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RESEARCH ARTICLE

Signaling Through FGF Receptor-2 Is Required for Lens Cell Survival and for Withdrawal From the Cell Cycle During Lens Fiber Cell Differentiation

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Fibroblast growth factors (FGFs) play important roles in many aspects of development, including lens development. The lens is derived from the surface ectoderm and consists of an anterior layer of epithelial cells and elongated, terminally differentiated fiber cells that form the bulk of the tissue. FGF signaling has been implicated in lens induction, proliferation, and differentiation. To address the role of FGFs in lens development, we inactivated *FGF receptor-2* (*Fgfr2*) using a Cre transgene that is expressed in all prospective lens cells from embryonic day 9.0. Inactivation of *Fgfr2* shows that signaling through this receptor is not required for lens induction or for the proliferation of lens epithelial cells. However, *Fgfr2* signaling is needed to drive lens fiber cells out of the cell cycle during their terminal differentiation. It also contributes to the normal elongation of primary lens fiber cells and to the survival of lens epithelial cells. ***Developmental Dynamics 233:516–527, 2005.*** **© 2005 Wiley-Liss, Inc.**

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INTRODUCTION

Signaling by members of the fibroblast growth factor (FGF) family plays an important role in many aspects of vertebrate development. Naturally occurring mutations in humans, targeted deletion of FGFs and their receptors in mice, and extensive studies in several other systems demonstrate that FGFs are required for early embryogenesis, regional specification of the brain, limb formation and morphogenesis, normal bone development, and other important developmental events (Amaya et al., 1991; Deng et al., 1994; Floss et al., 1997; Celli et al., 1998; Yu et al., 2003).

In mammals, there are 22 FGFs with distinct patterns of expression (Ornitz and Itoh, 2001). FGFs signal through cell surface receptors that are encoded by four genes with different ligand binding specificity. Alternative splicing of the transcripts that encode the FGF receptors creates further variations in ligand binding (Ornitz et al., 1996; Xu et al., 1999; Ornitz and Itoh, 2001). In addition, cell surface heparan sulfate proteoglycans can alter the strength and specificity of FGF signaling (Ornitz, 2000; Pellegrini, 2001). The diversity of ligands, receptors, and coreceptors complicates studies of FGF function in vivo.

The lens is an advantageous system in which to study FGF function. FGFs

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have been implicated in several aspects of lens development, and the lens is a relatively simple structure in which cell proliferation and differentiation occur in spatially defined compartments (Fig. 1). The anterior surface of the lens is covered with a layer of cuboidal epithelial cells. Throughout most of life, cell proliferation occurs preferentially in a subset of these epithelial cells, located in the periphery of the epithelium near the lens equator. After they divide, most of these epithelial cells withdraw from the cell cycle and move posteriorly to become terminally differentiated lens fiber cells. As fiber cells differentiate, they rapidly increase in length and volume and accumulate high levels of crystallins, the proteins that account for the transparency and high refractive index of the lens. After they complete the process of elongation, fiber cells partially fuse with their neighbors and degrade all membrane bound organelles (Bassnett, 2002; Shestopalov and Bassnett, 2003).

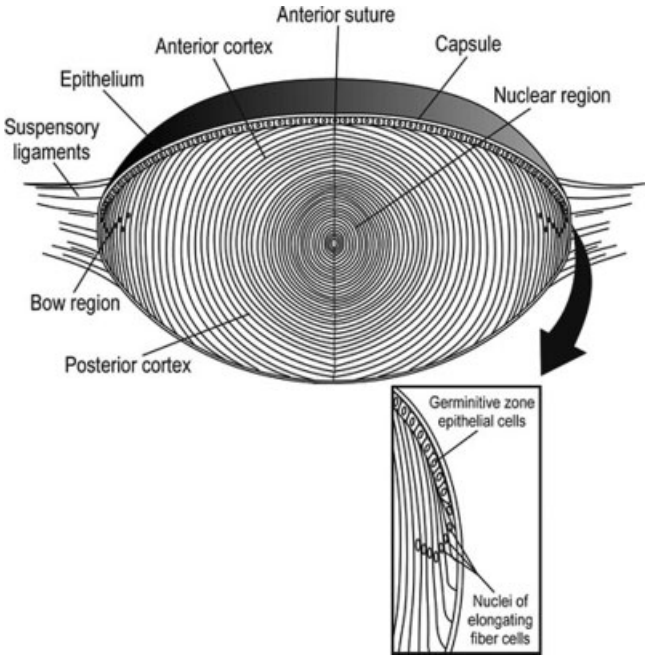


Fig. 1. Diagram of the lens. Lens epithelial cells are present at the anterior surface of the lens, nearest to the cornea. Cell proliferation occurs in a subset of epithelial cells in the "germinative zone" near the lens equator. After these cells divide, they become fiber cells (inset). Fiber cells cease dividing, elongate, and accumulate α-, β-, and γ-crystallins. Lens growth continues throughout life by the continual addition of fiber cells.

There are at least four FGF receptors expressed in the embryonic lens from three genes: *Fgfr1*, *Fgfr2b*, and *2c*, and *Fgfr3*. Previous studies have not detected the expression of *Fgfr4* in embryonic lenses, although this receptor can be detected at low levels in postnatal lens epithelial cells (our unpublished data). Mice lacking *Fgfr4* appear to have normal lenses (Weinstein et al., 1998). Figure 2 summarizes the relative distribution of the mRNAs encoding each of the FGF receptors that are known to be expressed in the embryonic lens (Matsuo, 1993; de Iongh et al., 1996, 1997). Each receptor is present to some degree in the lens epithelium, the site of cell proliferation, and at the lens equator, where fiber cell differentiation is initiated.

There are several lines of evidence that support a role for FGFs in lens growth and development. Treatment of cultured lens epithelial explants with FGF1 or FGF2 stimulates cell proliferation at lower doses and fiber cell differentiation at higher doses (Chamberlain and McAvoy, 1987, 1989; McAvoy and Chamberlain, 1989; Schulz et al., 1993; Le and Musil, 2001). Overexpression of FGF1, 3, 4, 7, 8, or 9 in the lens fiber cells of transgenic mice causes the neighboring epithelial cells to transform into fiber-like cells in vivo (Lovicu and Overbeek, 1998; Robinson et al., 1998).

Although experiments in which dominant-negative FGF receptors were overexpressed in the lens support a role for FGFs in lens fiber cell differentiation, these studies do not address whether Fgfr2 is required for this process. Fiber cell differentiation was disrupted in transgenic mice that use the αA-crystallin promoter to express a truncated form of *fibroblast growth factor receptor-1* (*Fgfr1*) in lens fiber cells (Chow et al., 1995; Robinson et al., 1995; Stolen and Griep, 2000). Because many different FGFs bind Fgfr1 and truncated receptors may heterodimerize with other FGF receptors to block signaling, these results have been interpreted as supporting a role for FGFs in fiber cell differentiation. However, the αA-crystallin promoter is expressed only after the onset of fiber cell differentiation, leaving open the question of whether FGF signaling is required to initiate fiber cell differentiation. In this regard, the overexpression of dominant-negative *Fgfr1* in lens epithelial cells and early fiber cells using the *Pax6* promoter or retroviral vectors did not prevent secondary fiber cell differentiation (Faber et al., 2001; Huang et al., 2003). In addition, overexpression of secreted, dimeric extracellular domains of *Fgfr1* did not inhibit fiber cell differentiation (Govindarajan and Overbeek, 2001). These results raise the possibility that the abnormalities in fiber cell differentiation seen when dominant-negative *Fgfr1* constructs are overexpressed in fiber cells might be due to a nonspecific mechanism. Finally, no lens or eye abnormalities have been reported in mice lacking FGF1 (Miller et al., 2000), FGF2 (Dono et al., 1998; Ortega et al., 1998), FGF1 and FGF2 (Miller et al., 2000), FGF3 (Mansour et al., 1993), FGF5 (Hebert et al., 1994), FGF6 (Floss et al., 1997), FGF7 (Guo et al., 1996), FGF14 (Wang et al., 2002), FGF17 (Xu et al., 2000), or FGF23 (Shimada et al., 2004). Therefore, although it is clear that FGFs can promote lens epithelial cell proliferation and fiber cell differentiation, it has been harder to discern the extent to which they normally perform these functions in vivo.

Tissue-specific deletion of FGF receptors using the Cre-*loxP* system (Gu et al., 1993) is a method to address the

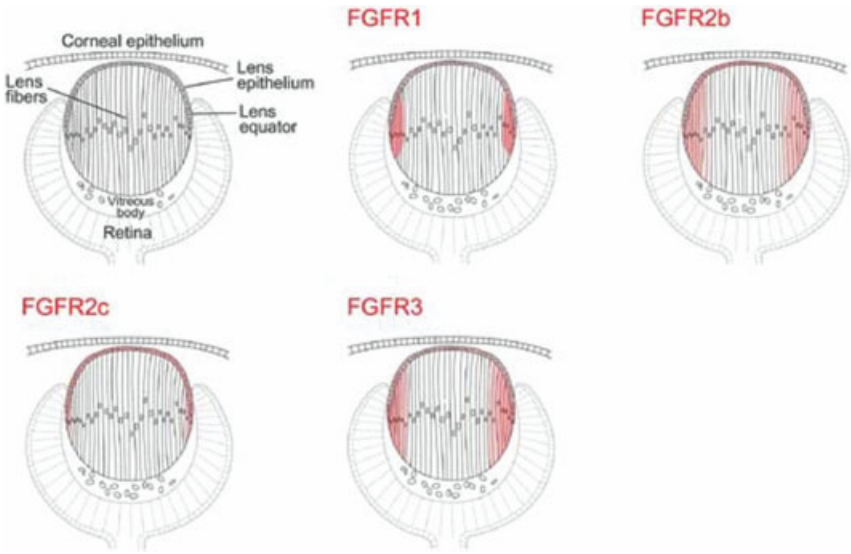


Fig. 2. Schematic diagram of fibroblast growth factor (FGF) receptor mRNA distributions during lens development. FGF receptors (FGFR) 1, 2, and 3 are expressed to some degree in the lens epithelium, where cell proliferation occurs, and at the equator, where fiber cell elongation is initiated.

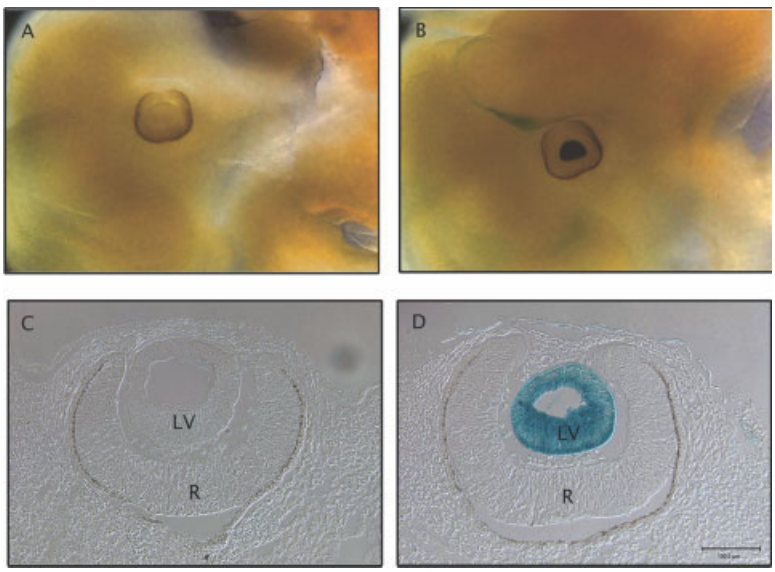


Fig. 3. In the genetic background used in these studies, Cre recombinase deletes a target gene in all lens cells by at least embryonic day 10.5. Cre-positive ROSA26 embryos were stained for the reporter β-galactosidase, which confirms the Cre-mediated deletion in the lens of targets flanked by loxP sites. **A–D:** Cre-negative (A,C) and Cre-positive (B,D) ROSA26R *Fgfr2*$^{loxP/loxP}$ littermates. B,D: Expression is seen in the lens of Cre-positive mice in whole-mount embryos (B) and in frozen sections of the eye (D). LV, lens vesicle; R, neural retina. Scale bar = 100 μm in D (applies to A–D).

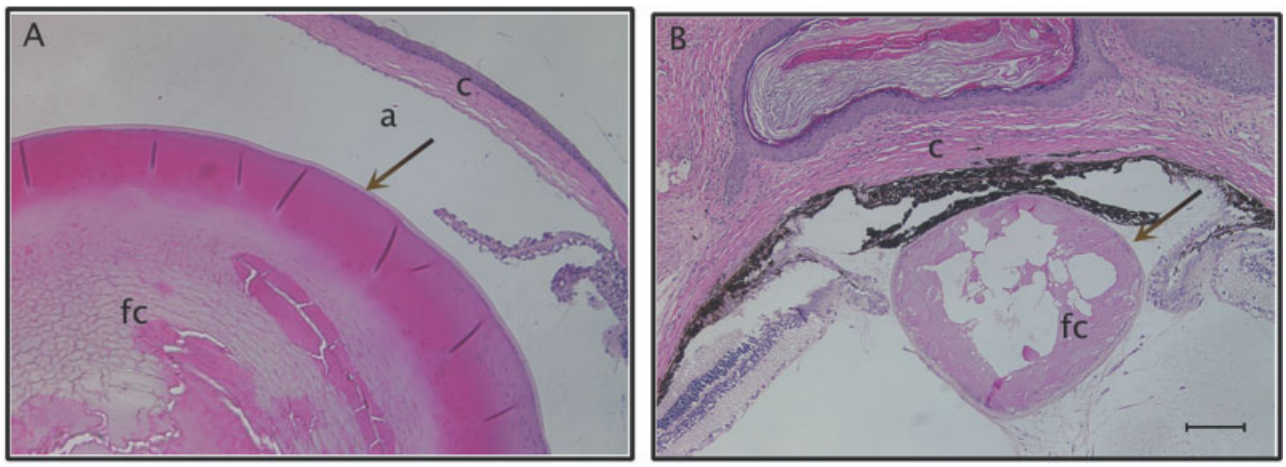


Fig. 4. *Fgfr2* signaling is essential for normal lens and eye morphology in adult mice. **A:** *Fgfr2*$^{loxP/loxP}$, Cre-negative mice have normal eyes. **B:** *Fgfr2*$^{loxP/loxP}$ Cre-positive mice have small lenses with disorganized, vesiculated fiber cells, and a paucity of epithelial cells. The anterior chamber in the Cre-positive animals is filled with pigmented cells and the corneal endothelium is absent. Abundant fibrotic tissue surrounds an epithelial vesicle that appears to be lined with keratinized corneal epithelial cells. Arrows point to the lens epithelia. a, anterior chamber; c, cornea; fc, fiber cells. Both images are at the same magnification. Scale bar = 100 μm in B (applies to A,B).

role of FGFs in lens formation, proliferation, and fiber cell differentiation. Previous studies of embryos with germ line deletion of *Fgfr3* or *Fgfr4*, or chimeric mouse embryos with deletion of *Fgfr1* (Zhao et al., manuscript submitted for publication) or *Fgfr2* (Li et al., 2001) did not report lens phenotypes. In the present experiments, we targeted floxed *Fgfr2* using a Cre transgene that can delete target genes in all lens cells beginning at embryonic day (E) 9.0 (Ashery-Padan et al., 2000). Our results, together with earlier reports, demonstrate that *Fgfr2* is not essential for lens formation, the initiation of fiber cell differentiation, or for normal levels of epithelial cell proliferation. However, lens fiber cells lacking *Fgfr2* failed to properly withdraw from the cell cycle, and the formation of the primary lens fiber cells was delayed. Lenses lacking *Fgfr2* also had markedly higher levels of apoptosis in epithelial and fiber cells and degenerated postnatally. These data show that signaling through *Fgfr2* contributes to lens fiber cell terminal differentiation and mediates an important survival signal in lens epithelial cells.

RESULTS

To address the role of members of the FGF family in lens fiber cell differentiation, our laboratories are using conditional and germline knockouts to delete critical domains of the genes for each of the FGF receptors that are expressed in the lens, singly and in combination. In this study, we examined the consequences of inactivating *Fgfr2* at the lens placode stage using the Cre/*loxP* system.

We used a line of transgenic mice in which the Pax6 regulatory region, including the P0 promoter, drives the expression of Cre recombinase in the cells that give rise to the lens and ocular surface epithelia (corneal, conjunctival, and eyelid epithelia) from E9.0 onward (Le-Cre; Ashery-Padan et al., 2000). Le-Cre mice were mated to mice carrying floxed alleles of *Fgfr2* (Yu et al., 2003). Heterozygous progeny from these matings were crossed to obtain mice that were $Fgfr2^{+/+}$, $Fgfr2^{loxP/+}$, and $Fgfr2^{loxP/loxP}$ with or without the expression of Cre recombinase. Previous studies showed that the Le-Cre construct deleted genomic sequences flanked by *loxP* sites in the lens placode by E9.5 and prevented lens formation in $Pax6^{loxP/LacZ}$ embryos (Ashery-Padan et al., 2000). Cre has also been shown to efficiently delete the critical exons of *Fgfr2* (Yu et al., 2003). We confirmed the recombination pattern by breeding Le-Cre–positive, $Fgfr2^{loxP/loxP}$ mice with ROSA26 Cre-reporter mice (Soriano, 1999). In $Fgfr2^{loxP/loxP}$; LeCre; ROSA26 embryos, all cells in the lens were positive for β-galactosidase by E10.5 (Fig. 3).

Adult Mice With Conditional Knockout of *Fgfr2*

Adult $Fgfr2^{loxP/loxP}$ mice without Cre had normal eye morphology, as did mice expressing Cre that were wild-type ($Fgfr2^{+/+}$) or heterozygous, ($Fgfr2^{+/loxP}$) (Fig. 4A). However, mice that expressed Cre and were homozygous for the targeted gene ($Fgfr2^{loxP/loxP}$) showed an abnormal eye phenotype in adult animals. The eye was small, and the eyelids were fused by abundant subepithelial fibrotic tissue. Cross-sections of eyes from adult mice showed that the lens was very small or, in some cases, absent. When the lens was present, the lens capsule appeared normal, the lenses had very few epithelial cells and the fiber cells were severely disorganized and vacuolated (Fig. 4B). These eyes had no anterior chamber or morphologically identifiable corneal endothelium. Instead, the small lenses were located close to the cornea, and the space that would normally have been the anterior chamber was filled with pigmented cells. The corneal epithelium appeared keratinized and was located in a cystic structure that was enclosed within the fibrotic tissue beneath the fused eyelids.

Eye Development After Targeting *Fgfr2* in the Lens and Ocular Surface Epithelia

At E17.5, the external appearance of Cre-positive, $Fgfr2^{+/+}$, and $Fgfr2^{+/loxP}$ animals was normal, with fused eyelids and eyes of normal size and shape (Fig. 5A,B,D,E). However, the eyes of Cre-positive, $Fgfr2^{loxP/loxP}$ animals had hypoplastic eyelids that were not fused and pupils that were irregularly shaped, instead of round (Fig. 5C). In rodent eyes, the pupil is often of abnormal shape when the lens is smaller than normal. Sections through these eyes confirmed that the Cre-positive, $Fgfr2^{loxP/loxP}$ lenses were smaller than those in the Cre-positive wild-type or $Fgfr2^{+/loxP}$ mice, although the epithelial cells of the null lenses appeared normal and the fiber cells were well organized (Fig. 5F). All lenses were surrounded by the fetal vasculature, although the capillaries of the tunica vasculosa lentis (TVL), the capillary network that surrounds the posterior half of the fetal lens, were often dilated in the Cre-positive, $Fgfr2^{loxP/loxP}$ eyes. Eyes with conditional knockout of *Fgfr2* in the lens frequently had abnormal folds in the neural retina (asterisk in Fig. 5F), a possible consequence of the small size of the lens, which normally fills most of the interior of the eye in rodents. Therefore, animals lacking *Fgfr2* are capable of forming small, normal appearing lenses. However, as these animals age, the lenses become progressively smaller, relative to the size of the eye, with disorganized and degenerating fiber cells.

As described previously in mice chimeric for *Fgfr2* (Li et al., 2001) or that carried homozygous deletion of the b isoform of *Fgfr2* (De Moerlooze et al., 2000), eyes from Cre-positive, $Fgfr2^{loxP/loxP}$ embryos showed defective eyelid formation and failure of eyelid closure. Because LeCre is expressed within and around the lens placode, the surface ectoderm cells that give rise to the eyelids also lack Fgfr2. At postnatal day 1 (P1), Cre-positive, $Fgfr2^{loxP/loxP}$ pups had open eyelids, small lenses with flattened epithelial cells and disorganized fiber cells (data not shown). Because the outer surface of the corneas appeared normal at birth, it is likely that the fused eyelids and degenerated corneal epithelia seen in adult animals are secondary to inflammation and scarring resulting from the inability of the eyelids to close after birth.

Lens Differentiation in the Absence of Signaling Through *Fgfr2*

At E12.5, Cre-positive, $Fgfr2^{loxP/loxP}$ lenses were already smaller than the

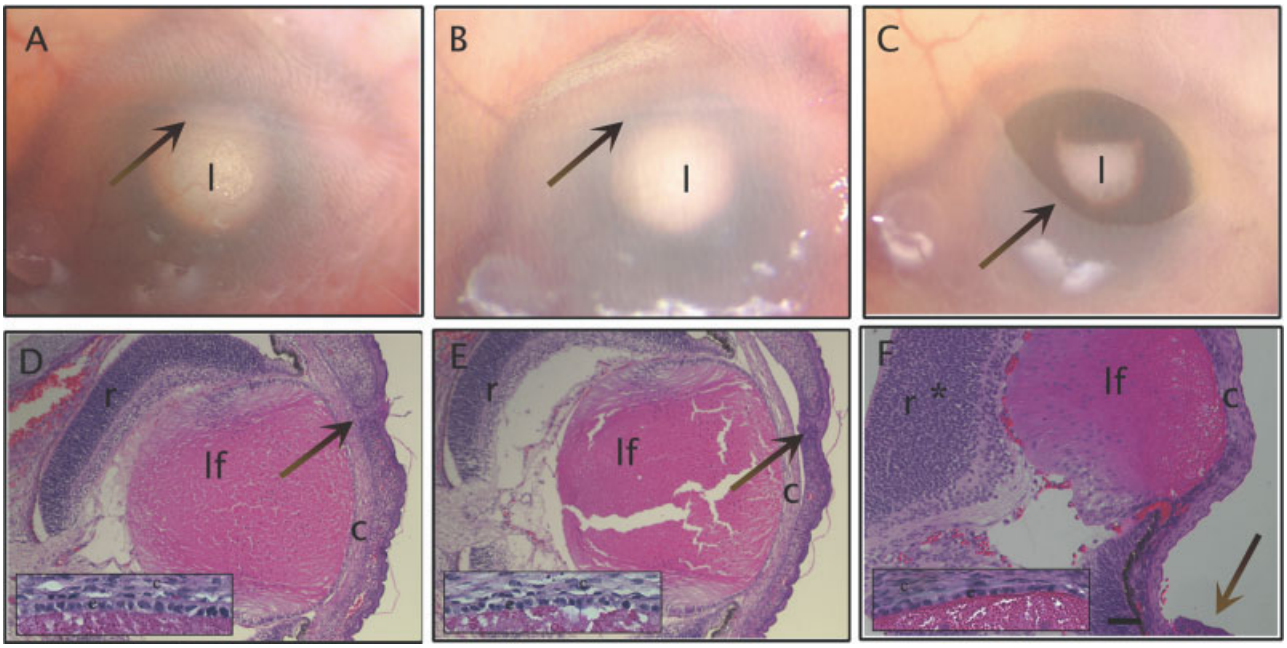
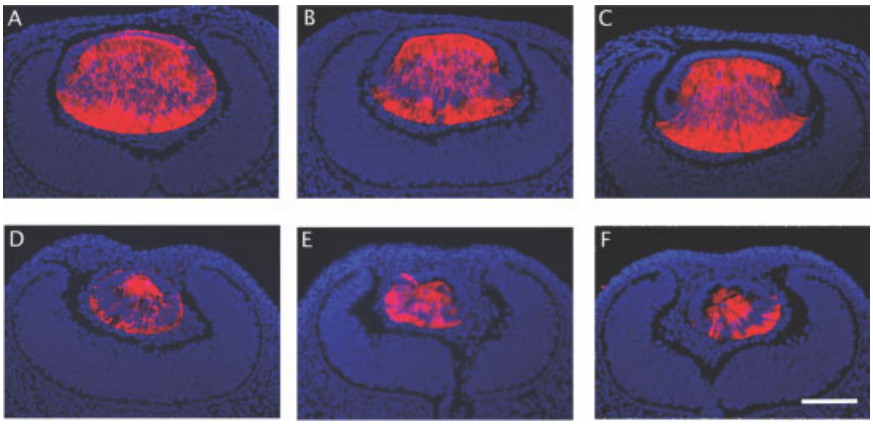


Fig. 5.



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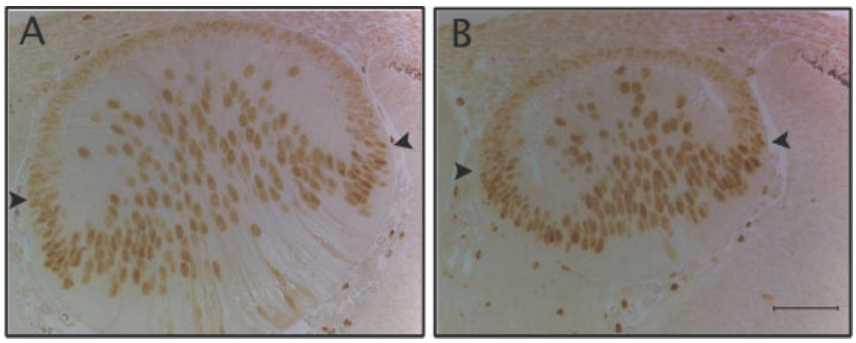

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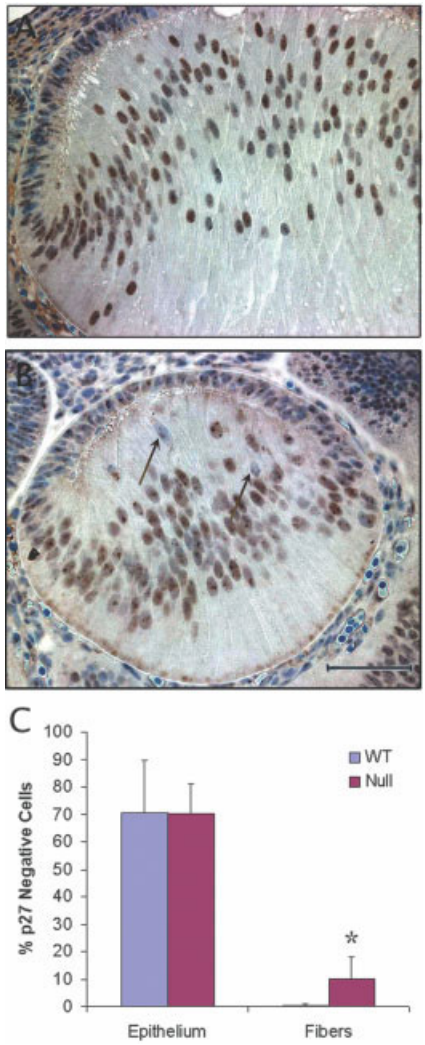

Fig. 8.

lenses of littermates that did not express Cre (Fig. 6). The morphology of the lens epithelial cells appeared normal, but the formation of the primary fiber cells was often delayed. Fgfr2 mRNA is first detected by in situ hybridization in rat lenses at E12 (de Iongh et al., 1997). At similar gestational ages, rat lenses are at a slightly earlier stage development than mouse lenses. Therefore, it is likely that expression of Fgfr2 begins in mouse lenses at or just before the beginning of fiber cell elongation, although this conclusion remains to be determined.

As part of their differentiation, lens cells produce abundant cytoplasmic proteins, called crystallins. α-Crystallin is normally expressed in both lens epithelial and fiber cells, whereas members of the β- and γ-crystallin families are expressed only in the fiber cells of mammalian embryos. Figure 6 shows that α-, β-, and γ-crystallins accumulated in the appropriate compartments in wild-type and null embryos. Crystallin gene expression depends on the transcription factor c-Maf (Kawauchi et al., 1999; Kim et al., 1999). Consistent with the normal expression of crystallins, we detected no change in c-Maf protein expression or nuclear localization in the null lenses when compared with wild-type littermates (Fig. 7). These results suggest that the program of gene expression that is characteristic of lens cell differentiation can occur without signaling through *Fgfr2*.

Just before they differentiate into lens fiber cells, epithelial cells near the lens equator withdraw from the cell cycle. This process depends on the expression of the cyclin-dependent kinase inhibitors $p27^{kip1}$ and $p57^{kip2}$, because in mice that lack these genes, fiber cells continue to proliferate and eventually undergo apoptosis (Zhang et al., 1998). Antibodies against $p27^{kip1}$ and $p57^{kip2}$ showed that, in Cre-positive lenses, these proteins were expressed in the appropriate locations (Fig. 8A,B for $P27^{kip1}$; data not shown for $P57^{kip2}$). There were also similar numbers of $p27^{kip1}$-negative epithelial cells in Cre-positive and Cre-negative embryos (70.2% vs. 70.3%, respectively; $P = 0.99$; Fig. 8C). However, in *Fgfr2* null lenses, 11% of fiber cells failed to stain for $p27^{kip1}$ compared with only 0.47% in the wild-type embryos ($P < 0.03$, Fig. 8C). A subset of fiber cells also failed to express $p57^{kip2}$ (data not shown). Decreased expression of these kinase inhibitor proteins suggests that withdrawal from the cell cycle is incomplete in the absence of signaling through *Fgfr2*.

Cell Proliferation and Death in *Fgfr2* Conditional Knockout Lenses

A previous study found that the overexpression of a dominant-negative form of *Fgfr1* reduced cell proliferation during early lens development (Faber et al., 2001). To determine whether the small size of the *Fgfr2* null lenses was due to reduced cell proliferation, we measured the fraction of epithelial cells that incorporated 5′-bromodeoxyuridine (BrdU) during 1 hr of labeling in Cre-positive or Cre-negative $Fgfr2^{loxP/loxP}$ embryos at E12.5 (Fig. 9). Lens epithelial cells of both genotypes incorporated BrdU to a similar extent: 39% in Cre-negative and 37% in Cre-positive lenses ($P = 0.55$). Therefore, at this stage of development, signaling through *Fgfr2* is not required to maintain epithelial cell proliferation (Fig. 9C). In contrast to mice that had an intact *Fgfr2*, Cre-positive, $Fgfr2^{loxP/loxP}$ lenses had a substantial number of BrdU-labeled nuclei in their fiber cells at E12.5 (arrows in Fig. 9B). The BrdU labeling index of Cre-negative lens fiber cells was 0.13%, whereas 3.5% of Cre-positive fiber cells were BrdU-positive ($P < 2 \times 10^{-5}$; Fig. 9C). This observation provides further evidence that lack of signaling through *Fgfr2* is as-

Fig. 5. Mice lacking *Fgfr2* form small lenses of normal morphology. A,B,D,E: At embryonic day 17.5, Cre-positive $Fgfr2^{+/+}$ (A,D) and $Fgfr2^{+/loxP}$ mice (B,E) had normal eyelids and lenses. **C,F:** The eyelids of Cre-positive $Fgfr2^{loxP/loxP}$ mice were not completely closed, and these eyes had smaller lenses than their wild-type and heterozygous littermates. Despite their smaller size, the lenses of Cre-positive $Fgfr2^{loxP/loxP}$ mice had epithelial and fiber cells that appeared similar to their wild-type and heterozygous littermates. The fetal vasculature was present around the lens in animals of all three genotypes, although these vessels were dilated in mice lacking *Fgfr2*. C,F: Cre-positive, $Fgfr2^{loxP/loxP}$ mice had asymmetric pupils (C) and folded retinas (F), presumably due to the smaller size of the lens. The arrows point to the eyelids. The insets in D, E, and F are higher magnification views of the lens epithelium. c, cornea; l, lens; lf, lens fibers; e, lens epithelia; r, retina; *, retinal fold. Scale bar = 100 μm in F (applies to A–F).

Fig. 6. *Fgfr2*-deficient lenses have normal crystallin expression at embryonic day (E) 12.5. **A–F:** Sections from Cre-negative $Fgfr2^{loxP/loxP}$ embryos are shown in A, B, and C and from Cre-positive $Fgfr2^{loxP/loxP}$ littermate embryos in D, E, and F. Cre-positive embryos have smaller lenses at E12.5 than their Cre-negative littermates. A,D: α-Crystallin is detectable in epithelial and fiber cells in Cre-positive (A) and Cre-negative (D) lenses. B,C,E,F: β-crystallins (B,E) and γ-crystallins (C, F) are present in the fiber cells of lenses of both genotypes. Antibody staining is in red, Hoechst-stained nuclei are in blue. Scale bar = 100 μm in F (applies to A–F).

Fig. 7. *Fgfr2*-deficient lenses have normal c-Maf protein levels and distribution at embryonic day (E) 12.5. The figure shows $Fgfr2^{loxP/loxP}$ E12.5 littermates. **A,B:** There was no apparent difference in c-Maf staining in Cre-negative (A) and Cre-positive (B) lenses. Antibodies to c-Maf weakly stained the nuclei of epithelial cells. Staining increased greatly as cells near the lens equator withdrew from the cell cycle. The point of transition between epithelial and fiber cells is marked by arrowheads. Scale bar = 50 μm in B (applies to A,B).

Fig. 8. A portion of the fiber cells in *Fgfr2*-deficient lenses fail to withdraw from the cell cycle. **A,B:** Cre-negative $Fgfr2^{loxP/loxP}$ (A) and Cre-positive $Fgfr2^{loxP/loxP}$ (B) lenses from E12.5 littermate embryos were stained with an antibody against $p27^{kip1}$. The overall staining patterns were similar in lenses of both genotypes. **C:** Most epithelial cell nuclei were not stained with the antibody, consistent with the persistence of cell proliferation in this region of the lens, whereas most of the nuclei of fiber cells were stained. A greater number of fiber cell nuclei were negative for $p27^{kip1}$ in Cre-positive embryos, than in Cre-negative embryos, suggesting that these cells failed to withdraw from the cell cycle. Arrows indicate $p27^{kip1}$-negative nuclei in the fiber cells of the Cre-positive lens. The asterisk indicates that the increase in $p27^{kip1}$-negative cells is statistically significant in the Cre-positive lenses ($P < 0.005$). WT, wild-type. Scale bar = 50 μm in B (applies to A,B).

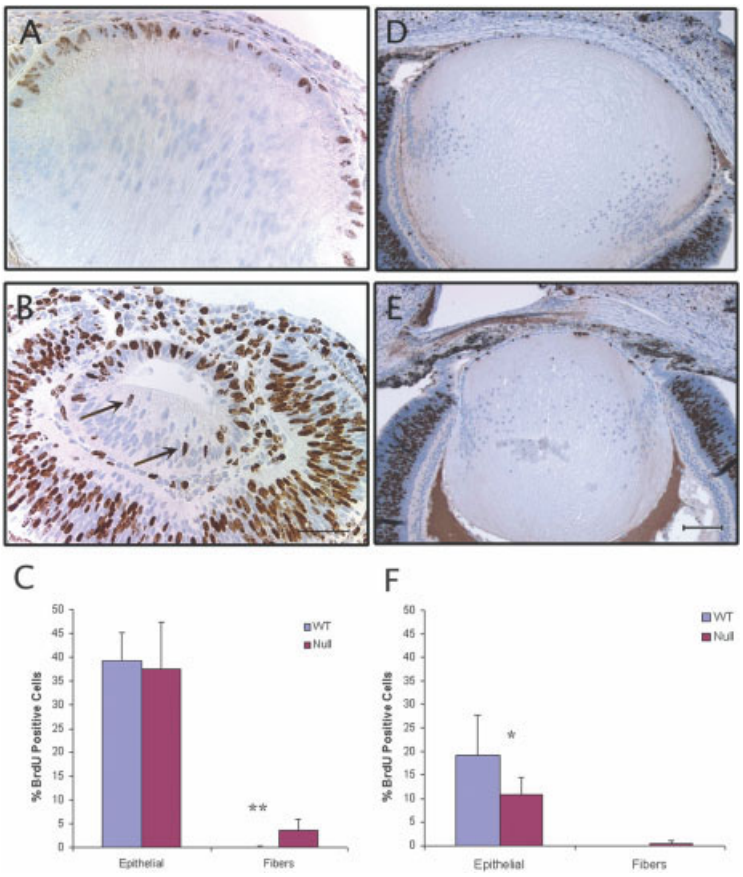

Fig. 9. Wild-type and *Fgfr2*-deficient lenses have similar rates of cell proliferation. **A,B:** Many epithelial cell nuclei were bromodeoxyuridine (BrdU) -positive in Cre-negative *Fgfr2*$^{loxP/loxP}$ (A) and Cre-positive *Fgfr2*$^{loxP/loxP}$ (B) embryonic day (E) 12.5 embryos. The Cre-positive lens was smaller and showed BrdU incorporation in the nuclei of a small number of fiber cells (arrows in B). **C:** There was no significant difference in the percentage of BrdU-positive nuclei in the epithelia of Cre-negative and Cre-positive lenses. However, a significantly greater number of fiber cell nuclei were positive for BrdU in Cre-positive than in Cre-negative lenses. **D,E:** At postnatal day 1, the BrdU labeling index was lower in both Cre-negative (D) and Cre-positive lenses (E), than in the lenses of E12.5 embryos. **F:** At this stage, the BrdU labeling index of epithelial cell nuclei was significantly higher in the Cre-negative than the Cre-positive lenses. Although a few BrdU-positive fiber cell nuclei were detected in Cre-positive lens fiber cells, the number was not significantly different from zero. The fiber cells appeared more disorganized in the Cre-positive lenses at this stage. *$P < 0.05$, **$P < 10^{-4}$. WT, wild-type. Scale bars = 50 μm in B (applies to A,B), 100 μm in E (applies to D,E).

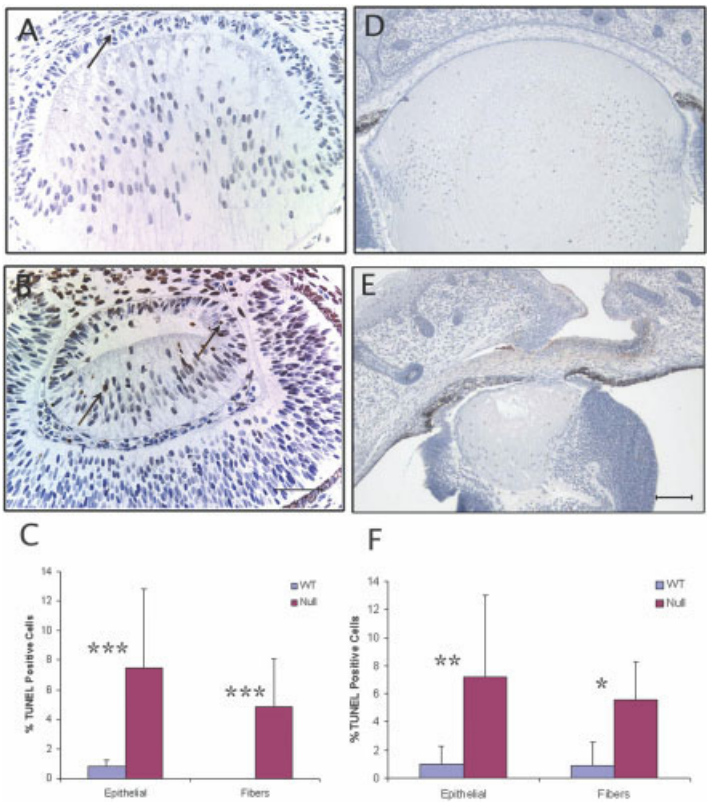

Fig. 10. Increased cell death in *Fgfr2*-deficient fiber and epithelial cells. **A,B:** Cre-positive embryonic day (E) 12.5 embryos had a higher percentage of terminal deoxynucleotidyl transferase–mediated deoxyuridinetriphosphate nick end-labeling (TUNEL) -stained epithelial and fiber cells (B) than their Cre-negative littermates (A). **C:** The apoptotic index (% TUNEL-positive nuclei) for Cre-negative and Cre-positive epithelial and fiber cells. **D,E:** At postnatal day (P) 1, there were also more TUNEL-stained nuclei in Cre-positive (E) than in Cre-negative lenses (D). **F:** The apoptotic index of Cre-positive and Cre-negative lenses was significantly different at P1 in lens epithelial and fiber cells. Scale bar = 50 μm in B; 100 μm in E. *$P < 0.05$; **$P < 0.01$; ***$P < 10^{-4}$.

sociated with the failure of lens fiber cells to withdraw from the cell cycle.

By postnatal day 1 (P1), there was an even greater difference in the size of *Fgfr2* null and wild-type lenses than at E12.5 and the fiber cells had become noticeably disorganized (Fig. 9D–F). Compared with E12.5 embryos, there was a decrease in BrdU incorporation in both Cre-negative and Cre-positive animals at P1, reflecting the slower growth rate of the older lenses. However, there was also a small but statistically significant decrease in the BrdU labeling index in the epithelial cells of the *Fgfr2* null mice at P1, compared with their wild-type littermates ($P = 0.04$; Fig. 9F). Therefore, some of the decreased size in the older *Fgfr2* null lenses is due to decreased epithelial cell proliferation. BrdU-positive fiber cells were present in the *Fgfr2* null animals at P1 (0.4%) but were not detected in fiber cells in wild-type lenses at this stage. Because BrdU-positive fiber cells were not found in most $Fgfr2^{loxP/loxP}$, Cre-positive lenses, the small number that was detected was not significantly different from zero.

Because *Fgfr2* null lenses were consistently smaller than wild-type, but showed indistinguishable rates of cell proliferation early during embryonic development, we used TUNEL staining to determine whether the smaller lens size could be explained by differences in the rates of cell death (Fig. 10). *Fgfr2* null lenses consistently showed higher numbers of TUNEL-positive epithelial and fiber cells at E12.5. Lens epithelia from Cre-negative lenses contained 0.8% TUNEL-positive nuclei, whereas epithelia from Cre-positive lenses had an apoptotic index of 7.5% (Fig. 10C; $P < 9 \times 10^{-5}$). Embryos with lenses that did not express Cre had no detectable apoptotic fiber cells, but 4.8% of the fiber cells in the $Fgfr2^{loxP/loxP}$, Cre-positive lenses were TUNEL-positive ($P < 1 \times 10^{-5}$; Fig. 10C).

The increased apoptotic index in the *Fgfr2* null lenses persisted at P1. Cre-positive $Fgfr2^{loxP/loxP}$ embryos had 7.2% TUNEL-positive epithelial cells, whereas epithelial cells in wild-type embryos had an apoptotic index of only 1.0% ($P < 0.05$). Similarly, *Fgfr2* null fibers cells had an apoptotic index of 5.4%, compared with 0.91% in the Cre-negative lenses ($P < 0.007$) (Fig. 10F).

DISCUSSION

FGF signaling has been implicated in lens induction (Faber et al., 2001) and the differentiation of lens fiber cells (Lang, 1999). However, the FGFs that normally contribute to lens formation and fiber cell differentiation remain to be identified (Lovicu and Overbeek, 1998; Govindarajan and Overbeek, 2001), and the functions of the different FGF receptors that are expressed in the lens are not known.

To better define the role of FGF signaling in lens development and the specific role of *Fgfr2* in this process, we used the Cre/LoxP system to target the gene for *Fgfr2* in the lens and ocular surface epithelia. Eyes of animals that expressed Cre, but were wild-type or heterozygous for the floxed allele of *Fgfr2* appeared normal, indicating that expression of Cre had no apparent effect on eye development. In lenses that expressed Cre and were homozygous for floxed *Fgfr2*, lenses formed and markers of fiber cell differentiation were expressed at the correct stages and in the correct locations, although the lenses were smaller than normal. This decrease in size was mostly due to increased apoptosis in epithelial and fiber cells, with little change in the rate of cell proliferation. In addition, the elongation of the primary fiber cells was delayed, possibly contributing to the smaller size of the lenses.

The absence of *Fgfr2* was associated with defects in the formation of the tissues that border the anterior chamber of the eye: the iris epithelia, iris stroma, corneal endothelium, and anterior chamber angle, as well as abnormalities in the TVL, the capillary network that surrounds the posterior of the lens. Because the Cre transgene is expressed in the lens and corneal epithelium but not in any of the tissues that border the anterior chamber or in the TVL (Ashery-Padan et al., 2000), the abnormal development of these tissues was presumably due to abnormalities in signaling by the lens or the corneal epithelium. Previous studies have shown that signals from the lens epithelium are required for the normal development of the tissues that border the anterior chamber (Beebe and Coats, 2000). It is possible that a comparison of the mRNAs expressed in the epithelial cells of wild-type and *Fgfr2* null lenses could provide clues about the nature of these signals.

Function of Different FGF Receptors in Lens Development

As described in the introduction, there are at least four FGF receptors expressed in the embryonic lens. However, it is not known whether each type of FGF receptor generates distinct signals that affect lens cells in unique ways, or whether all types of FGF receptors generate the same signal, with the sum of these signals contributing to lens cell proliferation, survival, and terminal differentiation.

In other tissues, FGF receptors may have distinct functions. The level of *Cbfa1*, a transcription factor that is important in osteoblast differentiation, can be directly regulated by *Fgfr1* (Zhou et al., 2000), but levels are not altered in the absence of *Fgfr2* (Yu et al., 2003). At the same time, unlike *Fgfr1*, signaling through *Fgfr2* regulates the proliferation of osteoprogenitor cells and the anabolic functions of mature osteoblasts (Yu et al., 2003). The selective activation of *Fgfr1* in murine prostate and human salivary tumor cells leads to rapid tumor growth due to increased proliferation, whereas activation of *Fgfr2* inhibits tumor growth, apparently by inhibiting the activity of the ERK/MAP kinase pathway (Zhang et al., 2001; Freeman et al., 2003). Activation of *Fgfr1* or *Fgfr2* also leads to different patterns of gene expression in prostate tumor cells (Freeman et al., 2003). Targeted deletion of combinations of FGF receptors in the lens may reveal more about the specificity of FGF receptor signaling.

FGF Signaling in Lens Induction

The results of previous studies showed that lens formation occurs normally in mice lacking *Fgfr1* (unpublished results from our laboratory and Zhao et al., manuscript submitted for publication), *Fgfr2* (Li et al., 2001),

or *Fgfr3* (Ornitz et al., 1996). These results argue that no single FGF receptor is required for lens formation.

However, it is likely that FGF signaling contributes to lens induction, because treatment of embryos with an inhibitor of the FGF receptor tyrosine kinase or overexpression of truncated *Fgfr1* interfered with (but did not prevent) lens formation (Faber et al., 2001). Because both treatments may block multiple FGF receptors, it appears that more than one FGF receptor normally contributes to lens formation and that the removal of any single receptor is compensated for by the remaining receptors. Therefore, in the case of lens induction, FGF receptors appear to have overlapping functions.

FGF Signaling in Lens Cell Proliferation

In the present study, the decreased size of embryonic lenses lacking *Fgfr2* was associated with a significant increase in cell death, but with no observable change in the BrdU labeling index at E12.5. This result was surprising, because the predominant effect of FGF signaling in most cultured cells is to promote proliferation. These results contrast with those obtained when a truncated form of *Fgfr1* was overexpressed during early lens formation (Faber et al., 2001). In this study, the smaller lenses that resulted were attributed to a modest decrease in the BrdU labeling index (Faber et al., 2001). Some of these transgenic lenses eventually degenerated. The authors stated that there was no evidence of increased cell death, although no tests were performed to detect apoptotic or necrotic cells (Faber et al., 2001). The truncated FGF receptor construct used by Faber et al. is believed to inhibit signaling through all FGF receptors (Amaya et al., 1991). Therefore, it is possible that inhibiting the function of multiple FGF receptors results in a phenotype that differs from that seen when only *Fgfr2* is deleted. Previous in vitro studies have shown that approximately 10 times less FGF is needed to stimulate lens cell proliferation than to promote fiber cell differentiation (McAvoy and Chamberlain, 1989; Schulz et al., 1993). Therefore, removal of *Fgfr2* may have left sufficient numbers of FGF receptors to support normal levels of lens cell proliferation. Deletion of combinations of FGF receptors in the lens is likely to clarify the role of FGF signaling in lens cell proliferation.

FGF Signaling Provides a Survival Signal for Lens Epithelial Cells

Our data suggest that signaling through *Fgfr2* contributes to the survival of lens epithelial and fiber cells. Fiber cell death may be a result of failure to properly withdraw from the cell cycle. However, this explanation does not apply to the epithelial cells, which do not undergo terminal differentiation and in which deletion of *Fgfr2* did not alter the percentage of cells stained for BrdU or $p27^{kip1}$. A previous report found increased epithelial cell apoptosis after extensive degeneration of lens fiber cells (Stolen and Griep, 2000). However, we observed increased apoptosis in lenses with relatively normal epithelial and fiber cell architecture. Therefore, signaling through *Fgfr2* appears to account for two different survival signals during lens development.

It may be significant that removal of *Fgfr2* caused increased epithelial cell death under conditions in which cell proliferation was not reduced. Reduced FGF signaling also leads to increased cell death during the limb and brain development (Sun et al., 2002; Chi et al., 2003). These observations are consistent with the view that cell viability often depends on signals received from nearby cells (Raff et al., 1994). Our data show that reducing signaling by a known mitogen can compromise cell viability without affecting cell proliferation.

FGF Signaling in Lens Fiber Cell Differentiation

Our studies revealed an important function for *Fgfr2* in fiber cell differentiation. In contrast to normal fiber cells, fiber cells lacking *Fgfr2* had reduced numbers of $p27^{kip1}$- and $P57^{kip2}$-labeled cells and showed appreciable numbers of BrdU-labeled cells. These observations indicate that signaling through *Fgfr2* is important for complete withdrawal of fiber cells from the cell cycle, an essential characteristic of their differentiation.

That both the b and c splice variants of *Fgfr2* are expressed in the lens complicates the process of identifying the FGFs that contribute to the terminal differentiation of lens fiber cells, because each isoform may be activated by different FGFs (Ornitz et al., 1996). Mice lacking the *Fgfr2b* isoform die at birth due to agenesis of the lungs (De Moerlooze et al., 2000). These animals have no eyelids, a finding that is consistent with the phenotype of mice lacking both isoforms of *Fgfr2* (the present study and Li et al., 2001) and the preferential expression of the b isoform in epithelial tissues. However, mice lacking *Fgfr2b* have eyes that otherwise appear to be in normal proportion to their smaller body size (De Moerlooze et al., 2000). This observation does not suggest significant lens fiber cell defects, which, in rodents, are usually associated with proportionally smaller lens and eye size. Mice lacking the c isoform of *Fgfr2* are viable and fertile, with misshapen heads and "slightly bulging" eyes (Eswarakumar et al., 2002). This eye phenotype is consistent with the cranial abnormalities in these mice and, again, suggests normal lens development. Therefore, either isoform of *Fgfr2* appears to be sufficient for normal lens development and fiber cell differentiation, although more detailed examination of the isoform-specific knockout animals would be required to confirm this impression. If either isoform of *Fgfr2* is sufficient for normal lens formation, it is likely that multiple FGFs contribute to terminal fiber cell differentiation, a conclusion that is in accord with other studies of FGF signaling in lens development (Govindarajan and Overbeek, 2001).

EXPERIMENTAL PROCEDURES

Animals

Mice in which the Pax6 P0 promoter drives the expression of Cre recombinase in the cornea, lens, and endocrine pancreas from E9.0 onward (Le-Cre; Ashery-Padan) were mated to mice that had *loxP* sites inserted in the introns downstream of exon 7 and

exon 10 of the *Fgfr2* gene (Yu et al., 2003). The heterozygous progeny from these matings were crossed to obtain mice that were Cre-positive or Cre-negative and $Fgfr2^{+/+}$, $Fgfr2^{+/loxP}$, and $Fgfr2^{loxP/loxP}$. Mice were genotyped using polymerase chain reaction with primers for Cre (Ashery-Padan et al., 2000) and *Fgfr2* (Yu et al., 2003). We found that mice with two copies of the Cre transgene sometimes have a lens phenotype, irrespective of the *Fgfr2* genotype (data not shown). Therefore, all matings were performed between Cre-positive and Cre-negative animals in a manner that ensured that only one copy of the transgene was present. In addition, Cre-positive $Fgfr2^{+/+}$ and $Fgfr2^{+/loxP}$ animals were examined to assure that they did not have a phenotype.

β-Galactosidase Staining

E10.5 whole embryos were fixed for 30 min at room temperature in 0.1 M phosphate buffer, 2% paraformaldehyde, and 0.2% glutaraldehyde then given three washes of 10 min each in 0.01% sodium deoxycholate, 0.02% Ipegal, 2 mM $MgCl_2$, and 0.1 M phosphate buffer, pH 7.3. Embryos were incubated overnight at 37°C, in 0.1 M phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Ipegal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml X-gal, then at 4°C for 48 hr in the same solution, and then rinsed three times in phosphate buffered saline (PBS) and one time in 3% dimethyl sulfoxide/PBS. Frozen sections were photographed with a Spot digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) attached to an Olympus BX60 microscope (Olympus America, Inc., Melville, NY). Whole embryos were photographed by using a Spot digital camera attached to a Zeiss Stemi 2000-C dissecting microscope (Carl Zeiss, Thornwood, NY).

Immunohistochemistry

Mouse lenses or heads from embryos were fixed in 10% buffered formalin overnight at room temperature, embedded in paraffin, and sectioned. For morphological studies, the slides were stained with hematoxylin and eosin. Antibody staining was performed on deparaffinized sections that were rehydrated in series of ethanols of decreasing concentration and then in PBS. Endogenous peroxidase activity was inactivated with 3% H_2O_2 for 30 min. Antigen retrieval was performed by treating the sections with 0.01 M citrate buffer, pH 6.0 (Zymed, South San Francisco, CA) and placing them in a Decloaking Chamber (Biocare Medical, Walnut Creek, CA) for 3 min. After blocking with 20% inactivated normal donkey serum for 30 min at room temperature, the slides were incubated with antibodies to c-Maf (1:250, Santa Cruz Biotechnology, Santa Cruz, CA), or p57 (1:200, Santa Cruz) overnight at 4°C. We titrated the p27 antibody (Santa Cruz) to determine the dilution that gave the best discrimination between stained and unstained nuclei (1:30,000 overnight at 4°C). For color development, we used a biotinylated secondary antibody and the anti-rabbit Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) for 1 hr at room temperature. Slides were treated with diaminobenzidine (DAB; Vector Laboratories), washed with PBS, and counterstained with hematoxylin (Vector Laboratories). The percentage of cells that were not labeled by antibodies to p27 was determined by counting labeled and unlabeled cells in sections from 7 to 10 lenses. For crystallin staining, deparaffinized sections were blocked with 0.5% bovine serum albumin and 1% Triton X-100 for 30 min at room temperatures. The sections were incubated with antibodies to α-crystallins, β-crystallins (1:3,000 dilution), or γ-crystallins (1:5,000 dilution; provided by Dr. J. Samuel Zigler Jr., National Eye Institute, Bethesda, MD) at 4°C overnight. After brief washes, the slides were incubated with Cy2 or Cy3-labeled anti-rabbit antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) for 1 hr at room temperature. The sections were then counterstained with 4′,6-diamidine-2-phenylidole-dihydrochloride (DAPI; Vector Laboratories).

Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridinetriphosphate nick end-labeling (TUNEL) labeling was done with an Apotag kit (Intergen Co., Purchase, NY). Deparaffinized slides were treated with 3% H_2O_2 for 30 min, followed by proteinase K treatment (20 μg/ml) for 15 min. Slides were incubated with TdT enzyme in equilibration buffer for 1 hr at 37°C. The reaction was terminated with stop/wash buffer for 10 min at room temperature. Anti–digoxigenin-peroxidase conjugate was added for 30 min at room temperature, followed by DAB treatment. Slides were counterstained with hematoxylin images recorded as described above, and the percentage of TUNEL-positive cells was determined.

For BrdU staining, pregnant or neonatal mice were injected with 50 mg/kg of body weight of 10 mM BrdU (Roche, Indianapolis, IN) and 1 mM 5-fluoro-5′-deoxyuridine (Sigma, St. Louis, MO) 1 hr before killing. A monoclonal anti-BrdU antibody (1:250, Dako, Carpinteria, CA) was used with a Vectastain Elite Mouse IgG ABC kit as described above. Sections were counterstained with hematoxylin and images were recorded as described above. The number of labeled and unlabeled cells was counted to determine the BrdU labeling index.

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