CELL INJURY, REPAIR, AGING, AND APOPTOSIS

The Patched 1 Tumor-Suppressor Gene Protects the Mouse Lens from Spontaneous and Radiation-Induced Cataract

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Accepted for publication
September 4, 2014.

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Age-related cataract is the most common cause of visual impairment. Moreover, traumatic cataracts form after injury to the eye, including radiation damage. We report herein that sonic hedgehog (Shh) signaling plays a key role in cataract development and in normal lens response to radiation injury. Mice heterozygous for Patched 1 (*Ptch1*), the Shh receptor and negative regulator of the pathway, develop spontaneous cataract and are highly susceptible to cataract induction by exposure to ionizing radiation in early postnatal age, when lens epithelial cells undergo rapid expansion in the lens epithelium. Neonatally irradiated and control *Ptch1*^{+/-} mice were compared for markers of progenitors, Shh pathway activation, and epithelial-to-mesenchymal transition (EMT). Molecular analyses showed increased expression of the EMT-related transforming growth factor β /Smad signaling pathway in the neonatally irradiated lens, and up-regulation of mesenchymal markers Zeb1 and Vim. We further show a link between proliferation and the stemness property of lens epithelial cells, controlled by Shh. Our results suggest that Shh and transforming growth factor β signaling cooperate to promote *Ptch1*-associated cataract development by activating EMT, and that the Nanog marker of pluripotent cells may act as the primary transcription factor on which both signaling pathways converge after damage. These findings highlight a novel function of Shh signaling unrelated to cancer and provide a new animal model to investigate the molecular pathogenesis of cataract formation. (*Am J Pathol* 2015, 185: 85–95; <http://dx.doi.org/10.1016/j.ajpath.2014.09.019>)

Cataract is the most frequent cause of blindness worldwide.¹ Development of cataract in adults is related to normal aging, but other factors are also involved, such as diabetes, smoking, and alcohol use, as well as exposure to UVB and ionizing radiations.² Cataract is also one of the most important causes of avoidable (preventable and treatable) childhood blindness,³ and often develops as a complication of radiotherapy.⁴ However, the exact mechanisms of radiogenic cataract are not entirely understood.

Inherited mutations of the Patched 1 (*Ptch1*) gene have been identified as responsible for Gorlin syndrome.⁵ Patients with this hereditary condition are hypersensitive to radiation and prone to develop multiple skin cancers, specifically basal cell carcinomas,⁶ occurring with greater incidence in portals of radiotherapy.⁷ They are also at risk for developing medulloblastoma, a specific type of brain tumor. Among many

minor criteria for diagnosis, ophthalmic abnormalities, including cataract, occur in 26% of patients.^{8,9}

Mice in which one copy of *Ptch1* is inactivated are characterized by activation of the sonic hedgehog (Shh) pathway and show increased susceptibility to spontaneous and radiation-induced tumors, providing an ideal *in vivo* model for studying the typical pathological conditions associated with Gorlin syndrome.^{10–12} In addition to the well-known role of hedgehog signaling in cancer, Shh signals direct cell proliferation, cell fate determination, epithelial-to-mesenchymal transition (EMT), and the rearrangement of cells by motility and adhesion changes

Supported in part by the Unit of Radiation Biology and Human Health, l'Energia e lo Sviluppo Economico Sostenibile, Intramural Program (M.M. and A.S.).

I.D.S., B.T., and P.G. contributed equally to this work.

Disclosures: None declared.

during embryogenesis, remaining largely expressed in the stem cell compartment of adult tissues.¹³ The vertebrate ocular lens, composed of epithelial and fiber cells, grows throughout life, implying the existence of a lens stem cell compartment. DNA labeling studies showed that most new lens cells arise in the germinative zone, a narrow cellular region that rings the lens epithelium toward the periphery of the anterior lens surface.^{14,15} After division, these cells withdraw from the cell cycle and terminally differentiate into fiber cells. Despite findings showing a role of Shh in mouse lens development¹⁶ and in lens regeneration of the adult newt,¹⁷ much remains to be understood about the role of this pathway in postnatal lens growth and its possible correlation with cataract development.

Herein, we show a specific role for the *Ptch1* gene in the maintenance of lens integrity with or without exogenous damage, and validate the *Ptch1*^{+/-} model as a tool to understand the molecular mechanism of cataract development. Altogether, our results support the hypothesis that Shh and transforming growth factor β (TGF-β) signaling pathways synergize with radiation damage to induce EMT, culminating in anterior subcapsular cataract (ASC) development. We also outline a role of the Nanog stemness gene in *Ptch1*-associated radiogenic cataract.

Materials and Methods

Mice

Mice lacking one *Ptch1* allele (*Ptch1*^{+/-neo6/7}; named *Ptch1*^{+/-} throughout the text) on CD1 background were bred and genotyped as described.¹⁰ Care of experimental animals was in accordance with the Italian legislation on animal experimentation. Experimental protocols were reviewed by the Institutional Animal Care and Use Committee.

Mice Irradiation and Monitoring

Ptch1^{+/-} and wild-type (WT) littermates of both sexes were irradiated using a Gilardoni CHF 320 G X-ray generator

(Gilardoni, Mandello del Lario, Italy), as described.¹² Animals were exposed to a single dose of 3 Gy at different postnatal (P) days (P2, P10, P56). Additional groups were left untreated as controls. Experimental groups are summarized in Table 1.

Mice were observed daily throughout their life span. The time of cataract onset (unilateral and bilateral) (Table 1) was recorded. On decline of health, mice were sacrificed and necropsied.

Histological, IHC, and Morphometric Analyses

Normal and cataract-bearing eyes were processed for histological analysis by standard techniques at the end of their lifetime or when bilateral cataract became evident; eyes were also collected from mice sacrificed before termination of the experiment. Eye sections (2 μm thick) were cut in a plane perpendicular to the anteroposterior eye axis and stained with hematoxylin and eosin. Immunohistochemistry (IHC) was performed on the following groups: i) eyes from satellite groups (*n* = 6 per genotype) irradiated at P2 and sacrificed at short-term after irradiation (ie, 0.5 hours for analysis of γ-H2AX foci (a biomarker of DNA double-strand breaks), 4.5 hours for detection of p53 and p21 immunoreactivity, and 6 hours for apoptotic analysis by activated caspase-3 antibody); ii) eyes from unirradiated mice at P2, P4, P6, and P10 or P2-irradiated mice sacrificed at different times after irradiation (P4, P6, and P10) for Ki-67 proliferation analysis and nestin immunoreactivity; and iii) fully developed cataracts. The following antibodies were used: Ki-67 (NCL-Ki67p; polyclonal; dilution 1:800; Novocastra Laboratories, Newcastle, UK); γ-H2AX (05-636; monoclonal; dilution 1:200; Upstate Biotechnology Inc., Lake Placid, NY); cleaved caspase-3 (9661; polyclonal; dilution 1:100; Cell Signaling Technology, Inc., Danvers, MA); E-cadherin (3195; polyclonal; dilution 1:200; Cell Signaling Technology, Inc.); N-cadherin (13116; polyclonal; dilution 1:200; Cell Signaling Technology, Inc.); anti-nestin (ab81755; polyclonal; dilution 1:300; Abcam, Cambridge, UK); α-smooth muscle actin (A2547;

Table 1 Incidence of Cataract in *Ptch1*^{+/-} and *Ptch1*^{+/+} Mice Irradiated at 2, 10, and 56 Days of Age with 3 Gy of X-Rays or Left Unirradiated

Treatment	Age, days	Mouse line	No. of mice	Unilateral cataract, % (no./total)	Bilateral cataract, % (no./total)	Median latency, weeks
3 Gy	2	<i>Ptch1</i> ^{+/-}	42	45.2 (19/42)*†	14.3 (6/42)‡	6.9
		<i>Ptch1</i> ^{+/+}	48	8.3 (4/48)§	2.08 (1/48)	9.5
3 Gy	10	<i>Ptch1</i> ^{+/-}	33	0	0	—
		<i>Ptch1</i> ^{+/+}	47	0	0	—
3 Gy	56	<i>Ptch1</i> ^{+/-}	44	2.27 (1/44)	0	7
		<i>Ptch1</i> ^{+/+}	39	2.56 (1/39)	0	32
0 Gy	—	<i>Ptch1</i> ^{+/-}	34	8.8 (3/34)	5.88 (2/34)	34
		<i>Ptch1</i> ^{+/+}	53	0	0	—

All *P* values were calculated using Fisher's exact test.

**P* < 0.001 between unilateral cataract P2 irradiated *Ptch1*^{+/-} mice and unirradiated *Ptch1*^{+/-} mice.

†*P* < 0.0001 between unilateral cataract P2 irradiated *Ptch1*^{+/-} and *Ptch1*^{+/+} mice.

‡*P* < 0.05 between bilateral cataract P2 irradiated *Ptch1*^{+/-} and *Ptch1*^{+/+} mice.

§*P* < 0.05 between unilateral cataract P2 irradiated *Ptch1*^{+/-} mice and unirradiated *Ptch1*^{+/+} mice.

Ptch1, Patched 1.

dilution 1:400; Sigma-Aldrich, St. Louis, MO); p21 (ab2961; polyclonal; dilution 1:100; Abcam); and p53 (NCL-p53-CM5p; polyclonal; dilution 1:500; Novocastra Laboratories).

The IHC scoring (proliferation and apoptotic indexes) was performed by investigators blinded to treatment groups (I.D.S. and P.G.). The number of cells positive for Ki-67 or cleaved caspase-3 antibody was calculated as percentage of total lens epithelial cells (LECs). Nestin IHC expression intensity was assessed by HistoQuest automated IHC analysis system (TissueGnostics, Vienna, Austria).

Morphometric analysis of lens capsule in mild and severe cataract was performed on high-magnification digital images ($n = 6$). Capsule thickness was recorded using imaging software NIS-Elements BR version 4.00.05 (Nikon Instruments Europe B.V., Amsterdam, the Netherlands), and averages were calculated from approximately 400 measurements.

Lens area was determined from *Ptch1*^{+/-} and WT mice ($n = 6$ per genotype) at 21 days of age using imaging software NIS-Elements BR 4.00.05 and reported as means $\pm$ SD.

Real-Time Quantitative PCR

Mice of each genotype ($n = 6$) were left unirradiated and sacrificed at P2 or P10, or they were irradiated at P2 and sacrificed after 8 days. Eyes were enucleated, and lenses were removed and snap frozen in liquid nitrogen before storing at -80°C .

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Milan, Italy). Purified RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Milan, Italy), and 1 μg of total RNA was reverse transcribed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), then stored at -20°C until use. Primers were designed for nine target genes working under the same cycling conditions (Table 2). Real-time quantitative PCR (qPCR) was performed by StepOnePlus Real-Time PCR System (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems). Reactions were performed in triplicate. Relative gene expression was quantified using glyceraldehyde-3-phosphate dehydrogenase as house-keeping gene. The standard curve method was used to

normalize expression of the reference gene and to calculate the relative expression levels of target genes.

Western Blot Analysis

Proteins were extracted from lens pools ($n = 12$) of unirradiated *Ptch1*^{+/-} and WT mice at P2 and P10, and of mice irradiated at P2 and sacrificed after 8 days, then analyzed as described.¹⁸ After incubation with anti-Smad3 phospho-S423/S425 (ab51451; polyclonal; dilution 1:1000; Abcam) and β -actin (A5441; monoclonal; Sigma-Aldrich), specific proteins were visualized with ImageQuant LAS 500 system (GE Healthcare Europe GmbH, Milan, Italy).

Statistical Analysis

Cataract incidence, expressed as the percentage of mice with macroscopic unilateral cataract, was analyzed using a Fisher's exact test. Kaplan-Meier survival curves were compared, and log-rank test *P* values were calculated. IHC scoring, intensity, and morphometric and qPCR analyses are reported as means $\pm$ SD, and a *t*-test was used for determination of statistical difference between groups. $P \leq 0.05$ was considered statistically significant. Analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA).

Results

Ptch1 Deficiency Causes Cataract

To test the role of Shh signaling pathway deregulation in cataract development, *Ptch1*^{+/-} ($n = 34$) and WT ($n = 53$) mice were observed throughout their life span. *Ptch1*^{+/-} mice developed grossly visible cataract, recognizable as a white pinpoint focus in unpigmented eyes, with an incidence of 8.8% (3/34) and median latency of 34 weeks (Figure 1, A and B). The event was bilateral in two-thirds of positive mice. No cataract development was observed in WT mice (0/53; $P = 0.05$) (Figure 1C and Table 1), showing a specific role for *Ptch1* in the preservation of lens integrity.

Table 2 Primer Sequences Used for Real-Time Quantitative PCR

Gene	Forward primer	Reverse primer
<i>Gli1</i>	5'-GAGGACCTGGAGAGAGAGAGAA-3'	5'-CCAGCGGCAGTCTGTCTCA-3'
<i>Gli2</i>	5'-CGCAAGCGGGCTCTGT-3'	5'-TCCGAATCATGCGTTGTAGGT-3'
<i>Zeb1</i>	5'-CAGGACTCAAGACATCTCAGTGTTC-3'	5'-TGTGGGACTGCCTGGTGAT-3'
<i>Vim</i>	5'-GAGAGAGGAAGCCGAAAGCA-3'	5'-GCCAGAGAAGCATTGTCAACATC-3'
<i>Cdh1</i> (E-cadherin)	5'-AGAGCCGGACACGTTTCATG-3'	5'-TTGGCAGTGTCCCTCCAAA-3'
<i>Pax6</i>	5'-CCATGTTGGGCCGAACA-3'	5'-TGGGTGGCAAAGCACTGTAC-3'
<i>Sox2</i>	5'-CGCGCCGGTATTTATAATC-3'	5'-ACTTTTGTCCGAGACCGAGAA-3'
<i>Nanog</i>	5'-GCTCAGACACAGTGGAGTATCC-3'	5'-TCCAGATGCGTTACACAGATAG-3'
<i>Gapdh</i>	5'-CATGGCCTTCCTGTTCCTA-3'	5'-GCGGCACGTCAGATCCA-3'

Figure 1 Cataract development in Patched 1 (*Ptch1*^{+/-}) and wild-type (WT) mice after irradiation at P2. **A:** Macroscopically visible cataract is recognizable as a white pinpoint focus in the pink eyes. **B:** Stereomicroscopic image of eyes showing the typical lens opacity associated with cataract compared to normal lens appearance. **C:** Incidence of cataract in irradiated and unirradiated *Ptch1*^{+/-} and WT mice. **P* < 0.05, *****P* < 0.0001.

Ptch1 Deficiency Confers Increased Susceptibility to Radiogenic Cataract Mainly in the Early Stages of Postnatal Lens Morphogenesis

Ptch1^{+/-} mice display many characteristics associated with Gorlin syndrome, including hypersensitivity to ionizing radiation. To test whether ionizing radiation can be an inducer of cataract in this model, groups of *Ptch1*^{+/-} and WT mice were whole-body irradiated with 3 Gy of X-rays at different postnatal ages (ie, at P2, an early stage of postnatal lens development; at P10, when postnatal development is near to completion; and at P56, in young adults). Results show low or no induction of cataract when radiation was delivered at P10 or P56, irrespective of *Ptch1* status (Table 1). In contrast, *Ptch1*^{+/-} mice irradiated at P2 showed a high cataract incidence compared to unirradiated *Ptch1*^{+/-} mice (*P* < 0.0001). Of 42 mice, 19 (45.2%) developed cataract, and the event was bilateral in 6 (14.3%) of 19 positive mice. Notably, in 12 of 19 positive mice, cataract development was an extremely early event, detected at 3 weeks of age, although the total median latency was 6.9 weeks (Figure 1C and Table 1). At a highly radiosensitive P2 age, 8.3% of WT mice also developed cataract with median latency of 9.5 weeks. These data demonstrate that germline inactivation of one copy of *Ptch1* confers increased predisposition to radiation-induced cataract, with high susceptibility during the early stages of postnatal lens morphogenesis.

Radiation and Shh Deregulation Synergize to Enhance EMT

Histopathological and IHC analyses showed no differences between normally appearing lenses of adult *Ptch1*^{+/-} and WT mice (Figure 2, A–D). A single layer of cuboidal cells covering the anterior lens surface, the LECs (Figure 2A), showed high immunoreactivity for the adhesion molecule E-cadherin (Figure 2B). LECs showed lack of N-cadherin signal (Figure 2C), consistent with the specificity of this marker for fiber cells.¹⁹

All cataracts (spontaneous or induced by radiation in WT and in *Ptch1*^{+/-} mice) had similar morphological characteristics at histological examination (ie, they had histological features of ASC with different degrees of damage severity). In milder cases, LECs were characterized by loss of epithelial cell polarity (Figure 2, E and F), prominent cell vacuolization (Figure 2E), and coexistence of E-cadherin (Figure 2F) and N-cadherin (Figure 2G) expression, indicative of EMT activation in lens epithelium (Figure 2, C and G). In addition, disorganization of fiber cell structure was already evident (Figure 2, E and H). In more severe disease, LECs formed a plaque consisting of multilayered cells with spindle-shaped features, reminiscent of fibroblastic morphological features (Figure 2I). IHC analyses showed limited reactivity for E-cadherin and N-cadherin (Figure 2, J and K). Aberrant deposition of collagen was evident (Figure 2L), as was expression of the EMT marker, α -smooth muscle actin (Figure 2M). At this stage, lens fiber cells were dramatically compromised (Figure 2M). Capsule thickness correlated with disease severity (Figure 3, A–D). In milder cases, there was a twofold increase compared to normal lens (5.4 μ m versus 2.6 μ m; *P* < 0.0001) (Figure 3, A and B). This trend was confirmed in severe cataracts, in which average capsule thickness was sixfold compared to normal lens (14 μ m versus 2.6 μ m; *P* < 0.0001) (Figure 3, A and C). Altogether, these data allow classification of cataracts observed as ASCs, developing through a mechanism guided by EMT.

Shh Deregulation Drives LECs toward a More Proliferative and Undifferentiated Condition

During lens embryonal development, all LECs have been shown to actively proliferate.²⁰ However, during the first postnatal week, the lens is still in rapid growth, the number of mitotically active LECs changes profoundly, with significantly increased dividing cells between P1 and P3, and a decline is observed at P5.²¹

We evaluated the proliferative capacity of the LECs in *Ptch1*^{+/-} and WT mice at P2, the age of peak susceptibility

Figure 2 Patched 1 (*Ptch1*^{+/-}) and wild-type (WT) mice develop anterior subcapsular cataract (ASC). **A–D:** Representative images of lens epithelial cells (LECs) of a normal unirradiated mouse lens. **A:** Histological image of the typical simple cuboidal epithelium, showing strong E-cadherin immunoreactivity (**B**), and lack of N-cadherin expression (**C**). The **arrow** in **A** indicates the capsule. **D:** Representative image of a normal unirradiated mouse lens. **E:** Early-stage cataracts show vacuolated and elongated LECs and concomitance of E-cadherin (brown stain; **F**) and N-cadherin (brown stain; **G**) immunoreactivity. **H:** The typical disorganization of fiber cell structure is already evident. **I:** Severe ASC, characterized by a typical multilayered plaque (**arrows**) consisting of spindle-shaped cells, lack of E-cadherin (**J**) and N-cadherin (**K**) expression, deposition of collagen fibers (blue stain), as demonstrated by Masson's trichrome staining (**L**), and typical α -smooth muscle actin expression (red stain; **M**). **N:** Extensive disorganization of fiber cells. Scale bar = 10 μ m (**A** and **E**, insets).

to radiation injury, and at P10, a nonresponsive age. By using a Ki-67 proliferation marker, we show that at P2, many LECs are mitotically active (Figure 4, A and B). In contrast, at P10, only a few centrally located LECs are Ki-67 positive (Figure 4, C and D), confirming an age-related decline of proliferation in both WT and *Ptch1*^{+/-} LECs. To explore potential differences due to *Ptch1* allelic status, Ki-67-positive cells were quantified at P2, P4, P6, and P10 in WT and *Ptch1*^{+/-} mice. Figure 4E shows that the proliferation index of the LECs in *Ptch1*^{+/-} mice was significantly higher compared

to WT mice at P2, P4, and P6. There were more Ki-67-positive cells in the *Ptch1*^{+/-} lens at P10, although this difference was not statistically significant. This evidence suggests that the Shh pathway may mediate cell proliferation during lens development by acting as a mitogen, similar to its role in other developing tissues/organs (eg, the cerebellum).²²

Because *Ptch1*^{+/-} mice have increased LECs proliferation, we investigated whether their lenses are larger compared to WT mice. We performed morphometric analyses to measure lens area on sections from unirradiated *Ptch1*^{+/-} and WT

Figure 3 Representative images of the capsule thickness in normal lens (**A**), mild anterior subcapsular cataract (ASC; **B**), and severe ASC (**C**), with quantification (**D**). **** $P < 0.0001$.

Figure 4 Sonic hedgehog drives lens epithelial cells (LECs) toward a more proliferative and undifferentiated condition. Representative images of unexposed Patched 1 (*Ptch1*^{+/-}) LECs after immunostaining with an antibody direct against Ki-67 at P2 (A and B) and P10 (C and D). **E**: Kinetics of proliferation of unirradiated *Ptch1*^{+/-} and wild-type (WT) mouse LECs. Representative images of *Ptch1*^{+/-} LECs immunostained at P2 (F and G) and P10 (H and I) with anti-nestin antibody. Evaluation of DNA damage response using an antibody directed against γ-H2AX (J) or activated caspase-3 (K) 0.5 and 6 hours after P2 irradiation, respectively. **L**: Quantification of apoptotic cells in *Ptch1*^{+/-} and WT mouse lenses at 6 hours after irradiation. **M** and **N**: The p21 and p53 immunoreactivity evaluated 4.5 hours after P2 irradiation. **O** and **P**: Proliferating LECs immunostained with Ki-67 (O) and nestin immunoreactivity in neonatally irradiated mice (P2) at 10 days of age (IR/P10) (P). **Q**: Kinetics of proliferation of *Ptch1*^{+/-} and WT mouse LECs, quantified at different times after P2 irradiation; for comparison, the kinetics of LECs from unirradiated mice (E) are shown (dashed lines). There was a statistically significant difference versus unirradiated age-matched counterparts at all experimental time points. **P* < 0.05, ***P* < 0.01, and *****P* < 0.0001. Scale bars: 10 μm.

mouse lenses at 21 days of age. The results (averaged on six mice) showed no significant difference in lens area between the two genotypes (Supplemental Figure S1).

Because Shh has been implicated in maintenance of a stem cell condition,²³ we used an antibody against nestin, a typical marker of epidermal stem cells,²⁴ to characterize LECs at P2 and P10. We observed substantial differences in LECs at these postnatal ages. In fact, although all LECs at P2 strongly expressed nestin, independent of localization (central and germinative zone) (Figure 4, F and G), nestin expression was significantly reduced in the centrally located LECs at P10, whereas it remained similarly expressed in the germinative zone (Figure 4, H and I, and Supplemental Figure S2). This result indicates that essentially all LECs at P2 have stem/progenitor cell properties, which then regionally decline with mouse age.

Stem/progenitor cells are defined as the cells that execute the repair of damaged tissue.²⁵ To examine the mechanisms behind cataract susceptibility of LECs at P2, we evaluated their DNA damage response at short times after irradiation. By using an antibody against γ-H2AX, a marker of double-stranded breaks, we show that virtually all LECs showed many γ-H2AX foci at 0.5 hours after exposure, independent of *Ptch1* genotype (Figure 4J). Because double-stranded breaks precede cell death via activation of pro-apoptotic pathways, we performed immunostaining with cleaved caspase-3 antibody, the main executioner of apoptosis, at 6 hours after irradiation. Unexpectedly, despite widespread DNA damage in LECs, only sporadic caspase-3-positive cells were observed (Figure 4K), resulting in a low apoptotic index (approximately 2.5%), with no significant difference between

Figure 5 Sonic hedgehog (Shh) and transforming growth factor β signaling synergize with radiation damage to promote Nanog overexpression and induce epithelial-to-mesenchymal transition (EMT) in irradiated lens. Relative mRNA expression levels of Gli1 (A), Gli2 (B), and Nanog (C) in unirradiated *Ptch1*^{+/-} and wild-type (WT) unirradiated lens at P2 and P10, and in neonatally exposed mice (P2) at 10 days of age (IR/P10). D: Western blot analysis of phosphorylated Smad3 (p-Smad3) expression in unirradiated *Ptch1*^{+/-} and WT lens at P2 and P10, and in IR/P10. E: Band intensities of p-Smad3 were sampled three times and normalized against β -actin. F: mRNA expression levels of EMT-related markers Zeb1, Vim, and E-cadherin, and stemness genes Pax6 and Sox2 evaluated in IR/P10. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Ptch1^{+/-} and WT mice (Figure 4L). The low rate of radiation-induced apoptosis is in agreement with stem/progenitor cell properties of LECs at P2.

Consistent with the low apoptotic rate after irradiation, we show strong expression of p21 (Figure 4M), which acts as an inhibitor of apoptosis in several systems.²⁶ We also examined p53 levels in the mouse lens irradiated at P2 and found a moderate p53 expression (Figure 3N). This is consistent with previous results from our group in irradiated neonatal cerebellum,²⁷ and with recent findings obtained in different stem cell compartments showing that p53 is not a key player in the DNA damage response of stem cells and that X-rays induce p53-independent up-regulation of p21, preventing apoptosis and promoting cell cycle entry and expansion of stem cells.²⁸

We also analyzed LECs at longer times after P2 irradiation. Independent of genotype, irradiated mice sacrificed at P10 showed more Ki-67–positive LECs compared to unirradiated age-matched counterparts (Figure 4, O and C, respectively). Time course analysis at 4 and 6 days confirmed a significantly higher proliferation index in irradiated mice (Figure 4N), regardless of *Ptch1* allelic status. At 10 days, this difference was significant only in *Ptch1*^{+/-} mice. Indeed, the proliferation index of LECs from *Ptch1*^{+/-} mice was significantly higher compared to WT mice at all time points analyzed. Finally, we examined whether increased proliferation in

irradiated LECs correlated with nestin expression.²⁹ Notably, higher levels of nestin were detected at P10 (Figure 4O) compared to age-matched unirradiated mice (Figure 4H), resembling nestin expression at P2 (Figure 4F). Supplemental Figure S1 provides quantification.

The increased rate of proliferation and the higher expression of nestin suggest that radiation damage may stimulate recruitment into the cell cycle and prevent exhaustion of LEC self-renewal competence after irradiation at P2 in both *Ptch1*^{+/-} and WT mice. Nevertheless, the strong increase of cataract development only in exposed *Ptch1*^{+/-} mice supports the hypothesis that additional mechanisms are involved in *Ptch1*^{+/-} LEC radiosensitivity.

Cross Talk between Shh and TGF- β Signaling Pathways in Radiogenic Cataract Development

To test the hypothesis that the age-dependent sensitivity of LECs to irradiation is controlled by Shh-dependent regulation of the stem cell competence of LECs, mRNA levels of Gli1 and Gli2, the downstream effectors of the Shh pathway, were evaluated in unirradiated LECs by qPCR. Results show a high expression of Gli1 at P2 and a sharp decline at P10 (approximately 12-fold; $P < 0.0001$) (Figure 5A), indicative of Shh pathway inactivity irrespective of *Ptch1* status. At P2, *Ptch1*^{+/-} LECs showed a higher Gli1 mRNA level compared

Figure 6 Model of interaction between sonic hedgehog (Shh) and transforming growth factor β (TGF- β) signaling in irradiated lens epithelial cells, consistent with *in vivo* findings from this study. Radiation injury may up-regulate Shh expression directly or indirectly via TGF- β signaling. Gli2 is an early gene target of TGF- β /Smad signaling, independent of Shh signals, and Gli1 can be induced by TGF- β in a Gli2-dependent manner. At P2, both signaling pathways converge on Nanog in Patched 1 (*Ptch1*^{+/-}) injured lens tissue (**blue and red arrows**), facilitating a self-amplifying Shh/TGF- β pathogenic loop promoting and amplifying epithelial-to-mesenchymal transition (EMT), and inducing cataract development. When Shh is normally regulated (WT mice at P2) or inactive (P10), the self-amplifying Shh/TGF- β pathogenic loop is abrogated, and only indirect activation of Shh signaling via TGF- β (**blue arrows**) contributes to Nanog expression and EMT, resulting in low cataract induction.

to WT LECs ($P = 0.0364$), consistent with Shh pathway deregulation in *Ptch1*^{+/-} animals. High Gli2 mRNA levels were observed at P2 in both *Ptch1*^{+/-} and WT LECs (Figure 5B). Notably, Gli2 expression did not decline at P10, showing Gli2 independence from Shh signaling at this age. Again, *Ptch1*^{+/-} LECs showed significantly higher Gli2 levels relative to WT LECs at both ages (P2, $P = 0.0454$; P10, $P = 0.0165$).

It is known that Shh signaling regulates Nanog expression in different systems.^{30,31} To determine whether Nanog could play a role in age-related cataract induction, we measured its mRNA levels at P2 and P10. Nanog mRNA decreased sharply during this time window ($P < 0.0001$), consistent with the hypothesis of stemness rapid decline in mouse LECs during lens postnatal development (Figure 5C).

A common feature of ASC is induction of EMT by TGF- β ,^{32,33} and cross talk between Shh signals and TGF- β in different pathological conditions has been observed.^{34–36} Therefore, it was important to assess the balance of Shh and TGF- β signaling during postnatal lens development with or without radiation insult. Because TGF- β signals through phosphorylation of the Smad3 transcription factor of the TGF- β superfamily ligands, we investigated expression of phosphorylated Smad3 (p-Smad3). Western blot analysis results show a trend toward increased p-Smad3 expression from P2 to P10. Moreover, p-Smad3 expression was not significantly different between *Ptch1*^{+/-} and WT LECs (Figure 5, C and D).

Expression levels of Shh and TGF- β are known to increase after radiation injury.^{37,38} We, therefore, evaluated the expression of Gli1 and Gli2 mRNAs and of p-Smad3 protein at P10, 8 days after irradiation. Gli1 mRNA was up-regulated relative to LECs from age-matched unirradiated mice (Figure 5A). Remarkably, *Ptch1*^{+/-} LECs showed a 24-fold increase of Gli1 expression after radiation injury ($P < 0.0001$) compared with only a 3.5-fold increase in WT LECs ($P < 0.0001$). Relative to Gli1, Gli2 mRNA expression was only marginally increased (approximately 1.3-fold), again with significantly higher levels in *Ptch1*^{+/-} mice (Figure 5B). p-Smad3 protein was also significantly up-regulated in irradiated *Ptch1*^{+/-} mice (Figure 5, D and E), showing differential activation of TGF- β /Smad signaling.

A trait of ASC formation is EMT of LECs into myofibroblasts.³³ To determine whether irradiated *Ptch1*^{+/-} and WT LECs show differences in markers associated with EMT, we examined, by qPCR, the expression of EMT-related genes Zeb1 and Vim.^{39,40} Figure 5F shows significantly increased expression of the EMT regulator Zeb1 and the mesenchymal marker Vim in irradiated *Ptch1*^{+/-} LECs. Moreover, expression of the cell adhesion molecule E-cadherin was significantly lower in LECs from *Ptch1*^{+/-} irradiated mice, consistent with increased expression of mesenchymal markers. These results suggest that interaction of the Shh and TGF- β /Smad signaling pathways can induce

EMT and promote fibrogenesis in radiation-injured *Ptch1*^{+/-} mouse lens.

Cells that undergo EMT are known to acquire stem cell-like properties.⁴¹ We, therefore, evaluated mRNA levels of Pax6 and Sox2 stem cell markers in irradiated WT and *Ptch1*^{+/-} LECs. Indeed, we found the expression levels of these stemness-associated genes significantly increased in *Ptch1*^{+/-} compared to WT mice. Finally, analysis of Nanog expression after P2 irradiation revealed a striking increase (approximately 130-fold) in *Ptch1*^{+/-} LECs at P10 ($P < 0.0001$) (Figure 5C), suggesting a central role of Nanog in *Ptch1*-associated cataractogenesis.

Discussion

Although Gorlin syndrome patients, who have a deregulated Shh pathway, are prone to ophthalmic abnormalities, including cataract, a role for Shh in cataract development has not been previously reported. We establish herein that mice heterozygous for the *Ptch1* gene, the Shh receptor, develop spontaneous cataract and are more sensitive than WT animals to the cataractogenic effects of ionizing radiation. The mechanisms of cataract formation are linked to DNA damage of LECs, which can result in defective cell cycle control and fibrotic reaction.⁴² Shh is involved in both processes, and as our results show, animals deficient in *Ptch1* protein are susceptible to cataract induction.

A common response to DNA damage in differentiated cells is activation of p53 and increased expression of p21 cyclin-dependent kinase inhibitor to arrest the cell cycle. However, we show that LECs irradiated at P2 evade apoptosis and proliferation arrest, suggesting a nonfunctional p53-p21 axis of the G₁/S checkpoint pathway,²⁸ and further strengthening the hypothesis that the lens epithelium includes a true stem cell population.⁴³

We show that radiation-induced cataractogenesis is subjected to temporal restriction. Although early studies have shown age-dependent responses to radiation,⁴⁴ the underlying mechanisms have remained elusive. In the rodent lens, the most rapid growth occurs during the first postnatal week, with increases in lens weight of up to 87% in a single day.⁴⁵ In principle, because radiosensitivity is higher in proliferating cells,⁴⁶ the temporal restriction observed might be attributable to age-related decline of proliferation in LECs during postnatal lens development. However, we observe that exposure of *Ptch1*^{+/-} mice to irradiation at P10 fails to induce cataract, despite evident ongoing proliferation in P10 lens. Likewise, although *Ptch1*^{+/-} mice have an increased percentage of proliferating cells in their lenses compared to WT mice throughout the first postnatal week, this is unlikely to explain a 5.4-fold cataract incidence in P2-irradiated *Ptch1*^{+/-} mice, which indicates that additional factors are likely to influence LEC susceptibility to radiogenic cataract.

It is unclear how *Ptch1* haploinsufficiency can predispose to spontaneous and radiation-induced cataract development.

The Shh mitogenic role in the developing mammalian lens is recognized, and previous data have shown that patterns of fiber cell differentiation are perturbed in mouse lens with constitutive activation of smoothened, a key signal transducer in the Shh pathway.¹⁶ However, there is a shortage of data about the Shh role during lens postnatal growth. Indeed, our work did not reveal grossly visible or histological abnormalities in the newborn or adult unirradiated *Ptch1*^{+/-} lens, except for spontaneous cataract development in 8.8% of mice. To explore Shh signaling involvement in lens postnatal growth, we measured Gli1 transcript levels as a readout of Shh pathway activation. Although at P2 we found expression of Gli1, with significantly higher levels in *Ptch1*^{+/-} mice compared to WT littermates, at P10 there was a sharp decrease of Gli1 expression, consistent with the reduction of number of proliferating cells in the germinative zone at this time of lens development. We also show that Gli1 expression was considerably up-regulated by irradiation and that increased Shh signaling correlates with proliferation of progenitor cells, suggesting that the Shh pathway could control mammalian lens regeneration after lens injury. Moreover, the large difference observed between Gli1 levels in exposed *Ptch1*^{+/-} compared to WT mice suggests a causal role for Gli1 in cataract. We note, in addition, that a different expression pattern was evident between Gli1 and Gli2, with Gli2 still highly expressed at P10.

Cross talk between Shh and TGF- β signaling in cancer has been recognized.³⁴ However, not much is known about its role in driving tissue fibrosis. TGF- β is required for embryonic eye development, driving, in particular, lens development and growth by the tight spatial and temporal regulation of lens cell proliferation and fiber cell differentiation.^{47–49} TGF- β also plays crucial roles during ocular fibrotic diseases, such as ASC development, in which LECs undergo EMT via TGF- β activation.^{33,42,50–52} Indeed, our data show that p-Smad3 expression increased both in *Ptch1*^{+/-} and WT mice, as reflected by 8.8% cataract incidence in WT mice irradiated at P2. However, TGF- β is also a potent inducer of Gli2, and consequently, of Gli1, independent of Shh/*Ptch1* signaling, and the cross talk between pathways mediated by Gli2 might increase significantly in mice with hyperactive Shh signaling.³⁴ Further descriptive and functional experiments will be required to prove or refute this hypothesis. Although this is something we intend to follow up, it is tempting to speculate that TGF- β /Smad signaling might contribute to the expression of Gli2, facilitating a self-amplifying Shh/TGF- β pathogenic loop that may promote and amplify the EMT process in the lens after radiation injury. Indeed, interaction between Shh and TGF- β signaling has been previously reported in human fibrotic lung disease,³⁵ and recent data have shown its role in mediating cyclosporine-enhanced gingival hyperplasia.³⁶

More important, a strong correlation was found between Shh deregulation, radiation damage, and expression of Nanog, which was greatly increased only in irradiated *Ptch1*^{+/-} mice. Although there has been little study on the

role of Nanog in cataractogenesis, a previous article investigating potential oncogenic functions of Nanog in transgenic mice expressing high levels of the NanogP8 homologue reported development of early cataract with high penetrance in lines expressing higher levels of the transgene.⁵³ It is presently unclear how high Nanog expression associates to enhanced cataract development, but unexpected phenotypes observed in K14-NanogP8 mice (eg, perinatal lethality, developmental defects, resistance to two-stage skin carcinogenesis, and wound-healing defects) suggested that high levels of NanogP8 may disrupt normal developmental programs and inhibit oncogenic processes by depleting stem cells. This implies that Nanog expression levels must be tightly controlled for proper development and response to injury. In addition, a recent report found that expression of matrix metalloproteinase-9, a direct target of Nanog,⁵⁴ is critical in TGF- β -induced ASC formation.⁵⁵ Although further studies will be needed to better elucidate Nanog's role in modulating cataractogenesis, there is evidence that Gli1 regulates Nanog expression in different systems.^{30,31} Moreover, TGF- β regulates Nanog and promotes EMT in many cancers^{56,57} and in non-cancer-related pathological conditions.⁵⁸ Therefore, in the interplay of Shh and TGF- β , Nanog might act as a critical player on which both signaling pathways converge in injured lens tissue (Figure 6).

Although Shh signaling is linked with regulation of injury repair and wound healing after tissue damage and with promotion of fibrosis in several tissues,^{51,59} its role in the pathogenesis of lens fibrosis is completely unknown. Results from the current study demonstrate, for the first time, a key role of Shh in spontaneous and radiation-induced cataract development, and suggest a cross talk between Shh and TGF- β converging on Nanog. We, in addition, provide a novel powerful animal model to investigate the mechanisms of cataract development. Further studies in this area will be needed because identification of the main molecular mechanisms involved in the induction of EMT and subsequent fibrotic alterations in lens cells may potentially help reduce cataractogenic effects after therapeutic radiation exposures, especially in survivors of childhood cancer who are at increased risk for ocular late effects, including cataract.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2014.09.019>.

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