Deficient FGF signaling causes optic nerve dysgenesis and ocular coloboma

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SUMMARY

FGF signaling plays a pivotal role in eye development. Previous studies using in vitro chick models and systemic zebrafish mutants have suggested that FGF signaling is required for the patterning and specification of the optic vesicle, but due to a lack of genetic models, its role in mammalian retinal development remains elusive. In this study, we show that specific deletion of Fgf11 and Fgf12 in the optic vesicle disrupts ERK signaling, which results in optic disc and nerve dysgenesis and, ultimately, ocular coloboma. Defective FGF signaling does not abrogate Shh or BMP signaling, nor does it affect axial patterning of the optic vesicle. Instead, FGF signaling regulates Mitf and Pax2 in coordinating the closure of the optic fissure and optic disc specification, which is necessary for the outgrowth of the optic nerve. Genetic evidence further supports that the formation of an Frs2α-Shp2 complex and its recruitment to FGF receptors are crucial for downstream ERK signaling in this process, whereas constitutively active Ras signaling can rescue ocular coloboma in the FGF signaling mutants. Our results thus reveal a previously unappreciated role of FGF-Frs2α-Shp2-Ras-ERK signaling axis in preventing ocular coloboma. These findings suggest that components of FGF signaling pathway may be novel targets in the diagnosis of and the therapeutic interventions for congenital ocular anomalies.

KEY WORDS: FGF, Frs2α, Shp2, Ras, Coloboma, Optic fissure, Optic disc, Optic nerve, Pax2, Mitf, Mouse

INTRODUCTION

Eye development follows an elaborate sequence of morphogenetic events, which are highly conserved between humans and mice. At mouse embryonic day 9.5 (E9.5) when the proximal optic vesicle connected to the diencephalon forms the optic stalk (OS), the distal optic vesicle contacting the head surface ectoderm folds into two layers: an outer layer of presumptive retinal pigmented epithelium (RPE) and an inner layer of developing neural retina (NR) (Bharti et al., 2006; Fuhrmann, 2010). This remodeling of optic vesicle into a cup-like structure at E10.5 leaves a transient groove opening at the ventral side of the eye, termed the optic fissure (OF), which will be progressively sealed from the proximal to the distal end through fusion of the lip areas of the ventral optic stalk and neural retina until E12.5 (Silver and Robb, 1979; Chang et al., 2006; Morcillo et al., 2006). The interface between optic stalk and neural retina, however, will be specified as optic disc (OD), a unique ocular structure where blood vessels enter the eye. Moreover, although optic disc is immediately adjacent to neuronal progenitor cells in neural retina, it consists exclusively of astrocyte progenitor cells (APCs), which express multiple axon guidance molecules. These extracellular cues direct the fasciculated axons of retinal ganglion cells to exit the eye at the optic disc as the optic nerve (ON), which connects the eye to the brain (Deiner et al., 1997; Dakubo et al., 2003; Morcillo et al., 2006; Hao, 2008). It has been proposed that aberrant closure of the optic fissure and defective formation of the optic disc in human fetuses can cause ocular coloboma and optic nerve dysgenesis, leading to childhood blindness (Gregory-Evans et al., 2004; Chang et al., 2006).

Multiple signaling pathways have been implicated in ocular coloboma and optic nerve hypoplasia. Early midline-derived Shh governs the proximal-distal patterning of optic vesicle by regulating Pax2 and Pax6 expression, which are necessary for the demarcation of the optic stalk, optic disc and neural retina (Macdonald et al., 1995; Chiang et al., 1996). This is followed after the onset of retinal neurogenesis by retinal ganglion cell-derived Shh, which further promotes proliferation of the optic disc and optic stalk (Dakubo et al., 2003). Studies of knockout mice reveal that Bmp4 expressed in the dorsal optic vesicle is crucial for dorsal-ventral patterning, whereas Bmp7 expressed in the ventral optic vesicle and the neighboring periocular mesenchyme is required for initiation of the optic fissure and specification the optic disc (Furuta and Hogan, 1998; Morcillo et al., 2006). Similarly, RA signaling has also been proposed to regulate the closure of the optic fissure by controlling ventral retina morphogenesis and periocular mesenchyme development (Matt et al., 2005; Molotkov et al., 2006; Matt et al., 2008; Lupo et al., 2011). Finally, mutations in Wnt signaling genes Lrp6, frizzled 5 (Fzd5) and β-catenin (Ctnnb1), which are known to regulate the dorsal-ventral patterning of optic vesicle and the specification of the RPE, all cause ocular coloboma (Liu et al., 2007; Liu and Nathans, 2008; Zhou et al., 2008; Fujimura et al., 2009; Westenskow et al., 2009). Many of these coloboma mutants, as well as human patients, exhibit dysregulation of Pax2, a key homeodomain protein required for the closure but not the initiation of the optic fissure (Torres et al., 1996; Alur et al., 2010; Cross et al., 2011). In addition, mutations in ocular transcription factors, e.g. Pax2, Chx10 and Mitf, also result in ocular coloboma (Robb et al., 1978; Silver and Robb, 1979; Scholtz and Chan, 1987; Hero, 1989; Torres et al., 1996; Barbieri et al., 2002; Mui et al., 2005). These animal models provide a framework within which to unravel the etiology of human coloboma and optic nerve defects.
Previous studies in chick and zebrafish models suggest that FGF signaling plays essential roles in eye development, but thus far none of the murine Fgf mutants have shown significant retinal defects. We recently reported that protein tyrosine phosphatase Shp2 (Ptpn11), a downstream mediator of receptor tyrosine kinase signaling, is required for embryonic neural retina specification and adult retinal survival (Cai et al., 2010; Cai et al., 2011). To further explore the signaling pathway upstream of Shp2, we have now generated a conditional knockout of two FGF receptors, Fgfr1 and Fgfr2, specifically in optic vesicle, which resulted in ocular coloboma and optic nerve dysgenesis. Although BMP and midline Shh signaling appear unaffected, Fgfr1/2 deletion disrupts Pax2 expression in the developing optic fissure and optic disc, which instead acquire ectopic expression of RPE marker Mitf. This failure of optic fissure and optic disc specification is also phenocopied by the specific deletion of Frs2α and Shp2, and by mutations that disrupt Fgfr-Frs2α or Frs2α-Shp2 interactions. By contrast, both Fgfr1/2 and Frs2α/Shp2 mutants can be rescued by constitutively active Ras-ERK signaling. Our results thus demonstrate a novel role for FGF-Frs2α-Shp2-Ras-ERK signaling in the development of the optic fissure, optic disc and optic nerve.

**MATERIALS AND METHODS**

**Mice**

Frs2α<sup>fl</sup>, Frs2α<sup>2f</sup>, Shp2<sup>fl</sup> mice have been previously reported (Zhang et al., 2004; Lin et al., 2007; Gotoh, 2008). Fgfr1<sup>F</sup> mice were derived from the recombinant ES cells kindly provided by Philippe Soriano (Mount Sinai School of Medicine, New York, NY, USA) (Hoch and Soriano, 2006). Fgfr2<sup>F</sup> mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) (Hoch and Soriano, 2006). Fgfr2<sup>G</sup> mice were kindly provided by Dr Vasant Dhodapkar (University of Iowa, Iowa City, IA, USA) (Hoch and Soriano, 2006; Cai et al., 2010). Fgfr2<sup>G</sup> mice were used to study FGF signaling in the development of the optic fissure, optic disc and optic nerve.

**Histology and immunohistochemistry**

Mouse embryos were staged according to the day the vaginal plug was first observed. The samples were fixed in 4% PFA overnight for frozen or paraffin wax-embedded sections before standard immunohistochemistry was performed as previously described (Pan et al., 2004; Pan et al., 2007). Serial sections (frontal or sagittal sections for ~E9.5–E11.5 embryos, transverse or horizontal sections for ~E12–E18 embryos) were used. The samples were fixed in 4% PFA overnight for frozen or paraffin embedded tissues.

**RESULTS**

Conditional knockout of Fgfr1 and Fgfr2 by Six3-Cre caused ocular coloboma and optic nerve dysgenesis

Previous RNA in situ hybridization experiments show that both Fgfr1 and Fgfr2 are expressed in embryonic retina, whereas transcripts for Fgfr3 and Fgfr4 appear undetectable (Visel et al., 2004). We thus used Six3-Cre to ablate Fgfr1 and Fgfr2 in optic vesicle to study FGF signaling in eye development (Furuta et al., 2000). We first carefully investigated the timing and specificity of Six3-Cre using the R26R reporter. As we previously reported, Six3-Cre;R26R embryos exhibited only sparse X-gal staining in the optic vesicle at E9.0 (Cai et al., 2010). At E9.5, however, strong X-gal staining was first observed in the ventral optic vesicle, the precursor region of the optic fissure, optic disc and ventral neural retina (supplementary material Fig. S1A–C). By contrast, the dorsal optic vesicle did not stain strongly for X-gal until E10.5 (supplementary material Fig. S1D–F). After that, transverse sections of the Six3-Cre;R26R embryos indicated that Six3-Cre activity was concentrated in the central retina, optic stalk and optic chiasm (Cai et al., 2010; Cai et al., 2011).

Fgfr1 and Fgfr2 single mutants were viable and fertile without ocular defects (data not shown). Examination of the adult Six3-Cre;Fgfr1<sup>fl/fl</sup>;Fgfr2<sup>fl/fl</sup> double mutants, however, revealed a ‘tear-drop’-shaped iris, which mimics uveal coloboma found in humans (Fig. 1A,E) (Chang et al., 2006). More strikingly, the optic nerve was either extremely thin or completely missing from the
Fig. 1. Ocular coloboma and optic nerve aplasia in the Six3-Cre;Fgfr1<sup>flox/flox</sup>;Fgfr2<sup>flox/flox</sup> mice. (A-H) The adult Six3-Cre;Fgfr1<sup>flox/flox</sup>;Fgfr2<sup>flox/flox</sup> animals displayed a teardrop-shaped iris (circled in yellow) and a loss of optic nerve (ON) (arrow in F). At E13.5, the optic fissure was completely closed in the control eye, but it remained open in the Six3-Cre;Fgfr1<sup>flox/flox</sup>;Fgfr2<sup>flox/flox</sup> embryos (arrow in H). (I-O) Sagittal sections of the E13.5 mutant eyes revealed ectopic Mitf expression in the open optic fissure (arrow in L), the hypoplastic optic stalk (circled in M) and a loss of optic nerve as indicated by lack of NF165 staining (circled in N). A sagittal section scheme is shown in O. (P-V) Transverse sections showed that the presumptive optic disc in Fgfr1<sup>flox</sup> mutant lost Pax2 and netrin 1 expression, but gained Mitf expression (S and T, arrows). As a result, NF165-stained retinal ganglion cell axons were misrouted (U, arrowheads) and optic nerve was not formed (U, arrow). The transverse section through the optic disc region is shown in V. (W) (Left) Summary of the ocular phenotypes in the E13.5 Six3-Cre;Fgfr1<sup>flox/flox</sup>;Fgfr2<sup>flox/flox</sup> embryos. Adapted, with permission, from Bharti et al. (Bharti et al., 2006). The control eyeball is composed of an outside RPE layer (yellow) and an inside neural retina (green) layer, which fuse at the ventral optic fissure (faint red), but remained open in the posterior optic disc (red). In the Six3-Cre;Fgfr1<sup>flox/flox</sup> mutants, the ventral neural retina along the optic fissure were transformed to RPE, preventing the closure of the eyeball. A similar transformation of optic disc to RPE prevented the formation of optic nerve. NR, neural retina; OD, optic disc; OF, optic fissure; ON, optic nerve; OS, optic stalk; RPE, retinal pigmented epithelium. (Right) Three-week-old eye balls were dissected to remove RPE covering the back of the eye, revealing abnormal pigmentation in mutant optic disc and optic fissure (arrows). Scale bars: 100 μm in I,L; 25 μm in J,K,M,N; 100 μm in P-U.
To determine the molecular mechanism of Fgfr1/2 mutant defects, we next investigated the extensive transcriptional network underlying retinal differentiation. Rx and Six3 are transcription factors required for eye field specification and Otx1 marks RPE, optic disc and ciliary margin. Their expressions in the Fgfr1/2 mutants expanded into the presumptive optic stalk (Fig. 2A-F'). In E13.5 control embryos, retinal progenitor marker Sox2 labeled the neural retina and optic disc tissue, whereas Pax6 was present only in the neural retina and RPE. In the Fgfr1/2 mutants, however, the presumptive optic disc region exhibited ectopic Pax6 expression at the expense of Sox2 expression (Fig. 2G-J', arrows). Arrows, Fig. 2G,H, arrows. Consistent with this, the axial polarity markers Vax1, Tbx5, Vax2, Radh3, Nls2, Bf1 and Bf2 were all expressed normally (Fig. 3G-L', arrows). By contrast, the neural retinal marker Chx10 was already reduced in the ventral retina adjacent to the optic fissure (Fig. 3O', arrows). Importantly, whereas control optic fissure expression was Pax2 at E11.5, the RPE marker Mitf was ectopically expressed by the Pax2-positive cells in the mutant optic fissure (Fig. 3R', arrows). As shown above in Fig. 1, Fgfr1/2 mutant optic fissure expression was lost in the Pax2 expression and acquired ectopic pigmentation. This apparent transition from Pax2/MITf co-expressing progenitors to purely Mitf-positive RPE in ventral retina cannot be explained by simple infiltration of RPE in the wake of the failed fusion of optic cup, but instead points to a genuine fate change of neural retina to RPE tissue. Collectively, these results suggested that the lack of Fgfr1/2 signaling disrupted the development of the optic fissure without affecting the general patterning of the optic vesicle.

### Table 1. Ocular coloboma and optic nerve (ON) dysgenesis in FGF signaling mutants

<table>
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<th>Ocular phenotype</th>
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<th>ON aplasia</th>
<th>ON hypoplasia</th>
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<td>0%</td>
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<td>Six3-Cre:Fgfr1ff/Fgfr2ff</td>
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<td>88%</td>
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<tr>
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<td>70%</td>
<td>30%</td>
<td>40</td>
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<tr>
<td>Six3-Cre:Frs2af/f;Shp2ff</td>
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<td>57%</td>
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<tr>
<td>Six3-Cre,Frs2af/f;Shp2ff;LSL-KrasG12D</td>
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**FGF signaling defects were reproduced by Frs2a and Shp2 double, but not single, mutants**

To further understand the molecular mechanism of FGF signaling in eye development, we next explored its intracellular signaling cascade. As early as E10.5, before the onset of Mitf
misexpression, a clear reduction in phospho-ERK staining was observed in the ventral optic vesicle, the presumptive optic disc, the ventral optic stalk and the optic chiasm (Fig. 4A-D,G-J, arrows). At E13.5, when control embryos exhibited strong elevation in ERK phosphorylation in the Pax2-positive optic disc, Fgfr1/2 ablation abolished both phospho-ERK and Pax2 staining in the presumptive optic disc region (Fig. 4E,F,K,L, arrow). ERK signaling is also known to promote cell proliferation and survival. Consistent with this, the presumptive optic disc region in the Fgfr1/2 mutants showed a loss of cell cycle regulator cyclin D1 and cell proliferation marker Ki67, but there was a significant increase in apoptotic marker cleaved caspase 3 and TUNEL staining at E11.5 and E13.5 (Fig. 4M-T, arrows). These results supported the theory that Fgfr1/2 was required for ERK signaling in the optic fissure and optic disc.

Tyrosine phosphorylation of Frs2 by FGF receptors is known to create docking sites for the adaptor protein Grb2 and protein tyrosine phosphatase Shp2, both of which are important for activating Ras-ERK signaling. Surprisingly, although we confirmed the Six3-Cre-mediated ablation of Frs2a both by RNA in situ hybridization and immunohistochemistry (supplementary material Fig. S3A-D, arrows), ERK phosphorylation was unchanged in the E10.5 Six3-Cre;Frs2aflox/flox optic vesicle (supplementary material Fig. S3E,I, arrows). Phosphorylated ERK was eventually downregulated in the central retina at E13.5, but the Pax2-positive optic disc and NF165-positive optic nerve were both formed (supplementary material Fig. S3F-H,J-L, arrows). Consistent with this, no ocular coloboma was observed in Six3-Cre;Frs2aflox/flox animals (data not shown). As we have previously reported (Cai et al., 2010), Six3-Cre;Shp2flox/flox mutant similarly did not display any defects in embryonic development, including the closure of optic fissure and the formation of optic disc (supplementary material Fig. S3M-P, arrows; data not shown).

To further investigate the functional significance of Frs2a and Shp2 in retinal development, we next generated a combined deletion of these two genes. Interestingly, the Six3-Cre; Frs2aαflox/flox;Shp2flox/flox mutants now reproduced the ocular coloboma and optic nerve dysgenesis phenotype observed in the Six3-Cre; Fgfr1flox/flox;Fgfr2flox/flox mutants (Fig. 5A-E, arrows; Table 1). By immunostaining, we confirmed that both Shp2 and phospho-ERK were lost in the ventral optic vesicle at E10.5 (Fig. 5F-G, arrows). In the E13.5 frontal sections, the Frs2a/ Shp2 mutants appeared to maintain the correct dorsal-ventral polarity as indicated by the ventral expression of Vax2, but the Pax2-expressing optic disc structure was absent (Fig. 5H, I). Similarly, ectopic Mitf expression was again induced in the ventral optic cup and the NF165-labeled optic nerve was severely hypoplastic (Fig. 5J, K). Moreover, transverse sections of the Frs2a/ Shp2 mutants showed that the presumptive optic fissure and optic disc, and the adjacent ventral retina regions lost the retinal progenitor markers Sox2, Fgf15, Chx10, Math5, Crx and 8m3b were excluded from presumptive optic disc and neural retina adjacent to the optic fissure. Dashed lines indicate the boundary of optic disc. Arrows indicate the presumptive optic disc/stalk (A-L') and neural retina (M-T') in mutants. NR, neural retina; OD, optic disc; OS, optic stalk. Scale bars: 100 μm.
**Frs2a and Shp2 acted epistatically to mediate FGF function in retinal development**

The above results show that ocular coloboma appeared only in the Frs2a/ Shp2 double mutants but not in the single mutants, which seemed to suggest that these two genes act redundantly in ocular development. That is, Frs2a can activate downstream ERK signaling even in the absence of Shp2, and vice versa. However, an alternative explanation is that Frs2a and Shp2 are indeed both essential for transmitting FGF signaling, but the conditional knockout of either Frs2a or Shp2 resulted in only a gradual loss of either protein, which by itself may not be rapid enough to abolish downstream ERK signaling before the optic disc and optic fissure are developed. In this model, simultaneous depletion of both the Frs2a and Shp2 proteins in the Frs2a/ Shp2 double mutants would accelerate the disruption of ERK signaling, resulting in ocular coloboma. This synergistic effect is thus similar to the classic genetic interaction between two hypomorphic systemic alleles on the same pathway, which produces a far more severe phenotype when combined.

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**Fig. 3.** The *Six3-Cre;Fgfr1^flox/flox;Fgfr2^flox/flox* mutant optic vesicle exhibit normal axial patterning but defective optic fissure development. (A-F) At E11.5, the loss of Fgfr1 and Fgfr2 signaling did not affect the expression of midline Shh, dorsal Bmp4, ventral Bmp7 and their downstream effectors Gli1 and phospho-Smad1. (G-L) Levels of the dorsal-ventral polarity markers Tbx5, Vax2, Radl3 and Nlz2, and the temporal-nasal markers BF1 and BF2 were unchanged in Fgfr1/2 mutants. (M-R) Although levels of the optic vesicle patterning genes Rx, Six3, Pax6 and Otx1 were also unaffected, levels of the neural retinal marker Chx10 and the optic disc marker Pax2 were downregulated in ventral retina, whereas the RPE maker Mitf was ectopically expressed in the ventral optic fissure in Fgfr1/2 mutants. Scale bars: 100 μm.
To test this hypothesis, we used the well-characterized \textit{Frs2a}^2F allele, which contained two point mutations in \textit{Frs2a} that disrupted the Shp2 binding (Fig. 6A) (Gotoh et al., 2004). We reasoned that if Frs2\(\alpha\) could act independently of Shp2, disabling the Frs2\(\alpha\) and Shp2 interaction in the \textit{Six3-Cre;Frs2a}^2F/flox;Shp2flox/flox mutants would not produce a more severe phenotype than the \textit{Six3-Cre;Frs2a}^flox/flox;Shp2flox/flox single mutants. However, if Frs2\(\alpha\) must recruit Shp2 to activate downstream signaling, the \textit{Six3-Cre;Frs2a}^2F/flox;Shp2flox/flox mutants would phenocopy the same ocular coloboma defects in the \textit{Six3-Cre;Frs2a}^flox/flox;Shp2flox/flox mutants. As shown in Fig. 6B-P, the \textit{Six3-Cre;Frs2a}^2F/flox;Shp2flox/flox mutants indeed lost both Shp2 and phospho-ERK staining in the central retina. As a result, Mitf was ectopically induced in the presumptive optic disc region, whereas Pax2, netrin 1 and NF165 were downregulated. Although the adult \textit{Six3-Cre;Frs2a}^2F/flox animals did not display any eye phenotype (data not shown), the \textit{Six3-Cre;Frs2a}^2F/flox;Shp2flox/flox mutants presented with ocular coloboma and optic nerve dysgenesis (Table 1). The phenotypic similarities between the \textit{Six3-Cre;Frs2a}^2F/flox;Shp2flox/flox and the \textit{Six3-Cre;Frs2a}^flox/flox, Shp2flox/flox mutants support the hypothesis that the recruitment of
Shp2 by Frs2α is essential in mediating FGF signaling during retinal development.

To further confirm the functional significance of Frs2 in FGF signaling, we next took advantage of a previously characterized Fgfr1ΔFrs allele (Hoch and Soriano, 2006), in which the Frs2 binding site was deleted in the Fgfr1 cytoplasmic domain (Fig. 7A). By generating the Six3-Cre;Fgfr1ΔFrs2/flox;Fgfr2flox/flox mutant, we essentially created a retinal specific Fgfr1ΔFrs2 mutant in a Fgfr2-null background. This strategy allows us to probe Fgfr-Frs2 interaction in a tissue-specific manner. The Six3-Cre;Fgfr1ΔFrs2/flox;Fgfr2flox/flox mutants completely phenocopied the Six3-Cre;Fgfr1Δflox/flox;Fgfr2Δflox/flox null mutants (Table 1). As shown in Fig. 7B-E, phospho-ERK staining was abolished in the presumptive optic disc region in the E13.5 Six3-Cre;Fgfr1ΔFrs2/flox;Fgfr2Δflox/flox mutants. Mitf was ectopically induced at the expense of Pax2 expression, which led to misrouting of NF165-positive retinal ganglion axons and an absence of the optic nerve (Fig. 7F,G,J,K, arrows). In sagittal sections, the mutant optic fissure remained open with clear expression of Mitf but not Pax2, whereas the residual optic stalk appeared hypoplastic without an NF165-positive optic nerve (Fig. 7H,I,L,M, arrows). Therefore, the recruitment of Frs2 to FGF receptor was essential for optic disc and optic fissure development.

**Constitutive Ras signaling rescues the ocular anomalies in the Fgfr1/2 and the Frs2α/Shp2 mutants**

Although FGF signaling is capable of activating multiple downstream signals in vitro, our study above showed that both the Fgfr1/2 and the Frs2α/Shp2 coloboma mutants converged upon a Ras-ERK signaling deficiency. To test whether the activation of Ras-ERK signaling alone can account for the role of FGF signaling in vivo, we next took a gain-of-function approach by employing an oncogenic LSL-KrasG12D allele (Tuveson et al., 2004), which can be induced by Cre recombinase to express a constitutively activated KrasG12D isoform (Fig. 8A). Induction of KrasG12D alone in the...
Six3-Cre; LSL-KrasG12D animals did not cause any overt phenotype (Cai et al., 2010; Cai et al., 2011). However, in the Six3-Cre;Frs2αflox/flox;Shp2flox/flox;LSL-KrasG12D and the Six3-Cre; Fgfr1flox/flox; Fgfr2flox/flox;LSL-KrasG12D mutants, Kras activation not only led to a recovery of phospho-ERK staining in the central retina, but also restored the formation of the optic disc and optic nerve, as indicated by Pax2, netrin 1 and NF165 staining (Fig. 8B-S, arrows). In adult animals, the occurrence of optic nerve dysgenesis or optic coloboma was significantly reduced in the Six3-Cre;Frs2αflox/flox;Shp2flox/flox;LSL-KrasG12D mutants and completely eliminated in the Six3-Cre;Fgfr1flox/flox; Fgfr2flox/flox;LSL-KrasG12D mutants (Table 1). These genetic rescues thus supported an Fgfr-Frs2α-Shp2-Ras-ERK signaling cascade in ocular development.

**DISCUSSION**

In this study, we provided mouse genetic evidence that ocular ablation of Fgfr1 and Fgfr2 caused coloboma and optic nerve dysgenesis, which has not been observed when FGF signaling is disrupted in any other vertebrate organisms. This result is particularly noteworthy, because unlike previous studies of FGF signaling in *Xenopus* and zebrafish (Take-uchi et al., 2003; Lupo et al., 2005; Picker and Brand, 2005; Picker et al., 2009), our Fgfr1/2 mutants did not display any axial polarity defects, suggesting that the observed ocular coloboma and optic nerve dysgenesis was not secondary to abnormal patterning of early optic cup. As indicated by the misexpression of the ocular astrocyte progenitor cell (APC) marker Pax2 and the RPE gene Mitf, loss of Fgfr1/2 signaling transformed the presumptive optic disc and optic fissure into RPE, suggesting a crucial role for FGF signaling in promoting the astrocyte progenitor cell fate versus the RPE fate. This function of FGF signaling is probably mediated by the reciprocal expression of Pax2 and Mitf, mutation of either one is known to cause ocular coloboma (Scholtz and Chan, 1987; Torres et al., 1996). Interestingly, the potentially antagonistic interaction between Pax2 and Mitf in optic disc and optic fissure formation contrasts with the early role of Pax2 in stimulating Mitf expression in optic vesicle development (Bäumer et al., 2003). Such function reversals are not without precedence, as Pax2 and Pax6 also transition from redundant to antagonistic interactions from early to late retinal development (Schwarz et al., 2000; Bäumer et al., 2003). Similarly, Chx10 and Mitf are co-expressed in early optic vesicle, but later become antagonistic in specifying the neural retina versus the RPE under the influence of FGF signaling, loss of which results in conversion of neural retina into RPE (Nguyen and Arnheiter, 2000; Horsford et al., 2005; Cai et al., 2010). The role of FGF signaling in preventing ectopic RPE formation appears to be unique to mouse and chick, as inhibiting FGF signaling has never produced ectopic pigmentation or coloboma in amphibian or fish. Conversely, none of the axial polarity phenotype observed when FGF signaling is inhibited in amphibian and fish is detected in our FGF signaling mouse mutants. Taken together, our study reveals a distinctive role of FGF signaling in higher organisms in promoting neuronal against glial fate during eye development.

It is notable that our Fgfr1/2 mutants displayed a restricted transformation into the RPE only at the optic disc/fissure and a limited neurogenesis defects only in the lip region of the ventral neural retina adjacent to the optic fissure. One possible explanation is the compensatory signaling by Fgfr3 and Fgfr4, which may be expressed at low levels in retina to mediate FGF signaling. However, we suggest that the developmental timing may also restrict the retinal defects in Fgfr1/2 mutants. From our Cre reporter assay, it was clear that the Six3-Cre deleter in the E9.5 optic vesicle was activated in a ventral-to-dorsal order. Consistent with this, the loss of phospho-ERK staining in the Fgfr1/2 mutants at E10.5 was also limited to the ventral optic vesicle that formed the future optic fissure and optic disc. We...
propose that the mutant optic fissure and optic disc regions underwent Mitf-mediated transformation into the RPE because they lost FGF signaling by E10.5, whereas the rest of retina that lost FGF signaling after E10.5 was unable to alter their retinal fate. In this model, FGF signaling is required for the initial demarcation of the optic vesicle, but it becomes dispensable for the maintenance of retinal progenitor cell fate after a limited time window. Our results therefore challenge the view that FGF signaling is required for retinal neurogenesis after E12, but instead support our previous study that FGF signaling is necessary only for the establishment of neuronal fate in optic vesicle at E10.5 (Cai et al., 2010).

The transient requirement of FGF signaling in early optic vesicle development is also supported by the lack of an ocular phenotype in the Frs2α and Shp2 single mutants. As we have previously described and have also shown in this study, Six3-Cre; Shp2flx/flx single mutants did not display any embryonic retinal defects (Cai et al., 2010). By contrast, we have previously demonstrated that the retinal specific ablation of Shp2 by Rx-Cre, a Cre deleter that acts earlier than Six3-Cre, resulted in the transformation of the neural retina into the RPE. Comparing the Six3-Cre; Shp2flx/flx and the Rx-Cre; Shp2flx/flx mutants showed that there was a significant perdurance of Shp2 protein after the onset of Cre activities, which resulted in a considerable delay in ERK signaling disruption. As a result, only Rx-Cre was able to abrogate Shp2-ERK signaling early enough to interfere with the determination of the neural retina fate (Cai et al., 2010). In this study, we similarly showed that the Six3-Cre; Frs2αflx/flx mutants did not significantly downregulate ERK phosphorylation until E13.5. In fact, only by depleting both Frs2α and Shp2 simultaneously in the Six3-Cre; Frs2αflx/flx; Shp2flx/flx mutants were we able to disrupt ERK signaling in the ventral retina at E10.5. This established a genetic interaction between Frs2α and Shp2, which form a binding complex in mediating FGF signaling in eye development. Such a binary complex is expected to be relatively resistant to a slow depletion in one of its binding partners, but the complex will disintegrate when the concentrations of both proteins are reduced below a certain threshold. Therefore, only Frs2α/Shp2 double mutants displayed ocular coloboma, albeit still at a slightly lower penetrance than that of Fgfr1/2 mutants (Table 1), which is likely because of the slower recycling rates of intracellular proteins when compared with membrane receptors. Our study thus presents a caution for future investigation of FGF signaling in embryonic development that protein perdurance may be an important confounding factor in interpreting conditional knockout results.

FGF receptors are known to recruit multiple adaptor proteins, including Frs2, Crk and PLCγ, to activate downstream signaling. In particular, recent studies suggest Frs2α, a lipid-anchored protein that is constitutively bound to FGF receptors, mediates some but not all FGF downstream pathways (Eswarakumar et al., 2006; Hoch and Soriano, 2006; Sims-Lucas et al., 2011). It has been previously reported that the Frs2αΔFrs2α/Frs2α mutant, which disrupts the Frs2α-Shp2 interaction, displayed microphthalmia or even anophthalmia (Gotoh et al., 2004). However, as Frs2αΔFrs2α is a systemic mutant, it is not clear whether these severe ocular phenotypes are due to cell autonomous defects or to abnormal tissue-tissue interaction. In this study, we have combined systemic deletion mutants with conditional alleles to disrupt protein-protein interactions in a tissue-specific manner. In the Six3-Cre; Fgfr1ΔFrs2/Frs2αflx; Fgfr2flx/flx mutants, for example, the Fgfr1ΔFrs2 mutation was compensated for by the Fgfr1flx and Fgfr2flx alleles in every tissue other than the retina, where these conditional alleles were ablated by Six3-Cre. This allowed us to demonstrate that the Fgfr1-1-Frs2 interaction within retinal progenitor cells was crucial in coloboma formation.
Similarly, the coloboma phenotype in the Six3-Cre;Frs2αflox/flox;Shp2flox/flox mutants revealed the importance of Frs2α-Shp2 interaction in the retinal-specific FGF signaling. Finally, we showed that constitutive Kras signaling was sufficient to prevent ocular coloboma in these FGF signaling mutants, demonstrating a remarkably specific pathway from Fgfr to Frs2-Shp2 to Ras-ERK signaling in retinal development.

In summary, our present study identifies deficient FGF signaling as a novel cause of ocular coloboma and optic nerve dysgenesis. We show that FGF-activated Ras-ERK signaling is essential for the development of the optic nerve, optic fissure and optic disc, the abnormalities of which have been implicated in not only ocular birth defects but also in adult glaucoma. It is notable that iris and optic disc colobomas have also been observed in some individuals with Noonan syndrome, who have gain-of-functions mutations in Ras-MAPK signaling pathway (Kleinhans et al., 1987; Ascaso et al., 1993; Rudolph et al., 2001; Legius et al., 2002; Carvalho et al., 2003; Tartaglia and Gelb, 2005). Interestingly, we have recently shown that deletion of the genes associated with Noonan syndrome, Shp2 and Nf1, caused lens development defects (Pan et al., 2010; Carbe and Zhang, 2011). These findings suggest that both the gain- and loss-of-function mutations in FGF-Ras-MAPK signaling may have deleterious consequences in human ocular development. Therefore, FGF signaling components should be considered in the diagnosis and therapeutic interventions for ocular anomalies.

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Z.C. and X.Z. conceived the project. Z.C., C.T. and H.L. performed the experiments. R.L., N.G., G.-S.F. and F.W. provided mouse mutants. Z.C. and X.Z. wrote the manuscript.
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