evidence that Hh activity is important in regulating lens formation.^{8–10} For example, over-expression of Hh in zebrafish results in suppression of lens formation.^{11,12} Similarly, exaggerated Hh activity through suppression of the Hh inhibitor, Xhip, in the prospective lens ectoderm in *Xenopus* also leads to loss of lens placode formation.¹³ Conversely, loss of Hedgehog signaling in non-neural ectoderm has been shown to result in a conversion of the pituitary to lens.^{10,14,15} The expression and function of an intact Hh pathway also is associated with the process of lens regeneration in the newt.¹⁶ These data suggest strongly that the appropriate control of Hh signaling in lower vertebrates is an important mechanism for regulating lens-specific gene expression and lens development.

In the mouse, Shh and, to a lesser extent, Ihh have been shown to have specific roles in eye formation, including development of the retina and the scleral mesenchyme.^{17–22} For example, studies in *Ihh* knockout (KO) mice have shown that there is a loss of Hh target gene expression in the periocular mesenchyme, and this results in defects in the posterior sclera, including a deformed ocular shape and fragile ocular globe. The *Ihh* KO mice also exhibit abnormalities of the RPE, including abnormal pigment distribution, as well as disruption in photoreceptor specification in the neural retina.

From the ¹Department of Pathology and Molecular Medicine, McMaster University Health Sciences Centre, Hamilton, Ontario, Canada; and ²Department of Craniofacial Biology and Department of Cell and Developmental Biology, University of Colorado Denver, Aurora, Colorado.

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*Each of the following is a corresponding author: Judith A. West-Mays, Department of Pathology and Molecular Medicine, McMaster University, Health Sciences Centre, Room 1R10, Hamilton, ON, Canada, L8N 3Z5; Telephone 905-525-9140, ext. 26237; Fax 905-525-7400; westmayj@mcmaster.ca.

Trevor Williams, Department of Craniofacial Biology and Department of Cell and Developmental Biology, University of Colorado Denver, Anschutz Medical Campus Mailstop 8120, RC-1 South Building, 11th Floor, Rm 111, Aurora, CO 80045; Telephone 303-724-4571; Fax 303-724-4580; Trevor.Williams@ucdenver.edu.

Shh has been found to be expressed in the retinal ganglion cells of the mouse retina and, when overexpressed in these cells, there is reduced retinal ganglion cell population, whereas mice with inhibited Shh activity have an increased retinal ganglion cell number.²² Thus, not surprisingly, *Shh* KO mice exhibit, in addition to cyclopia, a disrupted optic stalk and failed neural retina formation.²³ Correspondingly, *Ptch* has been shown to be expressed in the neural retina, RPE, and iris, and at low levels in the cornea of the mouse eye. The expression of *Ptch* also has been shown to overlap with Shh expression in the developing embryonic mouse eyelids.^{24,25} In contrast, *Ptch* expression has yet to be observed in the normal mouse lens.²⁵

Despite the findings for Hh in lens development in lower vertebrates and effects of Hh mutations on lens defects in humans, the role of Hh signaling, particularly expanded Hh levels, in the development and differentiation of the murine lens has not been investigated. The lens placode is derived from a region of surface ectoderm (SE) overlying the optic vesicle. It is detected first, at the morphological level, as a thickening of the SE. The invagination of the lens placode gives rise to a lens pit, which subsequently separates away from the overlying SE, giving rise to a hollow lens vesicle. This vesicle ultimately matures into a specialized polar lens structure, with an anterior lens epithelium and central lens fiber cell region.^{26–28} To determine how aberrant Hh signaling may influence lens development and differentiation, we created mouse mutants that exhibit constitutive Hh signaling in the SE of the head and lens. Previous studies have identified a mutation in *Smo*, termed M2, that prevents its interaction with, and repression by, *Ptch* proteins.²⁹ Subsequently, this mutation has been engineered into the *Rosa26* locus of the mouse genome downstream of a *LoxP*-Stop-*LoxP* cassette.³⁰ We combined this allele with an early ectodermal specific Cre recombinase transgene to activate *Smo*M2 expression from E9.5 onwards. We demonstrated that, while the lens placode does form in these mutants, there are major alterations in the expression of key regulatory molecules associated with aberrant differentiation and disorganized lens cell morphology. Our findings showed further that the developing mouse lens is capable of responding to altered *Smo* activity, suggesting that aspects of the Hh pathways that influence lens formation are conserved in vertebrate development.

MATERIALS AND METHODS

Generation of Activated *Smo* Mutant Mice

All animal procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Mice containing the activating mutation in *Smo*^{29–31} were obtained from the Jackson Laboratories, Bar Harbor, ME (STOCK Gt(*Rosa*)26^{Sor^{tm1}(*Smo*/EYFP)^{Amc}/J}, stock # 005130), and were crossed with transgenic mice expressing a new Cre-recombinase specific to the SE and its derivatives (*Crect*), to generate the activated *Smo* mutant mice. *Crect* mice use an ectodermal enhancer from *Tcfap2a* to drive Cre expression specifically in the ectoderm, and will be described elsewhere (Yang H, Melvin VS, and Williams T, manuscript in preparation). Noon on the day of vaginal plug detection was considered day 0.5 (E0.5) of embryogenesis. Mice were genotyped using DNA extracted from tail or yolk sac samples using the DNeasy tissue kit (Qiagen, Valencia, CA). Genotyping was performed using the primers oIMR0316, oIMR0872, oIMR1416, and oIMR3621, and the following conditions: 1 cycle of 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C for 1 minute and 72°C for 1 minute, and 72°C for 2 minutes to generate a 425 base pair (bp) product for the

WT *Rosa26* allele and 173 bp product for the EYFP component of the targeted locus. To detect the Cre allele, PCR was performed using the primers Cre1: 5'-GCT CCT TAG CAC CGC AGG TGT AGA G-3', Cre3: 5'-CGC CAT CTT CCA GCA GGC GCA CC-3', and the following conditions: 120 seconds at 95°C, 35 cycles of 95°C for 45 seconds, 67°C for 45 seconds, 72°C for 60 seconds, followed by 72°C for 10 minutes to generate a 421 bp product for the Cre allele.

Histology

Whole embryos were collected corresponding to Gt(*Rosa*)26^{Sor^{tm1}(*Smo*/EYFP)^{Amc}/J}; *Crect* mutants and wild type (WT) litter mates. Embryonic tissue was fixed in 10% neutral buffered formalin overnight at room temperature and then transferred into 70% ethanol until processing. Whole embryos (E12.5) or embryo heads (E15.5, E18.5, and P0) were processed and embedded in paraffin. Serial sections were cut at a thickness of 4 µm and used for hematoxylin and eosin (H&E) staining as well as immunofluorescent analysis. For all stages examined in the activated *Smo* study, sample sizes of 3 lenses from 3 animals were stained.

Immunofluorescence and TUNEL Assay

Indirect immunofluorescence was performed using the following primary antibodies: goat polyclonal green fluorescent protein (GFP) (1:250; Bioshop Canada, Burlington, ON), rabbit polyclonal *Pax6* (1:50; Covance, Princeton, NJ), mouse monoclonal *Pax6* (1:5; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), rabbit polyclonal *FoxE3* (1:1000; developed by Peter Carlsson, University of Goteborg, Goteborg, Sweden), rabbit polyclonal β-crystallin and rabbit polyclonal γ-crystallin (1:200; provided by Samuel Zigler Jr., Chief of lens and cataract biology section of the National Eye Institute, National Institute of Health, Bethesda, MD). The mitosis marker anti-phospho-Histone H3 (rabbit polyclonal; Upstate Biotechnology, Lake Placid, NY) was used to detect mitotic cells (1:30). Mouse monoclonal PCNA was used to detect cells in S phase of the cell cycle (1:750; Dako, Burlington, ON). We also used mouse monoclonal cyclin D1 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal p27^{kip1} (1:350; BD Transduction, San Jose, CA), goat polyclonal p57^{kip2} (1:100; Santa Cruz Biotechnology), rabbit polyclonal *prax1* (Covance; 1:100), goat polyclonal *c-maf* (1:200; Santa Cruz Biotechnology), and goat polyclonal Calretinin (1:25; Santa Cruz Biotechnology). Fluorescent secondary antibodies were either Alexa Fluor 488 (goat anti-mouse and goat anti-rabbit; Invitrogen Molecular Probes, Burlington, ON), or Alexa Fluor 568 (goat anti-mouse and donkey anti-goat; Invitrogen Molecular Probes), used at 1:200 for 1 hour at room temperature. Paraffin-embedded sections were deparaffinized in xylene, hydrated (through 100%, 95%, and 70% ethanol, followed by water), treated with 10 mM sodium citrate buffer (pH 6.0, boiling for 20 minutes) for antigen retrieval, blocked with normal serum, and incubated with primary antibodies overnight at 4°C. For colocalization studies, both primaries were mixed and incubated simultaneously, followed by both secondaries. Each stain included a negative control with no primary antibody. Terminal uridine deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was performed using the ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (Millipore-Chemicon, Billerica, MA), according to the manufacturer's instructions for fluorescent staining of paraffin-embedded tissue. Following immunofluorescence or the TUNEL assay, stained slides were mounted with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlington, ON) or Prolong Gold antifade reagent with DAPI (Invitrogen). All H&E and fluorescent stains were visualized with a microscope equipped with a fluorescence attachment, and images were captured with a high-resolution camera and associated software (Open-Lab; Improvision, Lexington, MA). Images were reproduced for publication with image-management software (Photoshop 7.0; Adobe Systems Inc., Mountain View, CA).

FIGURE 1. Verification of constitutively active smoothened allele expression in SE and SE derivatives. Sections of WT (A–C) and Crect activated Smo mutant (D–F) mouse eyes at E12.5 (A, D), E15.5 (B, E), and E18.5 (C, F) immunostained for GFP expression. Ptch1-LacZ (G, I) and Crect Smo-Ptch1-LacZ (H, J) at E12.5 (G, H) and P0 (I, J) were stained with X-gal to examine for Ptch1 expression as indicated by LacZ staining. The blue staining seen in the WT P0 samples in the surface ectoderm overlying the eye represents expression in the hair follicles associated with the eyelids. CE, corneal epithelium; LE, lens epithelium; C, cornea; Le, lens; R, retina; SE, surface ectoderm. All scale bars represent 100 μ m.

LacZ Assay

To examine Hh signaling pathway activity, activated Smo mutants were bred with Ptch1-LacZ reporter mice (Ptch1^{tm1Mps}).³² LacZ staining was performed to examine Ptch expression as a readout of Hh signaling. Embryonic tissue was fixed overnight in 4% paraformaldehyde (PFA) followed by cryoprotection in 30% sucrose. Tissue was embedded in ornithine carbamoyltransferase (OCT) for cryosectioning. Slides were washed in PBS and double distilled water (ddH₂O). An X-gal dilution buffer containing 5 mM potassium ferricyanide crystalline, 5 mM potassium ferricyanide trihydrate, 1 mM magnesium chloride, and PBS was warmed to 37°C, and 40 mg/mL X-gal stock solution was added to the dilution buffer, and applied to the slides containing 8 μ m cryosections. Slides were incubated overnight at 37°C and viewed the following day.

RESULTS

Verification of Constitutive Expression of Smo in SE and SE Derivatives

Constitutively active Smo was fused to a yellow fluorescent protein (YFP) in the Smo construct. This fusion protein allowed for the verification of properly expressed constitutively active Smo in the SE and SE derivatives (including the skin, eyelids, cornea, and lens). GFP and YFP differ by only a single

amino acid (due to a mutation at T203Y)³³ and, therefore, antibodies raised against full length GFP can be used to detect YFP expression. Immunostaining for GFP revealed expression of the constitutively active Smo allele in the SE and its derivatives, including the lens (Fig. 1). GFP staining was seen within the corneal epithelium and lens at E12.5, E15.5, and E18.5 in the Gt(ROSA)26Sor^{tm1(Smo/EYFP)Amc}/J mice containing the Crect transgene. These mutants will be referred to as “activated Smo mutants” throughout this study.

As an independent method to measure the activity of the Hh signaling pathway in the activated Smo mutant eye, we bred them with the Ptch1-LacZ reporter line and assayed for beta-galactosidase activity. Ptch1 is a transcriptional target of the Hh pathway and can be used as a readout for pathway activity.³² At E12.5 in otherwise wild-type mice, the mutant Ptch1 allele drove LacZ expression in the neuroepithelium of the brain and within the spinal cord (Fig. 1G). LacZ expression was not detected in the eyes of these mice (Fig. 1G). In contrast, in activated Smo mutants, expression was expanded. Not only was LacZ expression detected within the neuroepithelium and spinal cord of the mutants, but its expression also was detected within the entire lens and SE (Fig. 1H). Similar results were obtained at P0 with β -gal expression in the lens observed only in the presence of the activated Smo allele (compare Figs. 1I and 1J). Further analysis indicated that Ptch1-LacZ activity in the developing lens was observed only in a

TABLE. Summary of Differences in Marker Expression in Activated Smoothened Mutant versus Wild-Type Eyes

Marker	WT Spatial Localization	Stage at Which Difference from WT Was First Observed	Ectopic Expression in Fiber Cell Compartment of Mutant Lens	Additional Comments
Ptc1-LacZ	-Not present in lens or retina at PO -lacZ staining associated with hair follicles in eyelid; within neuroepithelium and spinal cord	E10.5-limited number of lacZ positive cells in the mutant lens pit and surrounding ectoderm E11.5 – widespread lacZ expression in mutant lens and surrounding ectoderm	+	-
<i>FoxE3</i>	LE	E12.5	+	-
<i>Pax6</i> (lens)	LE	by E15.5 (lens)	+	-
β and γ -crystallin	FCs	by E15.5	NA	Failed FC denucleation
<i>c-maf</i>	Lens TZ	by E15.5	+	-
<i>Prox-1</i>	LE and TZ	by E15.5	+	-
Cyclin D	LE and TZ	by E15.5	+	-
p27 ^{kip1}	LE (low levels) and TZ	by E15.5	+	-
p57 ^{kip2}	TZ	by E15.5	+	-
Phosphohistone H3 (lens)	LE and TZ	by E15.5	+	-
<i>Pax6</i> (retina)	GCL and nbl	P0	NA	Expression persists in mutant nbl at PO and also observed in IR
Calretinin (retina)	Ganglion and amacrine cells	P0	NA	Disorganized expression in the IR
PCNA (retina)	Ganglion cell layer, INL, onbl	P0	NA	Abnormal proliferation in IR
TUNEL	Some cell death in LV at E12.5, very little lens cell death at later stages. Small amount of cell death in INL and onbl	E15.5 (lens) P0 (Retina)	NA	-excessive cell death in LE and FCs -abnormal cell death in IR

LE, lens epithelium; FC, fiber cells; TZ, transition zone; LV, lens vesicle; NA, not applicable.

small number of cells (<5%) at E10.5, but was more widespread by E11.5, indicating the response to Smo activation occurred by E11.5, consistent with the timing of Cre transgene activity (the Table and data not shown). Note that the Cre transgene used in our study is not expressed in the retina, as revealed by the lack of GFP and Ptc1-LacZ expression in this tissue (Fig. 1). Taken together, these findings demonstrate Ptc1-LacZ expression occurred in the same regions of the eye where we observed GFP expression in the activated Smo mutants. The data indicate further that the cells of the lens have the capacity to respond to Hh signal transduction.

Activated Smo Mutants Exhibit Aberrant and Disorganized Lens Morphology

Since lens cell morphology resulting from excessive Hh signaling had yet to be explored in a mouse model we first performed a gross morphological examination of eyes of the Cre⁺ activated Smo mutants (Fig. 2). Analysis of E10.5-E12.5 embryos revealed that gross morphological defects were apparent most readily by E12.5, corresponding to the day after the major up-regulation of Ptc1-LacZ expression (Fig. 2D and data not shown). At E12.5 the lens was misshapen and displayed a much thicker lens epithelial region than that observed in the WT lens at the same stage (Fig. 2D, black arrow). An aberrant group of cells also was seen to be developing within the lumen of the lens vesicle of the Smo mutants (Fig. 2D, pink arrow). As development progressed to E15.5, the WT lens displayed a distinct lens epithelial and fiber cell region. However, in the Smo mutants, the lens epithelial

and fiber cell regions were disorganized, with the mutant lens protruding further away from the optic cup towards the cornea (Fig. 2E). The anterior portion of the mutant lens appeared to adhere to the overlying SE (Fig. 2E, black star), whereas the WT had completed its separation. This adhesion also was evident at E12.5, but was not as pronounced as in the E15.5 embryonic lens. By E18.5, extensive lens epithelial and fiber cell disorganization persisted in the mutant lens. The completely misshapen lens was smaller than a WT E18.5 lens, and continued to protrude even further away from the optic cup than was observed at E15.5 (Figs. 2C, 2F). Throughout these stages of embryonic development, vacuoles and cavities also were evident throughout the entire mutant lens region. These data indicate that a constitutively active mutation in Smo expressed in the surface ectoderm and derivatives causes defective and disorganized embryonic lens cell morphology.

Activated Smo Mutants Display Abnormal Patterns of Proliferation and Apoptosis within the Developing Lens

Hh signaling has been shown to regulate cell proliferation.¹ Thus, we assayed for the phosphorylated form of Histone 3 (PH3) as a readout for cells in the mitotic stage of the cell cycle (Fig. 3).³⁴ Proliferating cells were detected within the lens epithelial and fiber cell regions of the activated Smo mutant and WT lens at E12.5. When examining lenses of the mutants and WT at this early stage, it was evident that many more cells stain positively for PH3 in the mutant compared to the WT (Figs. 3A, 3D). WT lenses at E15.5 and E18.5 exhibited PH3

FIGURE 2. H&E stains of WT (A–C) and activated Smo mutant (D–F) lenses. At E12.5 (A, D) defective lens morphology in the mutant (D) was characterized by a thicker than normal lens epithelial region (black arrow) and a misshapen lens. An aberrant group of cells developed in the mutant lens vesicle lumen (pink arrow, D). By E15.5 (B, E), the WT lens displayed distinct lens epithelial and fiber cell layers, while the mutant lens was disorganized, with the lens protruding away from the optic cup. An adhesion of the lens to the overlying SE also was observed (black star, E). By E18.5 (C, F), the mutant lens was smaller than that of the WT, remained disorganized, and continued to protrude even further away from the optic cup (C, F). FC, fiber cells. All scale bars represent 100 μ m.

staining that was confined to the lens epithelial cell layer (Figs. 3B, 3C), while activated Smo mutants at equivalent stages displayed numerous PH3 positively stained cells within the lens epithelial and fiber cell regions (Figs. 3E, 3F). Similar results were obtained by staining for proliferating cell nuclear antigen (PCNA), which labels cells in the S phase of the cell cycle (data not shown).³⁵

Evolutionary models of eye degeneration, including the Iberian mole and the blind cavefish, share some lens-specific phenotypes with our mouse model.^{36–38} These models can be caused by excess Shh signaling and exhibit lens degeneration. Therefore, TUNEL reactions were carried out to examine cell death in WT and mutant lenses (Fig. 3). Lens vesicle closure is occurring around E12.5 during normal embryonic development and, thus, some TUNEL-positive staining can be expected at this stage in the region where this separation is occurring.²⁷ Normal amounts of apoptosis were seen in the lens epithelial regions of the WT and activated Smo mice examined at E12.5 (Figs. 3G, 3J). However, abnormal TUNEL staining was observed in the posterior region of the lens vesicle of the activated Smo mutants at this stage (Fig. 3J). As expected, no apoptosis was observed in E15.5 and E18.5 WT lenses (Figs. 3H, 3I). However, abnormal amounts of apoptosis were observed in the lens epithelial and posterior fiber cell region of the mutant lens at both of these stages (Figs. 3K, 3L). The Table provides a summary of the observed changes in the mutant, including those pertaining to the cell proliferation and cell death data.

Abnormal Patterns of Expression of Cell Cycle Proteins and Abnormal Cell Cycle Behavior Occur during Lens Development in Smo Mutants

To understand better the aberrant proliferative and apoptosis patterns observed in the activated Smo mutants, we performed

an investigation into the expression patterns of the cell cycle promoting factor cyclin D1, and also examined the expression of p27^{kip1} and p57^{kip2}, which are negative regulators of the cell cycle.³⁹ Cyclin D1 is required for the G1 to S phase transition in the cell cycle, and is expressed normally in proliferating lens epithelial cells and equatorial lens fiber cells, with expression absent from those fiber cells that have completed their differentiation process.^{40,41} Importantly, cyclin D1 also is a known target of the Hh pathway⁴² and, thus, we reasoned that its expression pattern may be altered due to the constitutive activation of Smo in our mutants. Although cyclin D1 expression appeared normal at E12.5 in the Smo mutants (Fig. 4C), its expression became aberrant with the progression of lens development. In the WT lens at E16.5, cyclin D1 was expressed in the proliferating lens epithelial cells and at the lens equator (Fig. 4B). At equivalent stages in the activated Smo mutants, however, cyclin D1 expression was scattered throughout the lens epithelial region and, interestingly, was expressed in the posterior fiber cell compartment (Fig. 4D), unlike WT lenses at this stage (Fig. 4B). A similar trend was seen at E18.5, with cyclin D1 expressed throughout the anterior and posterior lens compartments in the mutant lens (data not shown).

During development, the terminal differentiation of specific cell types requires that cells exit the cell cycle correctly. Cell cycle exit and terminal differentiation is of particular importance during lens development, as cells fated to differentiate into fiber cells must complete these pathways successfully.⁴³ The p27^{kip1} and p57^{kip2} are cyclin-dependent kinase inhibitors (CDKIs) belonging to the p21^{cip1} family, and are required for inhibition of kinases involved in the G1/S transition of the cell cycle.^{39,43,44} The p27^{kip1} is expressed normally in lens fiber cells, specifically at the transition zone of the lens, while p57^{kip2} is expressed in the lens epithelium and fiber cells, though its expression in the epithelium pales in comparison to

FIGURE 3. Patterns of proliferation and cell death are abnormal in developing activated Smo lens. Sections of WT and Smo mutant mouse (Smo) eyes at the indicated time points immunostained for expression of phosphohistone H3 or detection of TUNEL as shown. *White arrows* indicate proliferating cells (A–F) or TUNEL positive cells (G–L), respectively. Slides also were stained with DAPI to highlight nuclei (*blue*). All scale bars represent 100 μm.

its levels of expression at the transition zone of the lens.³⁹ The p27^{kip1} expression appeared normal in the earlier stages of lens development at E12.5, displaying expression throughout the lens vesicle of the WT and mutant lenses (Figs. 4E, 4G). However, with the progression of development to E15.5, p27^{kip1} was expressed aberrantly throughout the entire anterior lens epithelial regions and posterior fiber cell regions of the activated Smo mutant lens (Figs. 4F, 4H). The expression patterns of p57^{kip2} were similar to those seen for p27^{kip1}. Although p57^{kip2} expression appeared normal at E12.5 (Figs. 4I, 4K), its expression became quite irregular later in development at E15.5. The p57^{kip2} expression was concentrated at the transition zone of the WT lens, but was expressed throughout the majority of the anterior and posterior regions of the mutant lens (Figs. 4J, 4L). The expression patterns of p27^{kip1} and p57^{kip2} at E18.5 were similar to those seen at

E15.5, with expression of both CDKIs seen throughout the lens epithelial and fiber cell regions of the mutant lens (the Table and data not shown).

An Activated Smo Mutation Results in Abnormal Expression Patterns of Lens Epithelial Cell Markers

Pax6 and *FoxE3* are proteins that have important roles in lens development, and their expression becomes confined to the epithelial compartment of the differentiating lens. Immunofluorescent analysis of these two lens epithelial cell markers in the activated Smo mutants revealed defective patterns of expression. *Pax6* expression was examined at E12.5, E15.5, and E18.5 in the WT and mutant lens (Fig. 5). At E12.5, the expression pattern of *Pax6* in the WT and mutant lenses

FIGURE 4. Cell cycle promoting and inhibiting factors cyclin D1, p27^{kip1}, and p57^{kip2} are expressed ectopically in the activated Smo lens. Sections of WT (A, B, E, F, I, J) and activated Smo mutant (C, D, G, H, K, L) mouse eyes at E12.5 (A, C, E, G, I, K), E15.5 (F, H, J, L) and E16.5 (B, D) immunostained for expression of cyclin D (A–D, green), p27^{kip1} (E–H, green), or p57^{kip2} (I–L, red). Slides also were stained with DAPI to highlight nuclei (blue). White arrows (E, J) show localized expression of p27^{kip1} and p57^{kip2} within the transitional zone of the lens. White arrows (D, L) show expression of cyclin D and p57^{kip2} in cells within the lens epithelial region of the mutant. LE, lens epithelium; FC, fiber cells. All scale bars represent 100 μm.

FIGURE 5. *Pax6* and *FoxE3* are expressed ectopically in developing activated *Smo* lens. Normal sections of WT (A, B, C, G, H) and activated *Smo* mutant (D, E, F, I, J) mouse eyes at E12.5 (A, D, G, I), E15.5 (B, E, H, J), and E18.5 (C, F) immunostained for expression of *Pax6* (A–F, red) or *FoxE3* (G–J, red). Slides also were stained with DAPI to highlight nuclei (blue). White arrows (E, F, J) indicate *Pax6* (E, F) and *FoxE3* (J) staining within the lens epithelial cell region. All scale bars represent 100 μ m.

appeared similar, with *Pax6* expressed specifically in the lens epithelium (Figs. 5A, 5D). However, by E15.5, *Pax6* expression in the activated *Smo* mutants became disrupted. While the WT lens displayed a pattern of *Pax6* expression that was confined to the lens epithelial cell layer (Fig. 5B), the mutant lens at this stage showed *Pax6* expression throughout the entire lens region (Fig. 5E), a trend that persisted at E18.5 (compare Figs. 5C, 5F).

FoxE3 also was investigated in the developing lens at E12.5 and E15.5 (Fig. 5). By E12.5, the pattern of *FoxE3* expression already was disrupted in the activated *Smo* mutant lens. While its expression was confined to the lens epithelial cells in the WT lens (Fig. 5G), *FoxE3* expression not only was seen within

the lens epithelial region of the mutant lens, but its expression also had expanded into the posterior fiber cell region (Fig. 5I). A similar pattern of *FoxE3* expression was observed by E15.5 in the activated *Smo* lens, with expression evident throughout the lens epithelial and fiber cell regions (Fig. 5J). Thus, the ectopic expression of *FoxE3* in the *Smo* mutants preceded the ectopic expression of *Pax6* (see the Table).

Final Patterns of Fiber Cell Differentiation are Perturbed in Activated *Smo* Mutant Lenses

In the developing and adult vertebrate lens, the crystallins, including α A-crystallin, α B-crystallin, β -crystallin, and γ -crystal-

lin, are expressed in specific patterns within the differentiating fiber cells. Thus, to examine fiber cell development in the activated Smo mutants, expression of β -crystallin and γ -crystallin was explored (Fig. 6). γ -Crystallin expression appeared normal in the activated Smo mutants at E12.5, E15.5, and E18.5. Expression was observed in the posterior region of the lens vesicle at E12.5, and within the fiber cell region of the lens during the later stages of development (Figs. 6A–F). β -Crystallin, another excellent marker of fiber cell differentiation, is detected first within elongating primary fiber cells. In subsequent stages of embryonic development, its expression is initiated at the time when secondary fibers begin to differentiate away from the transitional zone of the lens.²⁷ Like that of γ -crystallin, β -crystallin expression appeared normal in the activated Smo mutants, with expression seen throughout the fiber cell region of the lens vesicle at E12.5 and throughout the fiber cell region of the lens during subsequent stages of embryonic development (Figs. 6G–L). Interestingly, although fiber cells in the activated Smo mutants expressed γ -crystallin and β -crystallin in a relatively normal manner, cells in this region showed clear cell morphological defects when viewed with DAPI. Specifically, in contrast to the wild-type situation at E15.5 and E18.5, the cells in the posterior region of the mutant lenses failed to lose their nuclei (Figs. 6E, 6F, 6K, 6L).

In the mouse, *Pax6* has been found to repress the transcription of genes encoding β -crystallin.^{45,46} Normally, β -crystallin is expressed in fiber cells, where *Pax6* expression has been down regulated, while the expression of *Pax6* remains confined to the lens epithelium.²⁷ Expression patterns of β -crystallin and *Pax6* were examined simultaneously by double immunostaining for each lens cell marker (Fig. 6). At E12.5, staining for *Pax6* and β -crystallin expression within the developing lens of the activated Smo mutants and WT litter mates appeared normal, with *Pax6* expressed in the developing lens epithelium and fiber cells and β -crystallin expressed within the fiber cell region (Figs. 6M, 6P). By E15.5, however, it is evident from examination of the WT lens, that *Pax6* expression had become confined to the lens epithelial region, while its expression within the β -crystallin-expressing fiber cell region had been down regulated (Fig. 6N). In the activated Smo mutants at this stage, *Pax6* expression was seen not only throughout the lens epithelial cell region, but also in the β -crystallin-positive fiber cell region (Fig. 6Q). Similarly, while these lens cell markers were expressed correctly in the E16.5 WT lens, *Pax6*-positive cells also stained positive for β -crystallin in the activated Smo mutants at this stage (Figs. 6O, 6R). This trend continued at E18.5 (data not shown).

The aberrant fiber cell phenotype of the activated Smo mutants was analyzed further by examining the expression patterns of *Prox1* and *c-maf*, both important in regulating fiber cell elongation and differentiation.^{47,48} By E13, *c-maf* becomes highly expressed at the equatorial region of the lens where proliferatively active anterior epithelial cells begin their differentiation process into secondary fiber cells.^{27,47,49} Expression of *c-maf* appeared normal in the activated Smo mutants at E12.5, with expression observed throughout the entire lens, appearing most pronounced throughout the fiber cell region (Fig. 7D). By E15.5, however, *c-maf* expression had become disrupted in the Smo mutants. While WT lenses displayed strong *c-maf* expression at the lens equator, and to a lesser extent throughout the anterior epithelium (Fig. 7B) *c-maf* expression was abnormal and expanded throughout the entire lens region of the activated Smo mutants (Fig. 7E). By E18.5 in the WT lens *c-maf* expression appeared highly concentrated at the lens equator, where secondary fiber cell differentiation is occurring, whereas *c-maf* expression again was abnormal and expanded throughout the entire lens region of the activated Smo mutants (Figs. 7C, 7F).

Similar to *c-maf*, *Prox1* also is expressed in the head ectoderm and lens placode early in development. With the progression of development to E12.5, *Prox1* is expressed throughout the entire lens region, and its expression is maintained in the lens epithelium and lens transition zone throughout embryonic development.^{27,50} Expression of *Prox1* in the activated Smo mutants at E12.5 was normal, and observed throughout epithelial and fiber cell compartments of the developing lens (Fig. 7J). In WT lenses at E15.5 and E18.5, *Prox1* was expressed correctly throughout the anterior lens epithelium and lens transition zone (Figs. 7H, 7I); however, its pattern of expression in the mutant lenses at these stages was expanded, encompassing the entire anterior epithelial and posterior fiber cell regions (Figs. 7K, 7L, and the Table).

An Activating Mutation in Smo Results in Retinal Disorganization and Degeneration at P0

Often, following the degeneration of the lens, retinal morphology and retinal cell survival can be impacted negatively. Also as shown in blind cavefish, which exhibit an expanded domain of Shh expression in the CNS, retinal degeneration follows extensive lens degeneration.^{51,52} Thus, due to the lens degeneration observed in the activated Smo mutants we examined the embryonic retina in this model. Gross retinal morphology, cell proliferation, cell death, and the expression of retinal specific markers were normal through E18.5 (Fig. 8). However, at P0, the latest stage the mice survive, retinal defects became apparent. For example, at P0 retinal lamination was disrupted in the activated Smo mutant retina and, unlike WT retinas, the mutant retina exhibited only a neuroblast layer (nbl) and no distinct ganglion cell layer (GCL, Fig. 8H). Retinal markers *Pax6* and Calretinin also were examined (Figs. 9A–H). By P0, *Pax6* is expressed strongly in the GCL and inner nuclear layer (INL) of WT mice, but expression is not observed within the outer neuroblast layer (onbl). In contrast, P0 Smo mutants lacked retinal lamination and a distinct GCL, along with the strong GCL *Pax6* expression domain. Instead, *Pax6* expression was maintained aberrantly in the nbl as well as in a small population of cells staining weakly positive for *Pax6* in the disorganized inner retina (IR) of these mutants (Fig. 9F). Calretinin, a calcium binding protein that labels amacrine and ganglion cells, also was examined. By P0, calretinin-expressing cells appeared to have lost their normal architecture in the activated Smo retinas, and expression was dispersed within the IR (Fig. 9H). Proliferation and apoptosis also were examined through staining with PCNA and TUNEL, respectively (Figs. 9I–P). While cellular proliferation in the activated Smo retinas appeared normal at E18.5 (Fig. 9M), at P0 an increase in proliferation was seen within the IR of the mutant (Fig. 9N). Patterns of apoptosis in the activated Smo retinas also appeared similar to those observed in WT litter mates at E18.5 (Figs. 9K, 9O). However, at P0, while a small number of cells label TUNEL-positive in the INL and outer nuclear layer in WT and mutant retinas, TUNEL-positive cells also were apparent in the disorganized IR of the activated Smo mutants. (Figs. 9L, 9P, and the Table).

DISCUSSION

Previous studies in vertebrates have shown that during early development Hh signals can regulate specification and fate of the head ectoderm into lens, and that appropriate levels of Hh signaling impact lens-specific gene expression and lens development. For example, in zebrafish, disrupted Hh signals can cause the formation of ectopic lenses at the expense of the adeno-hypophysis. Furthermore, when Hh signaling is exagger-

FIGURE 6. γ -Crystallin and β -crystallin are expressed in an appropriate spatial pattern, though crystallin-expressing FCs fail to de-nucleate and maintain *Pax6* expression. Sections of WT (A–C, G–I, M–O) and activated Smo mutant (D–F, J–L, P–R) mouse eyes at E12.5 (A, D, G, J, M, P), E15.5 (B, E, H, K, N, Q), and E18.5 (C, F, I, L, O, R) immunostained for expression of the γ -crystallin lens fiber cell marker (A–F, green), β -crystallin (G–L, red) or both Pax6 and β -crystallin (M–R, green and red, respectively). Slides also were stained with DAPI to highlight nuclei (blue). All scale bars represent 100 μ m.

FIGURE 7. *c-maf* and *Prox1* expression is expanded throughout the anterior and posterior lens of the activated *Smo* mutants. Sections of WT (A–C, G–I) and activated *Smo* mutant (D–F, J–L) mouse eyes at E12.5 (A, D, G, J), E15.5 (B, E, H, K), and E18.5 (C, F, I, L) immunostained for expression of *c-maf* (A–F, red), or *Prox1* (G–L, red). Slides also were stained with DAPI to highlight nuclei (blue). White arrows show *c-maf* and *Prox1* expression at the equatorial transitional zone. All scale bars represent 100 μ m.

ated in the ectoderm, lens placode formation is suppressed completely.^{11–13} However, the role of Hh signaling in mammalian lens development is less understood, even though it has been demonstrated that mutations in human Hh signaling components that result in activation of the Hh pathway can cause cataracts.⁶ In our study, we explored the consequence of excessive Hh signaling through constitutive activation of *Smo* in the surface ectoderm of the head, at a time by which the lens placode already is specified (E8.5). Our findings show that constitutive activation of *Smo* did not appear to impact negatively early development of the lens, including the lens pit and vesicle stages, but did disrupt later stages of lens differentiation, with the first defect apparent at E12.5. By E15.5 the fiber cells of the mutant lens were shown to have remained

in the cell cycle and failed to differentiate terminally. Accompanying this was the persistent epithelialization of cells in the fiber cell compartment.

The ectopic expression of *FoxE3* in the fiber cell compartment was the earliest expression defect observed in the activated *Smo* mutants at E12.5. *FoxE3*, a forkhead transcription factor gene, is a critical regulator of lens vesicle separation, and importantly also has been shown to regulate the normal proliferation patterns in the vertebrate lens.⁵³ Interestingly, ectopic and sustained *FoxE3* expression in the posterior region of the lens has been shown to cause a vacuolated lens with fiber cells that maintain a partial epithelial phenotype,⁵⁴ similar to the phenotype observed in the activated *Smo* lens. In this previous model, the α -A-crystallin (*Cryaa*) promoter was used to

FIGURE 8. Retinal lamination is lost between E18.5 and P0. Sections of WT (A–D) and activated Smo mutant (E–H) mouse eyes at E12.5 (A, E), E15.5 (B, F), E18.5 (C, G), and P0 (D, H) stained with H&E. inbl, inner neuroblast layer; IPL, inner plexiform layer. All scale bars represent 100 μm.

FIGURE 9. Activated Smo mutants show disorganized retinal morphology, and expression of *Pax6* and Calretinin, while exhibiting abnormal retinal cell proliferation and death by P0. Sections of WT (A–D, I–L) and activated Smo mutant (E–H, M–P) mouse eyes at E18.5 and P0. Slides were immunostained for expression of *Pax6* (A, B, E, F) and Calretinin (C, D, G, H), as well as PCNA (I, J, M, N) and TUNEL (K, L, O, P). White arrows indicate irregular proliferation and cell death in the IR of the mutants. All scale bars represent 100 μm.

drive ectopic, transgenic expression of *FoxE3* in differentiating mouse lens fibers. This resulted in an increase of mRNAs normally enriched in lens epithelial cells, as determined by microarray profiling, and was consistent with an epithelialization of the transgenic fibers. Similar to our activated Smo mutants, some aspects of fiber differentiation were unaffected, such as the expression of the crystallins. However, loss of fiber cell organelles and antimitotic signaling were affected. These data suggest that the early ectopic expression of *FoxE3* in the activated Smo mutants may be responsible for the subsequent defects observed in the fiber cells.

Like *FoxE3*, *Pax6* expression becomes confined to the lens epithelium after E12.5.⁵⁵ Although *Pax6* was found to be expressed in a normal manner in the Smo mutants at E12.5, its expression pattern became ectopic by E15.5, with staining evident throughout the epithelial and posterior fiber cell regions. The ectopic expression of *Pax6* also may have contributed to the altered fiber cell phenotype. For example, it has been demonstrated in *Pax6* transgenic mice, which over express *Pax6* in lens fiber cells, that there is a disorganized and vacuolated fiber cell region. These fiber cells failed to lose their nuclei, leading to disrupted fiber cell elongation and differentiation,⁴⁶ features reminiscent of the phenotype observed in our activated Smo mutants. Ectopic expression of *Pax6* also was shown to reduce drastically the expression of β B1-crystallin in the fiber cells of the transgenic lenses.⁴⁶ This outcome is not surprising, since *Pax6* has been shown to repress directly the expression of β B1-crystallin.^{46,56,57} However, in our activated Smo mutant model, β B1-crystallin and γ -crystallin, two markers of fiber cell development, elongation and differentiation,^{27,58,59} were expressed in a spatially correct pattern throughout the fiber cell region. Since the fiber cell region of the activated Smo lenses showed co-expression of *Pax6* and β B1-crystallin, it is possible that the levels of *Pax6* in our mutants are below the threshold necessary to repress crystallin gene expression.

Another possibility for the sustained expression of β B1-crystallin in the fiber cells of the activated Smo lens is that an additional, positive regulator of crystallin gene expression, *c-maf*, also was found to be expressed ectopically in the mutant lens, away from its normal localization in the equatorial region of the lens. *C-maf* has been shown to be critical for proper lens fiber cell differentiation and elongation, and importantly, in the regulation of β and γ -crystallin expression.^{47,49} *C-maf* homozygous null mutants also were shown to exhibit cessation in primary fiber cell differentiation and elongation by E12.5, as well as defects in secondary fiber cell development, including reduced expression of β -crystallin and absent γ -crystallin.^{47,49} Kawauchi et al. also reported that *c-maf* regulates directly the expression of γ -crystallin.⁴⁹ Thus, the ectopic expression of *c-maf* in the posterior cells of the activated Smo lens may be responsible for maintaining the β B1-crystallin and γ -crystallin expression observed, despite the persistent expression of the repressor, *Pax6*.

The constitutive activation of Smo also resulted in defective patterns of proliferation and cell death. Staining with the proliferation-specific markers PH3 and PCNA revealed proliferating cells in the fiber cell region of the mutant lens, at embryonic stages in which no proliferation should be observed. Hh signaling has been shown to regulate cell proliferation and differentiation in other systems. For example, activation of the Hh pathway in the regenerating newt lens was shown to cause an increase in lens vesicle cell proliferation.¹⁶ Aberrant expression of cell cycle regulators also was observed in our Smo mutant lens model. In particular, cyclin D, which is known to be downstream of Hh⁴² and functions in the G1 phase of the cell cycle to promote proliferation, normally is localized within the lens epithelium and fiber cells at the lens

equator.^{40,60,61} However, in the activated Smo mutants the domain of cyclin D, expression was expanded such that it was observed ectopically throughout the posterior fiber cell compartment. Interestingly, a study by Rowitch et al. illustrated that ectopic Hh pathway activation in central nervous system precursor cells during embryogenesis resulted in increased proliferation, and maintenance of these cells in an undifferentiated state.⁶² We postulate that a similar mechanism is operating in the activated Smo mutants, resulting in alterations in cell cycle regulation and fiber cell differentiation.

The p27^{kip1} and p57^{kip2}, both belonging to the family of p21^{cip1} CDKIs, cooperate with each other to promote proliferating lens epithelial cells at the lens equator to withdraw from the cell cycle, and begin their differentiation process into lens fiber cells. These CDKIs work by inhibiting kinases required for the G1-S phase transition.^{43,63,64} At E15.5 of development, both CDKIs are highly expressed at the transition zone of the lens, where they work to inhibit G1 phase cyclins, allowing these cells to exit the cell cycle and differentiate into secondary fiber cells.^{43,65,66} In the mutant lens at E15.5 and onward, however, p27^{kip1} and p57^{kip2} were expressed ectopically into the fiber cell region, mirroring the ectopic Cyclin D1 expression discussed previously. Not surprisingly, *Prox1*, which promotes the expression of p27^{kip1} and p57^{kip2}, also was shown to have escaped its normal spatial localization within the transition zone of the lens,^{50,67} and was expressed ectopically throughout the anterior and posterior of the mutant lenses, similar to the expression pattern observed for each CDKI examined in our study. Together, these data suggest that ectopic *Prox1*-driven expression of p27^{kip1} and p57^{kip2} is functioning to counteract the cyclin D proliferation caused by altered Smo activity.

The activated Smo lenses also exhibited lens degeneration, illustrated by positive TUNEL staining observed throughout the lens epithelial and fiber cell regions onwards of E15.5. The inappropriate expression of cell cycle promoting and inhibiting factors in the lens fiber cell compartment may have been a determinant in the apoptotic response. Additionally, as outlined previously, Hh signaling has been shown to suppress lens cell fate during lens placode formation,¹¹⁻¹³ and a consequence of the prolonged and exaggerated Hh signaling may be lens cell death, as an attempt to suppress further development of the lens. Nonetheless, lens degeneration did appear to have an impact on the retina in the activated Smo mutants. While the mutant retina exhibited normal morphology throughout the majority of embryonic development, in late embryonic and early postnatal stages, following lens degeneration, aberrant retinal morphology became evident. For example, by P0, the retina displayed abnormal amounts of apoptosis and a loss in laminar organization. Despite this, retinal cell markers, including *Pax6*, and calretinin were expressed. Interestingly, retinal degeneration in the blind cavefish does not occur due to a defect in proliferation, or as a result of incorrect patterning of critical retinal development markers, including *Pax6*, but rather as a result of programmed cell death in the retina subsequent to lens apoptosis and a loss of protective signals offered from the lens to the retina during development.^{51,52} It is important to reiterate that the Cre used in our studies (Cre^{ct}) is active only in the surface SE and its derivatives, and not in the retina. Thus, the fact that these retinal defects occurred following the peak of intense lens cell death and degeneration suggests that the degenerating lens in our Smo mutants had a role. The lens may have lost its protective effect on the retina or, alternatively, provided a new signal that induced retina degeneration.

In conclusion, our studies have illustrated that active Hh signaling in the mammalian lens can lead to changes in cell cycle regulation, and expression of critical molecules respon-

sible for lens development and differentiation. On one hand, sustained Hh signaling in fish and amphibians can lead to the suppression of lens formation or lens degeneration during development. Therefore, our findings raise the possibility that the mammalian eye anlagen has retained components of the regulatory network that might suppress embryonic lens formation if the lens is positioned inappropriately within the head. Conversely, the Hh signaling pathway has been shown to be critical for normal lens regeneration in the newt, even though the pathway has not been associated with normal lens development in this species. Therefore, it is possible that the Hh pathway could function in mammalian wound healing following lens injury or be used in lens regeneration. Further studies will be required to examine if the Hh pathway is associated with mammalian lens repair and regeneration. Finally, the Cre⁺ Smo^{+/−} M2 mouse also may serve as a model to study aspects of BCNS (Gorlin syndrome), known to affect the lens and cornea. In this context, it may be valuable to determine if *Foxe3* activation also is associated with BCNS in ocular pathology.

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