FUNCTIONS OF THE FGF SIGNALING PATHWAY DURING THE OPTIC FISSURE CLOSURE

By

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ABSTRACT

The mammalian eye represents an excellent model system to study tissue morphogenesis and cell fate determination in the nervous system. During mammalian eye development, the optic fissure (OF), a transient opening on the ventral side of the optic cup, provides an entry site for the mesenchymal cells to migrate into the eye to form hyaloid vessels for blood supply in the developing eye. The OF is then closed but leaves a permanent opening in the posterior, which is known as the optic disc, following the migration of the mesenchymal cells. The failure in the OF closure causes the formation of coloboma, which affects 2.6 babies per 10,000 births. However, the molecular and cellular mechanisms that control the OF closure are still obscure. In my dissertation study, I have carefully documented, for the first time, the process of the OF closure. Then, I have used the conditional knockout of *Fgfr1* and *2* specifically from the developing eye to demonstrate that FGF signaling is required for controlling the OF closure. The eyes which are absent of functions of *Fgfr1* and *2* develop coloboma. Furthermore, I have shown that FGF signaling regulates the proliferation, cell fate switches and morphological changes of OF progenitor cells. Finally, FGF signaling is also required for the formation of the optic disc and the maintenance of the optic stalk. Therefore, the knowledge gained from this study has provided novel insight into the cellular and molecular mechanisms underlying the OF closure and possibly coloboma formation in humans.

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ABBREVIATIONS

bHLH: basic helix-loop-helix

BMP: bone morphogenetic protein

DV: dorsal-ventral

FGF: fibroblast growth factor

FGFR: FGF receptor

GCL: ganglion cell layer

Hh: hedgehog

INL: inner nuclear layer

NR: neural retina

NT: nasal-temporal

OC: optic cup

OF: optic fissure

ONL: outer nuclear layer

OS: optic stalk

OV: optic vesicle

RGC: retinal ganglion cell

RPE: retinal pigmented epithelium

Shh: sonic hedgehog

TGF- β : transforming growth factor beta

TUNEL: terminal transferase dUTP nick end labeling

VAD: vitamin A deficiency

CHAPTER ONE

BACKGROUND

I. Overview of the vertebrate eye development

The mammalian eye is a complex structure (Fig. 1-1): The whole eye is wrapped by a protecting wall composed of the sclera on the back and the cornea on the front, which is transparent to allow the entrance of light. In addition, the anterior segment of the eye consists of the ciliary body which controls the curvature of the lens, the iris which determines the diameter of the pupil to adjust the amount of light entering the eye, and the lens which is the refractive structure focusing light on the back of the eye. The back of the eyeball is mainly occupied by the retina, the most important sensory structure of the eye. The retina is composed of two layers, the retinal pigmented epithelium (RPE) and the neural retina (NR). The RPE is a single-layered structure that nourishes adjacent photoreceptor cells and also regulates their function. The neural part of the retina is composed of six types of neurons and one type of glia cells, which is laminated into three layers, the ganglion cell layer (GCL), the inner nuclear layer (INL) and the outer nuclear layer (ONL). In the GCL layer, retinal ganglion cells (RGCs) are the only type of cell which relay electric information to the brain. Three types of interneurons, amacrine cells, bipolar cells

and horizontal cells, as well as one type of glia cells, Müller cells, reside in the INL. The ONL layer is composed of rod and corn photoreceptors, which sense light and convert it to electronic signals that are further processed by the interneurons and relayed to the brain through the axons of RGCs.

The adult eye develops from the eye primordium (Fig. 1-2), a single specified area in the neural tube at the forebrain region. This single eye field then splits by bilateral evagination to the surface ectoderm to form the optic vesicle (OV) on both the left and right sides. The OV invaginates to form the optic cup (OC) under the induction of the surface ectoderm, while the surface ectoderm itself also invaginates to form the lens vesicle, the lens primordium, and the remaining ectoderm develops into the cornea. The OC is a two-layered structure: the proximal layer (the layer contacting the surrounding mesenchymal cells) develops into the single-layered RPE, while the distal layer (the layer facing the eye chamber) develops into the multilayered neural retina. At the beginning of the OC stage, the invaginating optic neural epithelium leaves a cleft on the ventral side, known as the optic fissure (OF). The OF allows the entrance of the surrounding mesenchymal cells into the eye chamber to form the hyaloid vessel, the main blood supply for the developing eye. Soon after the mesenchymal cells have migrated in, the OF closes to form the continuous retina, leaving a permanent opening, the optic disc, at the posterior end. In the developing retina, multipotent retinal progenitor cells proliferate and differentiate into six types of retinal neurons and one type of glia cell, which are laminated into three retinal layers (Chow and Lang 2001; Adler and Canto-Soler

2007; Harada et al. 2007) .

Early eye development: from eye field specification to optic vesicle formation

While the first morphological evidence of eye formation in vertebrates is a bilateral expansion of the anterior region of the neural plate, it has been known for a long time that eye anlage is specified prior to the OV formation (Chow and Lang 2001; Esteve and Bovolenta 2006; Adler and Canto-Soler 2007). Fate mapping studies in amphibians have shown that the prospective eye progenitor cells are initially intermixed with telencephalic precursors and diencephalic precursors (Wetts and Fraser 1989). Local organizers, Wnts in the anterior region and FGFs and Wnt antagonists in the posterior region, are believed to regionalize the forebrain structure. Distinct levels of Wnt activity contribute to the regional specification of the anterior neural plate: the highest and the lowest levels promote diencephalon and telencephalon specification, respectively, while the intermediate level determines the eye field between the two regions (Wilson and Houart 2004). FGF signaling has been shown to regulate the positioning of retinal progenitor cells within the definitive eye field by modulating ephrin signaling (Moore et al. 2004). Thus, the specification of the eye field depends on the locally produced signals.

Not only does the position of cells determine the retinal cell fate, cell movements also contribute to the eye field formation. After cells have been positioned in the eye field, concomitant expression of a series of transcription factors further drives the morphogenetic movements of the eye field. These transcription factors include *Pax6*, *Rx1-3*, *Six3*, *Otx2*, *Lhx2*, *Optx2*, *ET* and *tll* (Chow and Lang

2001; Zuber et al. 2003; Stigloher et al. 2006). The studies on their homologs in *Drosophila* have provided insight into how they control the eye formation. These transcription factors have been shown to act as a self-regulatory network with hierarchical components and multiple steps of feedback regulations. For example, *twin of eyeless* (*toy*), a homolog of *Pax6*, acts at the apex of the network, while *eyeless* (*ey*), another *Pax6* homolog, is placed downstream of *toy*, followed by *eyes absent* (*eya*), *sine oculis* (*so*, a homolog of *Six3* and *Optx2*), and then *dachshund* (*dac*) (Chow and Lang 2001). Recent studies in vertebrates have further confirmed the existence of a similar regulatory network in eye specification (Zuber et al. 2003). *Six3*, *Pax6*, *Rx1*, *Lhx2* and *ET* are coexpressed in the presumptive eye field; simultaneous expression of these transcription factors along with *Otx2* is sufficient to induce ectopic eyes outside the nervous system through a regulatory feedback loop (Zuber et al. 2003). In the future, it will be important to reveal how the highly conserved transcriptional network controls the morphological movements of the eye field.

The bisection of the single eye field into the bilaterally positioned optic vesicles is controlled by the movements of, and signals produced by, axial tissues (prospective hypothalamus and underlying prechordal mesoderm) (Chow and Lang 2001; Wilson and Houart, 2004). Normally, prospective hypothalamic cells move rostrally within the neural plate to push medially positioned eye field cells laterally. In zebrafish, disruption of such movement results in cyclopia/holoprosencephaly phenotype (Hammerschmidt et al. 1996; Heisenberg et al. 1996; Heisenberg and

Nusslein-Volhard 1997; Marlow et al. 1998; Varga et al. 1999). The importance of the axial tissues becomes evident by the production of instructive signals such as *TGF-β* and hedgehog. C*yclops* (*cyc)*, a zebrafish Nodal-related member of the *TGF-β* superfamily, is expressed in the prechordal mesoderm and is necessary for the generation of two symmetric eyes (Macdonald et al. 1995). Hedgehog family signaling molecules are expressed in the ventral midline of the forebrain and underlying prechordal mesoderm. In both humans and mice, loss of *Shh* function results in cyclopia, in which only one eye forms (Chiang et al. 1996). Further studies in zebrafish and chick have shown that *Shh* functions downstream of *cyc* to control the bisection of the eye field (Wilson and Houart 2004; Esteve and Bovolenta 2006). So far, it has been shown that the bisection of the eye field requires the signals from the nearby tissues, but it remains largely unclear how the Shh-TGF-β signaling network controls the bisection process.

Although many transcription factors are expressed in the eye field, only *Rx*, and *tll* are essential for the formation of OV from the neural epithelium since loss of *Rx3* and *tll* functions cause defects in the OV evagination by disrupting normal active cell migration in the eye field (Hollemann et al. 1998; Loosli et al. 2003; Rembold et al. 2006). Following the bisection of the eye field, the transcription factors, which have been discussed earlier for their important roles in the specification of the eye field, change their expression patterns from the center of the brain to the laterally located OVs, indicating their late function in the eye development. Therefore, inductive signals and intrinsic factors function in a concerted manner to control both

the specification and bisection of the eye field.

The optic cup development

The formation and the specification of different structures of the OC are closely regulated by the surrounding tissues: the surface ectoderm induces the extending optic vesicle to invaginate to form the two-layered optic cup and further specify the inner layer of the optic cup as the neural retina; while the extraocular mesenchyme promotes the RPE fate in the outer layer of the optic cup by antagonizing the NR-promoting signals from the surface ectoderm (Chow and Lang 2001; Graw 2003; Adler and Canto-Soler 2007). The important roles of the surface ectoderm and the extraocular mesenchyme in the optic cup development have been demonstrated by tissue transplantation and explant culture experiments. As early as 1939, Holtfreter observed that without the surface ectoderm, the development of explanted amphibian anterior neural plates arrested at the OV stage, and consequently the neural retina failed to form. This observation has recently been confirmed in chicken and mice (Hyer et al. 1998a; Nguyen and Arnheiter 2000b). Similarly, microdissected chicken OVs do not develop RPE in the absence of surrounding extraocular tissues (Fuhrmann et al. 2000), underscoring the importance of the extraocular mesenchymal cells in the RPE development. The studies from different organisms have indicated that FGF and TGF-β signals, which are emanated from the surface ectoderm and the extraocular mesenchyme, respectively, are responsible for the initial regional specification of the OC. In explant cultures, Fgf1 and Fgf2 have

been shown to be capable of mimicking the effects of the surface ectoderm to induce neural retina formation from the layer that is close to the growth factor sources (Park and Hollenberg 1989; Pittack et al. 1991; Guillemot and Cepko 1992; Hyer et al. 1998a; Nguyen and Arnheiter 2000b), while activin, a member of TGF-β family, could mimic inducing activity of the extraocular mesenchyme on RPE formation (Fuhrmann et al, 2000). Thus, the specification of the neural retina and the RPE requires the signals from the adjacent tissues.

As mentioned earlier, the transcription factors that are required for the specification and bisection of the eye field continue their expression in the OV. Indeed, they play important roles in different aspects of the OC development. For example, *Pax6*, *Lhx2* and *Hes1* are essential for the invagination process of the OV since mutations in *Pax6*, *Lhx2* and *Hes1* develop OVs, but fail to constrict proximally to form OCs (Grindley et al. 1995; Porter et al. 1997; Lee et al. 2005; Canto-Soler and Adler 2006). In addition, *Pax6* is also required for the survival of the OV cells (Grindley et al. 1995; Canto-Soler and Adler 2006). As in the specification of the eye field, inductive signals and intrinsic transcription factors work cooperatively to support the OC development.

During the OC development, the eye undergoes many complex morphological modifications (Fig. 1-2). For example, although the RPE wraps around the neural retina in the adult eye (Fig. 1-2 A4, B3), it is restricted to the dorsal proximal area at the OV stage (Fig. 1-2 A2, B1), but later gradually spreads ventrally to completely surround the neural retina. In a similar fashion, the originally ventral-distally located

neural retina is wrapped inside. Similarly, the OS consists of dorsal and ventral walls at the OV stage and early OC stage; when the axons of the RGCs project in, the ventral OS neural epithelia cells differentiate into astrocytes which intermingle extensively with the projecting axons to form the optic nerve, while the dorsal wall of the OS moves ventrally to form the outside shield of the optic nerve. Finally, the most dramatic difference of the developing ventral optic cup from the dorsal optic cup is the existence of the optic fissure, which later disappears by fusion, leaving the ventral retina morphologically identical to the dorsal retina (Fig. 1-2B) (Chow and Lang 2001, Harada et al. 2007).

Establishment of the polarities of the optic cup

Although they are morphologically symmetrical, adult vertebrate eyes are highly polarized in three axes, anterior-posterior (AP), dorsal-ventral (DV) and nasal-temple (NT). Such asymmetry ensures the positional information of the vision to be accurately presented in the brain by regulating correct axonal targeting patterns of RGCs. The establishment of this retinal polarity can be traced back to the OV stage and is tightly controlled by coordinated functions of several signaling pathways and intrinsic factors (Chow and Lang 2001, Adler and Canto-Soler 2007, Harada et al. 2007).

The NT polarity is established earlier than the DV polarity, and its establishment requires both the signals from the surrounding tissues such as FGF and intrinsic factors such as transcription factors. In zebrafish, the telencephalic

primordium produces *Fgf8*, which determines retinal patterning along the NT axis by inducing nasal and/or suppressing temporal retinal cell fates (Picker and Brand, 2005). Misexpression of *Fgf8* induces nasalization of the retina by suppressing temporal retinal cell fates, while inhibition of FGF signaling leads to temporalization by inhibiting nasal retinal cell fates. In both nasalized and temporalized retinas, the axons of RGCs are misprojected to the midbrain, demonstrating the importance of this early patterning process for late topographic map formation. However, it remains unclear that FGF signaling represents a general mechanism for controlling the NT polarity of the retina. Four transcription factors have been identified to be required for the NT polarity of the retina, including two winged-helix genes, *BF1/Foxg1*and *BF2/Foxd1*, and two homeobox genes, *SOHo1* and *GH6*. Misexpression of any one of the four transcription factors could cause defects in the NT polarity and aberrant projection of RGC axons (Yuasa et al. 1996; Schulte and Cepko 2000; Takahashi et al. 2003; Herrera et al. 2004). *EphAs* are expressed in a high-to-low TN gradient to guide ganglion cell axons to project inversely to the AP axis in the superior colliculus or optic tectum, and their expression is regulated directly or indirectly by the aforementioned transcription factors (Feldheim et al. 2000; Peters and Cepko 2002; McLaughlin et al. 2003). It remains unclear whether EphAs are also regulated by FGF signaling and how intrinsic factors and FGF signaling interact with each other to control NT patterning in the retina.

Similarly, several transcription factors are required to determine and/or maintain the DV polarity in the retina. *Tbx5* and *Vax2* play important roles in

specifying the positional identity along the DV axis. Overexpression of *Tbx5* in the developing chicken eye leads to downregulation of ventral markers (*Vax*, *Pax2*, *EphB2*, *EphB3*) as well as ectopic expression of dorsal markers (*EphinB1* and *EphrinB2*), and alters the projection of the RGC axons (Koshiba-Takeuchi et al. 2000). On the contrary, misexpression of mouse *Vax2* or chick *Vax* genes in chick developing eyes ventralizes the retina through downregulation of *Tbx5*, *EphinB1*, *EphrinB2* and upregulation of *Pax2*, *EphB2*, *EphB3* (Schulte et al. 2005), while knockout *Vax2* dorsalizes the retina (Barbieri et al. 1999; Barbieri et al. 2002). These studies indicate that *Tbx5* and *Vax2* determine the DV polarity by regulating cell fate specification. As expected, the DV polarity of the retina is also regulated by different signaling pathways. Dorsally expressed *Bmp4* promotes dorsal cell fates by promoting *Tbx5* expression and repressing *Vax2* expression, and this dorsal BMP signaling is further restricted to the dorsal side by the ventrally localized BMP antagonist, *Ventroptin* (Koshiba-Takeuchi et al. 2000; Sakuta et al. 2001). Retinoic acid, Hh and FGF signaling pathways have been shown to function collaboratively to specify ventral retinal cell fates by promoting *Vax2* and *Pax2* expression (Lupo et al. 2005). Therefore, signaling pathways and transcription factors work synergistically to control retinal patterning along the DV axis.

In order for the retina to be patterned normally along three different axes, it would be expected that the development of these different axes is coordinated. Interestingly, some of the important regulators for axis formation in the retina show graded expression on different axes, and mutations in the genes cause defects on the

specification of more than one axis. For example, *Vax2* and *Pax6* mutant eyes show both DV and NT patterning defects, which render them top positions on the polarity regulatory transcription factor hierarchy (Huh et al. 1999; Sakuta et al. 2001; Baumer et al. 2002; Mui et al. 2002; Harada et al. 2007). In addition to its requirement for the NT polarity, FGF signaling is also required for DV patterning in the retina (Lupo et al. 2005). So far, it remains a mystery how the AP polarity of the retina is determined and what its relationship is with the other two axes.

Lamination and retinal cell specification

The mature vertebrate retina consists of six types of neurons and one type of glial cell, which are organized into the three layers. Terminally differentiated retinal cells are generated from multipotent progenitor cells in an orderly, while overlapping, fashion, with ganglion cells generated first, followed by amacrine cells, horizontal cells and cone photoreceptors, and ending with bipolar cells, rod photoreceptors and Müller cells (Marquardt 2003). The retinal progenitor cells have been suggested to pass through a series of competence states, during each of which the progenitor cells are competent to generate only a subset of retinal cell types. This competence model is mainly based on the observations that the early-stage retinal progenitor cells keep producing early-born retinal neurons (ganglion cells, amacrine cells) instead of late-born neurons (bipolar cells and rod photoreceptors) when they are put in a late-stage retinal differentiation environment (Cepko et al. 1996; Alexiades and Cepko 1997; Belliveau and Cepko 1999; Livesey and Cepko 2001). Thus, the competence states

seem to be intrinsically defined, but they must be influenced by extrinsic environments during the course of retinogenesis. However, the molecular mechanisms regulating the changes of the competence states during the early development remain largely unclear.

Although the detailed molecular mechanisms controlling individual retinal cell fate specifications are still obscure, a large body of knowledge has been accumulated about the important roles played by intrinsic transcription factors, especially homeobox and basic helix-loop-helix (bHLH) transcription factors, during retinal neuron differentiation (Marquardt 2003; Harada et al. 2007). One of the interesting aspects about the cell fate-determining transcription factors is that many of them also play important roles in early eye development. For example, *Pax6* and *Six3* are continuously expressed in retinal progenitor cells and mature amacrine cells, and *Chx10* continues its expression in bipolar cells (Burmeister et al. 1996; Marquardt et al. 2001). Knockout of *Chx10* results in loss of bipolar cells in mature retina (Burmeister et al., 1996), indicating its essential role in bipolar cell development. Using overexpression and conditional knockout strategies, it has been shown that *Pax6* and *Six3* are required for the retinal progenitor cells to maintain multipotency as well as for the specification of amacrine cells (Marquardt et al. 2001; Inoue et al. 2002). Another homeobox transcription factor, *Crx*, is essential for the differentiation of photoreceptor cells (Furukawa et al. 1997). bHLH transcription factors are also important players in retinal cell-fate specification. For example, *Math5* is essential for RGC commitment, *Math3* and *NeuroD* for amacrine cells and rod photoreceptors,

Mash1 for bipolar cells, *Hes1* and *Hes5* for Müller cells (Marquardt 2003; Harada et al., 2007). Genetic studies show that these transcription factors control the specification of different neuronal cell fates in the retina through promoting a specific retinal cell fate and simultaneously suppressing the other alternative cell fate choices. For example, loss of RGCs in the *Math5*-deficient retinas is accompanied by an increase in the number of amacrine cells and photoreceptor cells (Brown et al. 2001). Furthermore, it seems that homeobox and bHLH types of transcription factors cooperate with one another to control retinal cell fates. When cooperating with *Pax6*, *Math3* promotes the amacrine cell fate (Inoue et al., 2002); while cooperating with *Chx10*, *Math3* is able to promote the bipolar cell fate (Hatakeyama et al. 2001). Since several transcription factors have been shown to promote or suppress a particular retinal cell fate, it is important to understand how they execute their functions at the molecular level as well as their functional relationships.

As in other aspects of eye development, the specification of different cell fates in the retina is also regulated by extrinsic signals. Among these signals are *GDF11* (Kim et al. 2005), *Notch* (Tomita et al. 1996; Jadhav et al. 2006)) and *Shh* (Neumann and Nuesslein-Volhard 2000). *GDF11*, a member of TGF-β superfamily, regulates the temporal window of the competence of the progenitors to differentiate into RGCs since loss of *GDF11* results in an increase of the RGCs at the expense of amacrine cells and photoreceptors (Kim et al., 2005). *GDF11* controls the competence by controlling the duration of the expression of bHLH and homeobox transcription factors, such as *Pax6* and *Math5* (Kim et al., 2005). *Notch1* is expressed in all the

retinal progenitor cells and functions to maintain these cells in a progenitor state in the early retinal developmental stage but promotes the formation of Müller cell identity in the later stages (Jadhav et al., 2006). Similarly, inactivation of *Hes1*, a Notch effector, results in premature cell-cycle exit and differentiation of progenitor cells (Tomita et al., 1996). During late retinal development, *Shh* is secreted by newly postmitotic RGCs and promotes further production of RGCs by adjacent retinal progenitor cells, which is one of the mechanisms that ensures wave-like progression of retinal differentiation from the central to peripheral retina(Neumann and Nuesslein-Volhard 2000).

The organization of the retinal cells into distinct laminae is essential for the assembly of functional neuronal circuits, however, it is still poorly understood how the lamination of the retinal cells is achieved. Genetic studies in zebrafish have revealed that homophilic cell adhesion mediated by *N-cadherin* is essential for the lamination process of retinal development (Masai et al., 2003). In addition, atypical protein kinase C (*aPKC*) and *nok*, a member of membrane-associated guanylate kinase, are localized to the adherens junctions at the apical ends of the retinal neuroepithelia cells and are required for the maintenance of adherence junctions and thereby proper lamination (Horne-Badovinac et al. 2001; Wei and Malicki 2002).

II. Coloboma

Coloboma is derived from the Greek word *koloboma*, meaning mutilated or

curtailed. The term 'coloboma' can be used on a wide variety of ocular malformations that have a notch, gap, hole, or fissure in any ocular structures. Usually it is used to refer to uveal coloboma, which is caused by the failure of the optic fissure closure during development, and affects one or multiple of the following structures: the iris, ciliary body, retina, choroidea, and optic nerve (Chang et al. 2006).

Epidemiology

The prevalence of coloboma varies among different populations: 0.75 in China, 2.6 in the United States, 0.7 in France, 0.5 in Spain and 0.41 in Hungary, per 10,000 births. Coloboma contributes significantly to blindness, with 5-10% of blindness of European children caused by coloboma. Although most coloboma cases are caused by genetic reasons, environmental effects such as vitamin A deficiency have also been proposed to increase the risk of coloboma (Chang et al. 2006).

Human genetics of coloboma

Human genetic studies show that coloboma exhibits considerable genetic heterogeneity, which indicates the complex molecular mechanisms underlining the optic fissure closure. Coloboma may occur in isolation or may be associated with systemic syndromes. The most common syndrome associated with coloboma is the CHARGE syndrome, which is the constellation of coloboma, heart defects, atresia of choanae, retarded growth, genital anomalies, and ear anomalies and deafness. 15-30%

of microphthalmia/coloboma patients have CHARGE syndrome. Genetic studies indicate that *CHD7*, which encodes a chromodomain-containing DNA helicase, is responsible for 59% of CHARGE syndrome cases (Vissers et al., 2004). Another syndrome frequently associated with Coloboma is the papillorenal syndrome, which is caused by mutations in *PAX2* gene, and patients of which have renal malformation as well as coloboma. Mutations in *CHX10*, *MAF*, *PAX6*, *RX*, *SHH*, *SIX3*, *OTX2* and *SOX2* genes, which are known to be important for normal eye development, have also been reported to be found in coloboma patients (Chang et al. 2006).

Optic fissure development

The formation of the OF is a result of asymmetrically oriented invagination movement of the OV, which begins from its ventral distal region and points to the mediodorsal region (Fig 1-2). As a consequence, the OF is often seen as a morphological hallmark of the ventral retina, and indeed its development is tightly associated with the development of the ventral retina. It seems that the fates of the cells in the OF as well as the ventral retina are specified well before the invagination begins (Uemonsa et al. 2002). Using chick *in vitro* explant cultures and *in vivo* transplantation experiments, it has been shown that, without the influence of surrounding tissues, the 10-somite stage OV explants can automatically develop into OCs with a ventrally located OF. The OF progenitor cell fate appears to be flexible at the 10-somite and earlier stages since they develop into OCs with or without an OF when transplanted before the 10-somite stage. However, the OF progenitor cell fate is

permanently specified after the 17-somite stage since the OV develops into an OC with a dorsally located OF, which is evidenced by *Pax2* expression, when inversely transplanted at the stage (Uemonsa et al. 2002). Thus, these findings demonstrate that the invagination of the OV for the OF formation and the specification of the OF progenitor cells are controlled by the signals from their surrounding tissues.

The closing process of the OF begins from the middle of the OF, and proceeds distally to the rim of the OC and proximally to the end of the OS. In mice, this process begins around embryonic day 11.5 (E11.5) (Morcillo et al. 2006). However, it remains unclear what signal(s) triggers the closing of the OF. One possibility could be that the signal(s) from newly formed RGCs initiates the OF closing process since the birth of RGCs and the OF closing take place at the same developmental time window. In the Math5 knockout mice, RGCs do not form at all, but the OF closure finishes normally (Brown et al., 2001), formally excluding the possibility that RGCs send the signals to control the initiation of the OF closure. Another possibility is that paraocular mesenchymal cells, which use OF as a route to enter the retina, provide the signals for closing the OF. However, there have been no reports so far to support this possibility.

The molecular mechanisms underlying the OF closure

Genetic studies in humans and mice together with molecular manipulations in chicken, zebrafish and *Xenopus* embryos have shed some light on the molecular

mechanisms regulating the OF closure. The development of the OF is closely related to the ventral retina as well as the OS in terms of gene expression and regulation. The OF progenitors express the OS markers, such as *Pax2* and *Vax1*, and the ventral OC markers, such as Vax2. Interestingly, mutations in *Pax2* and *Vax1* cause coloboma and OS defects in mice (Torres et al. 1996; Bertuzzi et al. 1999; Hallonet et al. 1999), while a mutation in *Vax2* leads to coloboma formation and DV polarity defects in mice (Barbieri et al. 2002). Following the OF closing, the optic fissure progenitors differentiate into either RPE cells or neural retina cells based on their position, and *Pax2* and *Vax1* expression gradually disappears from the ventral retina. The mutations in *Pax2*, *Vax1* and *Vax2* also cause the defects in regionalization, particularly setting up the boundaries among the OS, the NR and the RPE. Consistently, the defects in signaling pathways regulating regionalization of the OS and the ventral retina, such as Hh, BMP and retinoic acid (Kastner et al. 1994; Take-uchi et al. 2003; Morcillo et al. 2006), also result in coloboma formation, further underscoring the importance of correct regionalization of the OF in its closing. Here, I discuss the key transcription factors and signaling pathways in more detail:

Pax2* and *Pax6: In vertebrates, two members of paired-box homeodomain transcription factors, *Pax2* and *Pax6*, play critical roles in multiple steps of the eye development. *Pax2* is originally expressed in the OS and the ventral side of the OV. With the OV developing into the OC, *Pax2* expression is gradually restricted to the OF progenitors, the optic disc and the OS (Torres et al. 1996; Schwarz et al. 2000; Martinez-Morales et al. 2001). Consistent with its expression in the OF progenitors,

mutations in *Pax2* lead to the formation of coloboma, which could be due to the disturbed OC-OS boundary with the retinal cells extended to the OS area. In addition, due to developmental defects in the optic disc, the axons of retinal ganglion cells in the *Pax2* mutant mice project ipsilaterally instead of bilaterally (Favor et al. 1996; Torres et al. 1996; Otteson et al. 1998; Schwarz et al. 2000). *Pax6* is involved in ocular morphogenesis and is expressed in numerous ocular tissues during development (Walther and Gruss 1991; Nishina et al. 1999). In addition to aniridia, Peters anomaly, corneal dystrophy, congenital cataracts, and foveal hypoplasia (van Heyningen and Williamson 2002; Hanson 2003; Hever et al. 2006), loss-of-function *Pax6* mutations also cause coloboma through upregulation of *Pax2*, since Pax2 and Pax6 repress each other's expression (Azuma et al. 2003).

Vax1* and *Vax2: *Vax1* and *Vax2*, two closely related homeobox genes, show restricted but distinct expression domains in the developing eye, and their mutations cause coloboma but with different appearances. *Vax1* expression is restricted to the OS and the OF, similar to that of *Pax2*. *Vax1*-null mice develop coloboma with an obscure retina-optic nerve boundary (Bertuzzi et al. 1999; Hallonet et al. 1999). *Vax2* is a well-established ventral retina marker, and overexpression of *Vax2* in *Xenopus* and chicken leads to ventralization of the retina (Barbieri et al. 1999; Schulte et al. 1999). Loss of the function of *Vax2* in the mice results in coloboma without affecting optic nerve development, although trajectory of dorsal ganglion cells axons is abnormal (Barbieri et al. 1999; Barbieri et al. 2002). One recent study has also shown that *Vax1* and *Vax2* have redundant functions in the specification of the ventral retinal

cell fates (Mui et al. 2005).

The retinoic acid (RA) pathway: RA, the active form of vitamin A (retinol), is a pivotal regulator of morphogenesis and organogenesis, since vitamin A deficiency (VAD) severely affects embryonic development, causing defects in ocular, cardiac, respiratory and urogenital systems (Luo et al. 2006). Three retinaldehyde dehydrogenases (Raldh1-3) are involved in processing retinaldehyde into RA (Sandell et al. 2007). Interestingly, these *Raldh* genes have distinct expression patterns in the developing OC, with *Raldh1* in the dorsal side and *Raldh3* in the ventral side. Overexposure of developing eye primordial with RA results in upregulation of ventral retinal markers, suggesting that RA signaling is involved in retinal DV patterning (Hyatt et al. 1996; Lupo et al. 2005). One common phenotype for knockout mice of *Raldh*, and RA nuclear receptors, *RARs* and *RXRs,* is the shortening of the ventral retina (Kastner et al. 1994; Molotkov et al. 2006), further supporting the importance of RA signaling in the ventral retina development. In addition, *RXRa* knockout mice develop coloboma (Kastner et al. 1994), indicating a role of RA signaling in optic fissure development. Furthermore, ectopic administration of RA on the developing ocular primordium generates ectopic OF in the position near to the RA source (Hyatt et al. 1996). Thus, RA signaling is important for the specification of the ventral cell fates including OFs and for the closing of OF.

N-cadherin: A cell adhesion molecule, *N-Cadherin*, is ubiquitously expressed in the developing OC but is not restricted to the OF area. Its mutations in zebrafish lead to

the formation of the disorganized retina and the misrouted RGC axons, indicating that N-cadherin-mediated adhesion is important for retinal lamination and RGC axon targeting (Masai et al. 2003). In addition, *N-cadherin* mutants in zebrafish, *parachute* (*pac*), also develop coloboma probably due to cell movement defects, indicating the important role of cell adhesion in the OF closure (Masai et al. 2003).

The BMP pathways: BMPs, particularly *Bmp4* and *Bmp7*, have been implicated in eye development. *Bmp4* is expressed in the dorsal retinal, and its mutation leads to the ventralized retina in which the dorsal retinal cell fate is not specified, indicating that it plays an essential role in establishing the DV polarity (Koshiba-Takeuchi et al. 2000; Trousse et al. 2001; Behesti et al. 2006). Normally, *Bmp7* is expressed in the ventral midline and proximal region of the OV and then the OC. Depending on genetic backgrounds, *Bmp7*-null mice develop anophthalmia without the lens or microphthalmia lacking the OF and OD formation, which may be due to reduced expression of OF/OS genes including *Pax2* and *Vax1* (Morcillo et al. 2006). In addition to its role in OF formation, BMP signaling is also required for the closure of the OF. Overexpression of BMP antagonists, *noggin* and *Drm/Gremlin*, in the developing chicken OC cause coloboma formation, which further supports that BMP signaling is required for the normal development of the OF (Adler and Belecky-Adams 2002; Huillard et al. 2005).

The *Shh* pathway: As mentioned earlier, *Shh* is an essential midline signal for the bisection of the single eye field into two optic vesicles and for the proximal-distal

regionalization of eye structures through promoting *Pax2* expression and repressing *Pax6* expression (Chiang et al. 1996). When *Shh* is ectopically expressed in the developing OC, the OS region is expanded and the OF is widened at the early OC stage, indicating that *Shh* signaling is capable of modulating the OF cell fate specification (Zhang and Yang 2001). In humans, a mutation in *Shh* leads to non-syndromic microphthalmia with coloboma (Schimmenti et al. 2003). Thus, *Shh* signaling is required for the function and closing of the OF.

In conclusion, changes in multiple transcription factors and signaling pathways have been shown to cause the formation of coloboma, which indicates that the OF closure is also regulated by coordinated functions of these two groups of molecules. However, how these molecules are involved in regulating the closing process of the OF is far from clear.

III. FGF Signaling in the Eye

Overview of FGF signaling

FGFs represent a large family of growth factors that exist in a wide range of organisms from worms to humans. In invertebrates, *Drosophila* has three *Fgf* genes (*Branchless*, *Fgf8-like1* and *2*), while *caenorhabditis elegans* has two *Fgf* orthologs (egl-17 and let-756). In contrast, there have been 22 *Fgfs* identified in humans and mice, and they are subdivided into 7 subgroups based on their sequence similarity and

biochemical properties. Most Fgfs have an internal core region that shares 28 highly conserved and 6 identical amino-acid residues with 10 of these highly conserved residues involved in receptor binding. Most Fgfs (Fgf 3-8, 10, 15, 17-19, 21-23) have an N-terminal signal sequence for their secretion from the cell. Fgf9, 16, 20 do not have the signal sequence but are secreted, relying on a hydrophobic sequence on their N-terminal. Fgf1 and 2 have neither signal sequence nor hydrophobic sequence but they are nevertheless found on the cell surface or in the extracellular matrix, probably released by damaged cells or through exocytosis. Fgf11-14 do not have signal peptides, remain intracellular and function inside of the cell through a mechanism independent of receptor binding. Interestingly, Fgf2, together with Fgf3, has a protein isoform with a nuclear-localization signal, and these isoforms are localized in the nucleus although their biological functions are unclear (Powers et al. 2000; Ornitz and Itoh 2001; Itoh and Ornitz 2004).

In contrast to more than 20 ligands, there are only 4 receptor genes that exist in human and mouse genomes: *Fgfr1-4*. FGFRs are receptor tyrosine kinases that are composed of an extracellular ligand binding domain, a transmembrane domain and a split intracellular tyrosine kinase domain. The extracellular domain of FGFRs contains two to three immunoglobulin (Ig)-like domains. The alternative splicing of the third Ig-like domain generates IIIb or IIIc isoforms for Fgfr1-3, which further diversify the function of the FGF signaling system (Powers et al. 2000). Ligand binding results in receptor dimerization and phosphorylation, which trigger downstream signaling cascades including phospholipase C-γ (PLC-γ),

phosphatidylinositol-3 kinase (PI3K) and Ras-Mapk pathways (Tsang and Dawid 2004). Four FGFRs show distinct binding affinities to 22 ligands, and even isoforms of the same receptor have different specificities for different ligands (Powers et al. 2000; Itoh and Ornitz 2004). The isoform binding specificities play essential roles during organogenesis. A good example is the initiation of limb bud formation by Fgfr2: in the early developing limb bud, the epithelial splice form Fgfr2b is expressed in the ectoderm, while the mesenchymal splice form Fgfr2c is expressed in the limb mesenchyme (Xu et al. 1998; Lizarraga et al. 1999). The epithelial Fgfr2b is activated by Fgf7 and Fgf10 secreted by mesenchymal cells but can not be activated by Fgf8 secreted by the epithelium itself, which works specifically on mesenchymal Fgfr2c. By tightly controlling specific splicing events in different compartments and taking advantage of distinct binding affinities between two isoforms of Fgfr2, the limb bud forms. Proper functioning of the FGF pathway is indispensable to heparin or heparan sulfate (HS) glycosaminoglycans. HSs stabilize Fgfs from thermal denaturation and proteolysis, and also restrict their diffusion, while they regulate activities of Fgf-Fgfr complexes. As expected, mice with defects in HS biosynthesis phenocopy mice with defective FGF signaling (Powers et al., 2000).

Functions of FGF signaling during eye development

Among 22 members of mammalian *Fgfs*, some are exclusively expressed in embryos (*Fgf 3, 4, 8, 15, 17, 19*), while the others are expressed during both embryo and adult stages. The wide expression pattern and high evolutionary conservation indicate the importance of FGF signaling in embryonic development and homeostasis

of adult organs, and indeed genetic studies have declared the essential roles of several *Fgfs* and *Fgfrs* in heart, skeleton, hair, lung and neural system development as well as sexual determination (Ornitz and Itoh 2001; Coumoul and Deng 2003). FGF signaling is one of the key regulatory signaling pathways involved in many aspects of eye development ranging from the very beginning of the eye field formation to axon pathfinding (McFarlane et al. 1995; Chow and Lang 2001; Moore et al. 2004). As mentioned earlier, FGF signaling regulates the positioning of retinal progenitor cells within the definitive eye field by modulating ephrin signaling. Activating FGF signaling before gastrulation represses cell movements within the presumptive neural plate and retinal fate specification, while its inhibition promotes cell dispersal and increases eye field contribution (Moore et al. 2004). In *Xenopus*, FGF signaling controls RGC axon pathfinding (McFarlane et al. 1995; Webber et al. 2003). Another well-known function of FGF signaling in eye development is involved in specifying the neural retina. Much of this information comes from explant cultures of frog, chicken or mouse eye primordia: in the presence of Fgf1 or Fgf2, the presumptive RPE layer of the OC differentiates into the neural retina forming a mirror-imaged double neural retina, while addition of a neutralizing antibody to Fgf2 blocks neural differentiation of the presumptive neural retina (Guillemot and Cepko 1992; Pittack et al. 1997; Hyer et al. 1998a; Nguyen and Arnheiter 2000b). Overexpression of *Fgf9* in the presumptive RPE or proximal region of the optic vesicle leads to transdifferentiation of the PRE into the neural retina, which further supports the neural inducing activity of FGF signaling (Zhao and Overbeek 1999; Zhao and

Overbeek 2001). Although knockout mice for most members of *Fgf* family have been generated, no other eye defects in these knockout mice have been reported, except for *Fgf9* which show transdifferentiation of a small part of the neural retina to the RPE, suggesting functional redundancy among different *Fgfs* (Zhao et al. 2001). For example, although Fgf1, Fgf2 are known to be expressed in the surface ectoderm, single knockout or double knockout of these ligands do not show any overt eye defects. Since there are so many members in this family, it is difficult to dissect their functions in eye development simply by removing their functions one by one. An alternative approach to reveal the functions of FGF signaling during eye development is to disrupt the functions of their four receptors. Among the four receptors, *Fgfr3* and *Fgfr4* single or double knockout mice are viable and have normal looking eyes (Deng et al. 1996; Weinstein et al. 1998), indicating that the functions of FGF signaling is mostly carried out by the other two receptors. To overcome the embryonic lethality of *Fgfr1* and *Fgfr2* knockout mice (Yamaguchi et al. 1994; Arman et al. 1998), in this study, I have applied the Cre-LoxP system to conditionally knockout *Fgfr1* and/or *Fgfr2* from the developing eye to study their functions during eye development.

IV. The Cre-LoxP system

The homologous recombination-based gene knockout has greatly aided in understanding gene functions. Since the traditional knockout technology does not

allow us to temporally and spatially control the removal of gene functions to reveal their roles in a particular tissue at a given time, it is difficult to obtain information about the function of a gene in organogenesis and homeostasis of adult organs of those genes if its loss-of-function mutation results in early embryonic lethality. To circumvent the problem of the traditional knockout technology, the Cre-LoxP system has been developed to conditionally knockout a specific gene from specific cell types at a given time. In this system, Cre, a site-specific recombinase from P1 bacteriophage, is expressed under the control of a tissue or cell specific promoter, and a critical part of the target genes is flanked by LoxP sites, which is a 34bp consensus sequence with a specific orientation determined by the asymmetry of the core sequence in the middle. *Cre* specifically recognizes LoxP sequence and catalyzes the recombination between the two LoxP sequences. If the two LoxP sites are located on both sides of a sequence and oriented in the same orientation, this *Cre* catalyzed recombination results in a deletion of the sequence. So far, many tissue specific *Cre* lines and floxed alleles of many genes have been generated to study their functions in different developmental processes (Nagy 2000).

The drawback of the traditional Cre-LoxP system is that the knockout can not be controlled temporally. To circumvent this shortage, inducible Cre lines have been generated. One strategy to control the availability of the Cre recombinase activity temporally is to put the expression of the *Cre* protein under the control of an inducer. A good example of this strategy is *Mx1-Cre* line (Kuhn et al. 1995). Mouse *Mx1* gene is part of the defense system, which is silent in healthy mice, but can be

transiently activated by high amounts of transcription. In *Mx1-Cre* line, *Cre* normally does not express unless the mice are administrated with interferon α or synthetic double-stranded RNA, pI-pC. By controlling the accessibility of inducer to the mice, *Cre* activity is temporally controlled. Another strategy to temporally control the *Cre* activity is by using modified *Cre* variants (Lewandoski 2001). In this approach, *Cre*-coding region is fused with a modified ligand binding domain of estrogen receptor, which can be bound and activated by tamoxifen but not the endogenous steroid. Without tamoxifen, *Cre* is sequestered in the cytoplasm, and cannot reach the genomic DNA. Only when the mice are administrated with tamoxifen, can Cre be transferred into the nucleus, where it executes recombinase activity, thus controlling the knockout temporally.

The Cre-LoxP system has greatly helped people to understand functions of embryonic lethal genes in adult tissue homeostasis as well as embryonic development. In my dissertation study, I took advantage of this system to study functions of two embryonic lethal genes, *Fgfr1* and *Fgfr2*, during eye development.

Figure 1-1. Anatomy of the adult mouse eye

On the right is a cross-section of an adult mouse eye. The anterior compartment of the eye is composed of the cornea, the iris and the ciliary body. The lens resides in the middle of the eyeball. The posterior wall of the eye is occupied by the retina which is nourished by the choroidea and protected by the sclera. In the center of the retina, resides the optic disc, where the axons of the retinal ganglion cells exit the eye and travel through the optic nerve, the structure that connects with the optic disc, to the brain. On the right is a close-up look of the retina. The retina is composed of the neural retina (NR) and the retinal pigmented epithelium (RPE). Cell bodies of neurons and glia cells of the NR are well-laminated into three layers: the ganglion cell layer (GCL), the inner nuclear layer (INL) and the outer nuclear layer (ONL), which are separated by two synapse layers: the inner plexiform layer (IPL) and the outer plexiform layer (OPL). Outside of the ONL are the cell body segments of the photoreceptors, which are in direct contact with the RPE. The retina is nourished by the blood vessel-enriched choroidea, and further protected by the sclera.

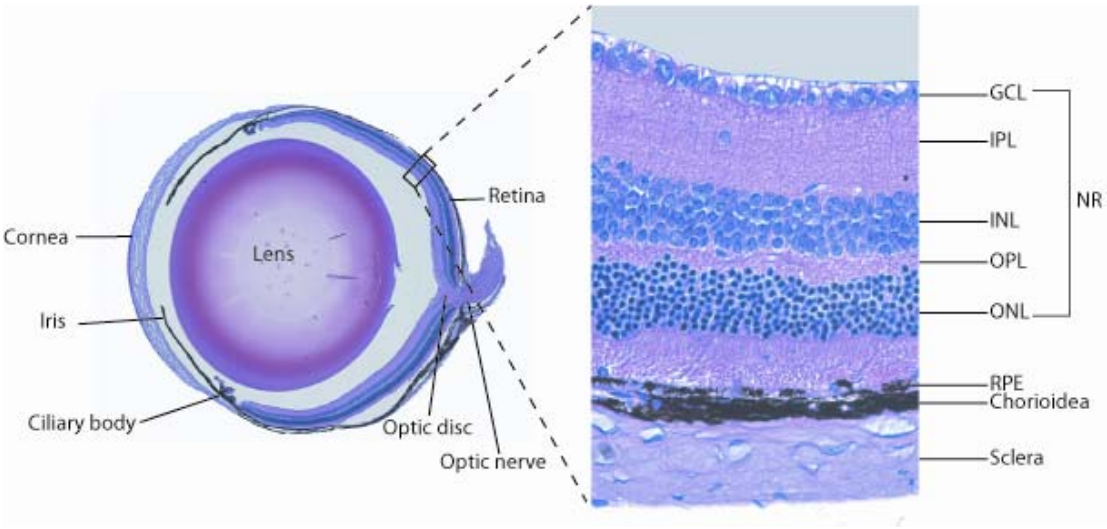

Cornea
Lens
Iris
Ciliary body
Retina
Optic disc
Optic nerve
GCL
IPL
INL
OPL
ONL
NR
RPE
Chorioidea
Sclera

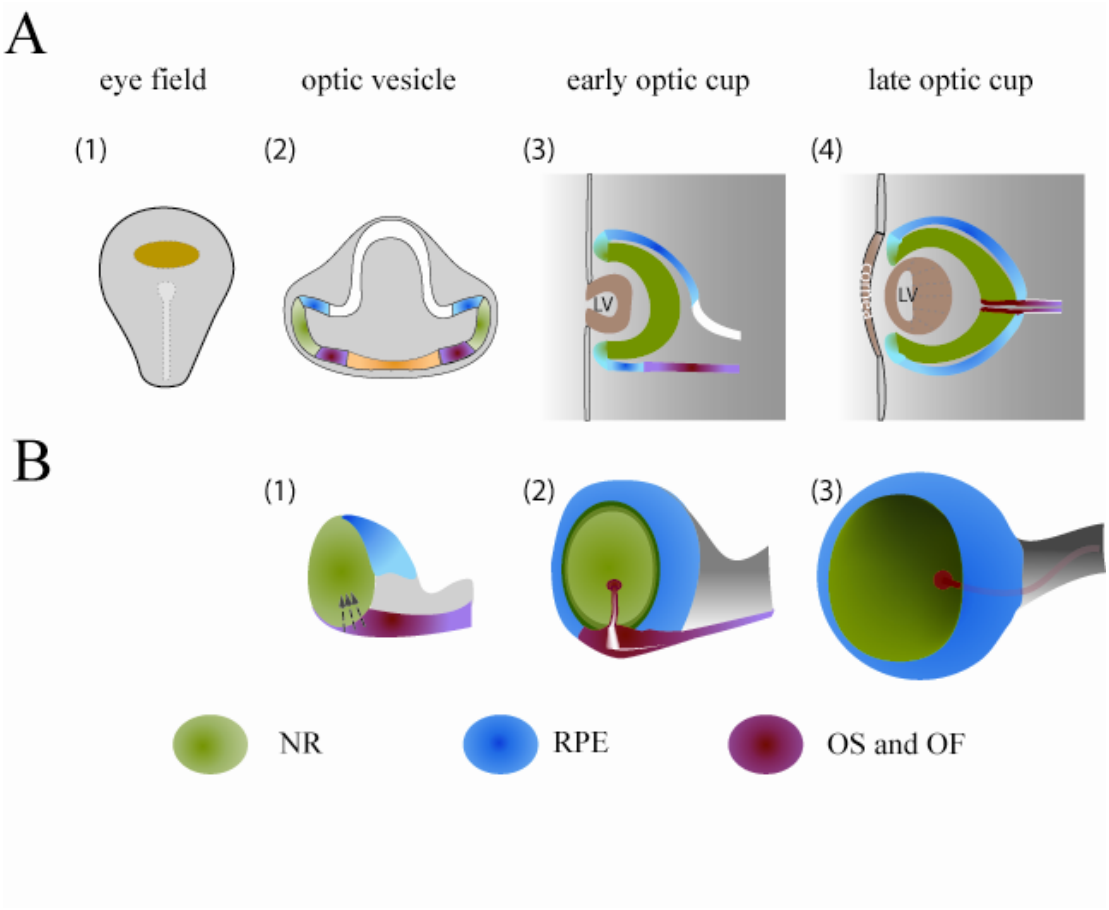
Figure 1-2. Schematics of the vertebrate eye development

(A) Overview of the eye development. (1) At the late gastrula stage, the eye primordium is a single specified eye field. (2) The bilateral evagination of the eye field forms the optic vesicles (OV). The most distal region of the OV (green) is the prospective neural retina (NR), which will directly contact the surface ectoderm later and invaginate, the dorsal distal region of the OV (blue) is the prospective RPE, which later will migrate and encircle the whole neural retina, and the ventral proximal region of the OV (red) is the optic stalk (OS), which connects the OV to the ventral forebrain (yellow). (3) When the OV reaches the surface ectoderm, it starts to invaginate to form the optic cup (OC). At the same time, the surface ectoderm also invaginates to form the lens vesicle (LV). (4) Completion of the invaginations establishes the overall structure of the eye. The originally distally located NR occupies the inside layer of the OC and the originally dorsal-distally located RPE migrates ventrally to wrap the NR and composes the outer layer of the OC, while the originally ventrally located OS is pushed to the midline of the OC. The invaginating LV eventually buds off the surface ectoderm, while the remaining surface ectoderm develops into the cornea.

(B) Perspective views of the developing eye emphasizing the development of the optic fissure (OF). (1) The invagination of the OV starts at the ventral-distal site and is oriented mediodorsally (indicated by arrows). (2) The invagination of the OV results in the OC, and leaves a transient opening on the ventral

side, the OF (arrow). (3) The OF closes at around E12, forming the morphologically symmetric OC leaving an exit for the axons of the retinal ganglion cells in the center of the retina, the optic disc (red dot in the center of the NR).

In both A and B, areas of the eye are colored differentially to indicate their eventual fates: green for the NR, blue for the RPE and red for the OS and the OF.


A
eye field
optic vesicle
early optic cup
late optic cup
(1)
(2)
(3)
(4)
LV
LV
B
(1)
(2)
(3)
NR
RPE
OS and OF

CHAPTER TWO

FGF SIGNALING CONTROLS THE OPTIC FISSURE CLOSING BY ORCHESTRATING PROLIFERATION, CELL FATE SWITCHES AND MORPHOLOGICAL CHANGES

I. Summary

Retinal coloboma is a congenital disease, which is caused by the failure to close the optic fissure. Although mutations in several genes have been known to cause coloboma, the molecular and cellular mechanisms underlying the failure of the OF closure remain largely unclear. In this study, we show that defective FGF signaling in the retina results in coloboma formation, and that FGF signaling normally controls the OF closure by regulating highly coordinated proliferation, morphological changes and cell fate switches of OF progenitor cells. The removal of *Fgfr1* and *Fgfr2* functions from the embryonic retina leads to reduction of retinal cell proliferation, gradual loss of the cell fate of the OF progenitors and the differentiation of all OF progenitors to RPE cells instead of both neural retinal cells and RPE cells. It is described here, for the first time, that the OF progenitors gradually differentiate into neural retinal and RPE cells, which undergo coordinated cell morphological changes in both the RPE layer and the neural retina layer in the OF lips just before the OF closure. The OF closure proceeds first with the fusion of the RPE layer, and then the

integration of the retinal cells. The coordinated morphological changes fail to take place in the FGF signaling defective retina. Finally, FGF signaling is important for the formation of the optic disc and the maintenance of the optic stalk. We propose that the coordinated cell proliferation, cell morphological changes and cell fate switches collectively contribute to OF closure, and the defective FGF signaling leads to coloboma formation by disrupting such highly orchestrated cellular events. Therefore, this study has provided novel insight into how coloboma develops.

II. Introduction

Retinal coloboma affects 2.6 babies per 10,000 births, and it occurs in isolation or is associated with developmental syndromes (Chang et al. 2006). The formation of retinal coloboma is due to the failure in the OF closure. During early eye development, the asymmetric invagination of the optic vesicle proceeding from the ventral side to the dorsal side results in the formation of the optic fissure, a cleft on the ventral optic cup (Chang et al. 2006). The fissure, extending from the distal edge of the optic cup to the optic stalk, represents a temporary passage for mesenchymal cells to migrate into the developing eye chamber and then form hyaloid vessels for blood supply for the developing eye. Soon after the mesenchymal cells finish their migration, the OF begins its closing process from the middle point and proceeds distally and proximally to close the fissure, leaving a permanent opening, the optic disc (OD), in the proximal end for optic nerves to exit the eye. After completing OF

closure, the retina in the ventral optic cup is continuous and is morphologically identical to the dorsal part.

Genetic studies in humans and mice have identified a number of mammalian genes associated with coloboma formation, encoding transcription factors and signaling molecules (Chang et al. 2006). Transcription factors include *Chd7*, *Rybp*, *Pax2*, *Pax6*, *Vax1* and *Vax2*. *Chd7*, a causative gene of the CHARGE syndrome, which includes coloboma in its phenotype, encodes a chromodomain-containing DNA helicase (Vissers et al. 2004), while *Rybp*, encoding a zinc finger protein interacting with polycomb complexes, is also involved in coloboma formation (Pirity et al. 2007). In addition, two closely related *Pax* genes, *Pax2* (Sanyanusin et al. 1995; Favor et al. 1996) and *Pax6* (Azuma et al. 2003), and two closely related *Vax* genes, *Vax1* (Hallonet et al. 1999) and *Vax2* (Barbieri et al. 2002), have been implicated in coloboma formation. Both Chd7 and Rybp are involved in remodeling chromatin to facilitate gene expression, while the *Pax* and *Vax* proteins directly bind to the promoters of specific target genes to control their expression. The signaling molecules include *Shh* (Take-uchi et al. 2003; Morcillo et al. 2006), retinoic acid signaling (McGannon et al. 2006), and *JNK1* and *JNK2* (Weston et al. 2003). As expected, transcription factors and signaling pathways function in a concerted manner to control the OF closing process. Indeed, *Shh* regulates *Pax2* expression in eyes of both fish and mammals (Dakubo et al. 2003)((Macdonald et al. 1995), while *JNKs* initiate the expression of *BMP4* and *Shh* that induces *Pax2* expression and OF closure in mice (Weston et al. 2003). *BMP7* has been recently shown to work with *Shh* to

pattern the OF in a stepwise manner regulating expression of *Pax2* and *Vax* expression (Morcillo et al. 2006), whereas *Shh* signaling is required to maintain *Vax* gene expression in the OD (Take-uchi et al. 2003). During embryonic eye development, pax2 and pax6, which are expressed in the optic stalk and in the optic cup, respectively, reciprocally repress each other to maintain the boundary between the optic cup and the optic stalk (Schwarz M, et al., 2000; Favor J, et al., 1996; Torres M, et al., 1996; Otteson DC, et al., 1998). However, it remains largely unclear how cellular changes caused by mutations in the transcription factors and signaling molecules lead to the failure in the OF closure. In this study, we have carefully examined cell behaviors during the closing process of the OF in the developing eye to shed light on how the process might be controlled normally.

FGF signaling plays important roles in multiple stages of the eye development, including optic cup patterning, neural retina dorsal-ventral and nasal-temple specification, retinal neuron differentiation, axon growth and targeting (Hyer et al. 1998b; Galy et al. 2002; Zhang et al. 2003; Horsford et al. 2005; Martinez-Morales et al. 2005; Spence et al. 2007). Because *Fgf 1, 2, 4, 8, 9* and *15* are known to be expressed in the developing retina, it is difficult to determine their roles separately. Since there are only four *Fgf* receptor genes, *Fgfr1-4*, and *Fgfr3* and *Fgfr4* mutants are viable with no obvious eye defects (Deng et al. 1996; Weinstein et al. 1998), it is much easier to determine the potential roles of FGF signaling in eye development by removing *Fgfr1* and *Fgfr2* conditionally from the eye. In this study, we show that conditional knockout of *Fgfr1* and *Fgfr2* function specifically from the

optic cup and optic stalk causes the formation of coloboma, the degeneration of the optic stalk and the retardation of neural retina proliferation. We also have observed that retinal cell proliferation and dynamic changes in cell shapes appear to drive the OF closure. Since FGF signaling has been shown to be involved in controlling cell movements and shape changes in a variety of organisms ranging from *Drosophila* to mammals (Murphy et al. 1995; Beiman et al. 1996; Gryzik and Muller 2004; Schumacher et al. 2004; Stathopoulos et al. 2004; Chuai et al. 2006), our results suggest that FGF signaling is involved in controlling the OF closing process by regulating cell proliferation, cell morphological changes and cell fate determination. The potential cellular mechanisms contributing to coloboma formation revealed in mice might be useful for understanding how coloboma develops in humans.

III. Results

Fgfr1 and *Fgfr2* Double Conditional Knockout Eyes Develop Coloboma, Optic Nerve Aplasia and Mild Microphthalmia

To investigate the role (s) of FGF signaling during eye development, we used *Six3-Cre* Cre line and *Fgfr1* and *Fgfr2* conditional alleles to specifically remove *Fgfr1* and *Fgfr2* functions from developing eyes. In the *Six3-Cre* line, *Cre* begins its expression from around embryonic day 9.5 (E9.5), and functions throughout the developing optic cup and the optic stalk (Furuta et al., 2000), while *Fgfr1* and *Fgfr2*

conditional alleles generate loss-of-function null alleles when the LoxP flanked fragments are deleted by *Cre* (Yu et al., 2003; Xu et al., 2002). When *Fgfr1* or *Fgfr2* was removed individually from the developing eye by *Six3-Cre*, no overt eye defects were observed (data not shown). Because *Fgfr1* and *Fgfr2* have overlapping binding specificities for FGF ligands (Powers et al., 2000), the lack of detectable eye mutant phenotypes in *Fgfr1* or *Fgfr2* single conditional knockout mice could be due to possible functional redundancy between the two receptors. To test this possibility, we used the same *Six3-Cre* line to simultaneously remove functions of both the receptors from the developing eye. Since *Six3-Cre; Fgfr1*$^{fx/+}$*; Fgfr2*$^{fx/+}$, *Six3-Cre; Fgfr1*$^{fx/fx}$*; Fgfr2*$^{fx/+}$ and *Six3-Cre; Fgfr1*$^{fx/+}$*; Fgfr2*$^{fx/fx}$ mice do not show any eye defects, all these littermates to *Six3-Cre; Fgfr1*$^{fx/fx}$*; Fgfr2*$^{fx/fx}$ are referred hereafter to as control mice. At E13.5, the eyes of the *Six3-Cre; Fgfr1*$^{fx/fx}$*; Fgfr2*$^{fx/fx}$ double knockout mice (hereafter, referred to as *Fgfr* mutants) show two obvious retinal defects when compared with their control littermates. First, the *Fgfr* mutant eyes are slightly smaller than the control eyes (compare Fig.2-1 A, A' and 2-1B, B'). Second, the *Fgfr* mutant eyes show an obvious cleft on the ventral side (Fig. 2-1B' arrowhead), representing the unclosed OF. In a normal E13.5 wild-type eye, the OF should have fused completely with the eyeball being wrapped by a continuous RPE layer (Fig. 2-1A and 2-1A'). To determine if the remaining cleft on the ventral side is simply due to the delay in the eye development, we examined the *Fgfr* mutant eyes at later stages. In the HE stained control newborn eye sections, the optic disc, where the axons of the retinal ganglion cells exit the eye, and the optic nerve can be easily

identified (Fig. 2-1C, green arrowhead), while they are completely absent in any of the serial sections throughout the whole *Fgfr* mutant eyes (Fig. 2-1D). Instead, the unclosed OF remains in the *Fgfr* mutant newborn eye, leaving a permanent gap in the ventral retina (Fig. 2-1D, green arrowhead). Consistent with the idea that the unclosed fissure is not simply due to the developmental delay in the *Fgfr* mutant eyes, the enucleated P15 control eyeballs show a round pupil formed by the intact iris on the front and an optic nerve cord on the back (Fig. 2-1E), while the pupil of the *Fgfr* mutant eyeballs appears oval due to the lack of iris along the unclosed OF, and the optic nerve cord is missing from these eyes (Fig. 2-1F). Taken together, *Fgfr1* and *Fgfr2* double conditional knockout eyes develop coloboma, optic nerve aplasia and mild microphthalmia.

Coloboma Formation in the *Fgfr* Mutant Eyes Is Not Due to Dorsal-Ventral Patterning Defects.

Since the OF forms on the ventral side of the eye, any perturbation in the ventral retinal cell fate specification could lead to coloboma. Indeed, a deletion mutation of *Vax2*, a ventral retina cell fate determination transcription factor (Schulte et al. 2005; Barbieri et al. 1999), leads to coloboma in addition to dorsal-ventral (DV) polarity defects (Barbieri et al. 2002). In addition, FGF signaling has been shown to control tissue patterning in several mammalian tissues (Serls et al. 2005; Sun et al. 2002). Thus, our observation that *Fgfr* mutant eyes develop coloboma raises the

possibility that coloboma in these eyes results from the DV patterning defects caused by defective FGF signaling. To investigate this possibility, we examined the expression pattern of two well-established ventral retina markers, *Vax2* and *Raldh3*, by in situ hybridization. In the control optic cups, *Vax2* and *Raldh3* are expressed in the cells on the ventral side including OF progenitor cells (Fig. 2-2A and 2-2B). In the *Fgfr* mutant optic cups, *Vax2* and *Raldh3* appear to be present on the ventral side at similar levels to those in the control optic cups (Fig. 2-2C and 2-2D). These results indicate that *Fgfr* mutant eyes do not have obvious DV patterning defects, which could not be the reason for coloboma formation.

FGF Signaling Is Active in the Entire Retina and Is Severely Affected in the OF Region of the *Fgfr* Mutant Eyes.

To investigate how *Fgfr1* and *Fgfr2* are involved in the control of the OF closure, we first checked the expression patterns of the two receptors in the optic cups of the E11.5 embryos, in which the fissure begins to close. Since only *Fgfr1* and *Fgfr2* double, but not single, mutant eyes show defects, we expected both receptors to be expressed in the retina, particularly in the ventral optic cup. And indeed, both *Fgfr1* and *Fgfr2* mRNAs are present in the neural retina (Fig. 2-3A and2-3B) which is consistent with expression studies in rats and chicken (Wanaka et al. 1991; Tcheng et al. 1994). However, it appears that *Fgfr1* mRNAs are expressed at low levels in the RPE and two lips of the OF, while *Fgfr2* mRNAs are present in these cells at

higher levels. In addition, *Fgfr1* and *Fgfr2* mRNAs are also expressed in surrounding mesenchymal tissues, but the *Fgfr2* mRNAs appear to be higher (Fig. 2-3A and 2-3B). Four FGF receptors are members of a transmembrane receptor tyrosine kinase family (Powers et al. 2000). Upon ligand binding, FGFR tyrosine kinases are activated and can trigger several downstream signaling cascades, one of which is the Ras-mediated extracellular-signal-regulated-protein-kinase (ERK) pathway, which can be reliably monitored by the expression of phosphorylated ERK (pERK) (Tsang and Dawid 2004; Eswarakumar et al. 2005). It has been shown that ectopic expression of constitutively active Ras in the proximal region of the optic vesicle could mimic FGF signaling activity to convert the presumptive RPE to neural retina (Zhao et al. 2001), indicating that the function of FGF signaling on neural retina specification is mediated by the Ras-ERK pathway. To test whether this pathway is active in the developing optic cup in the normal situation and whether it is involved in the OF closure, we did p-ERK immunohistochemistry on the sagittal sections of E11.5 control optic cups. Consistent with the existence of several FGF ligands and receptors in the optic cup, pERK kinase is present in the entire retina including the OF, but the level is much lower in the RPE layer (Fig. 2-3C). In addition, it appears that FGF signaling has an activity gradient with a higher activity on the dorsal side.

To further determine how removal of *Fgfr1* and *Fgfr2* functions from the retina affects FGF signaling using the *six3-Cre* line, we first examined the Cre expression pattern in the control optic cups using a Z/EG reporter line. In the *Z/EG* reporter line, the removal of the *lacZ* gene flanked by two *LoxP* sites leads to GFP

expression so that the GFP expression pattern should reflect the *Cre* expression pattern (Novak et al. 2000). In the three pairs of the eyes carrying *six3-Cre* and *Z/EG* in the *Fgfr1* and *Fgfr2* double heterozygous background examined, GFP expression is quite mosaic, yet exhibiting obvious patterns (Fig. 2-3D). There are more GFP-positive retinal cells on the temporal side (45.1 ±14.9%; n=3 mice) than on the nasal side (22.7±8.4%; n=3 mice), while there are even more GFP-positive cells in the ventral-most (51.7±14.3%; n=3 mice) as well as the dorsal-most (67.2%±8.3%; n=3 mice) areas. The GFP expression patterns in the control retinas suggest that the six3-Cre line can efficiently remove the functions of *Fgfr1* and *Fgfr2* from the OF area, which may explain the coloboma phenotype of the double conditional *Fgfr* mutant eyes.

To then confirm whether the *six3-Cre* line can indeed efficiently remove the functions of *Fgfr1* and *Fgfr2* to block FGF signaling in the OF area as its expression patterns predict, we determined the expression patterns of pERK in the E11.5 *Six3-Cre; Fgfr1*$^{fx/fx}$*; Fgfr2*$^{fx/fx}$ optic cups. Interestingly, in the *Fgfr* mutant optic cup, pERK staining is almost completely eliminated from the OF area, an indication of severe reduction of FGF signaling in the OF progenitor cells (Fig. 2-3E). Surprisingly, pERK expression is only slightly downregulated or not changed at all in the rest of the mutant retinas (Fig. 2-3E). One explanation for the differences of the pERK level changes between the OF and the other parts of the retina is that OF progenitor cells rely mostly on *Fgfr1* and *Fgfr2*, while other parts of the retina could use the other two receptors *Fgfr3* and *Fgfr4* to compensate *Fgfr1* and *Fgfr2* functions. To test this

possibility, we have carefully determined GFP expression patterns in the optic cups of *Six3-Cre; Fgfr1*$^{fx/fx}$*; Fgfr2*$^{fx/fx}$*; Z/EG* mice, which carry the Cre-LoxP system reporter *Z/EG* in the *Fgfr* double mutant mice. To our surprise, the GFP expression in these *Fgfr* mutant optic cups is not only mosaic but also shows distinct patterns. Most GFP^{+} *Fgfr* mutant cells are accumulated in the OF region (58.0±13.9%; n= 3 eyes) and the temporal side (61.5±22.8% ; n= 3 eyes) of the *Fgfr* mutant neural retina, leaving the nasal side almost absent of the GFP^{+} cells (4.3±2%; n= 3 eyes) (Fig. 2-3F). Since we did not observe obvious TUNEL activity differences between control and mutant retinas (data not shown), we conclude that *Fgfr1* and *Fgfr2* are two important FGF receptors for transducing FGF signaling in the OF progenitors and that the function of FGF signaling in the OF progenitor cells is likely mediated by the Ras-ERK signaling pathway.

FGF Signaling Controls Proliferation of Retinal and Fissure Progenitor Cells

Six3-Cre; Fgfr1$^{fx/fx}$*; Fgfr2*$^{fx/fx}$ mice develop microphthalmia, suggesting that FGF signaling controls either proliferation or survival of retinal progenitor cells. To determine the proliferation status in both the control and the *Fgfr* mutant eyes, we labeled the S-phase cells in the retinas by incorporating a nucleotide analog, BrdU. In the control eyes, the retinal progenitor cells in the nasal and temporal quadrants are 59.2%$\pm$4.8% and 54.8%$\pm$6.9% BrdU-positive, respectively, while the OF progenitors are 22.2%$\pm$7.5% BrdU positive (three E11.5 control eyes) (Fig. 2-4A and 2-4A').

These results indicate that the retinal progenitor cells regardless of their temporal or nasal positions proliferate similarly (p=0.90), but they proliferate faster than the fissure progenitor cells (p=0.001). In contrast, in the *Fgfr* mutant eyes, the retinal progenitor cells in the nasal and temporal quadrants are 48.9%$\pm$6.3% and 23.1%$\pm$2.1% BrdU positive, respectively, which are significantly lower than those in the corresponding quadrants of the control eyes (p=0.04 and p=0.0007 for nasal and temporal quadrants, respectively; three *Fgfr* mutant E11.5 eyes) (Fig. 2-4B). Similarly, the OF progenitors in the mutant eyes are 10.1%$\pm$5.1% BrdU positive, which is significantly lower than those in the control eyes (p=0.04) (Fig. 2-4B'). These results demonstrate that retinal progenitor cells on the nasal and temporal sides as well as the OF progenitors on the ventral side proliferate significantly slower in the *Fgfr* mutant eyes than those corresponding cells in the control eyes, which may explain why the *Fgfr* mutant eyes are smaller than control eyes.

To further determine if FGF signaling also regulates the survival of retinal and OF progenitors, we used TUNEL labeling to detect dying cells in both the control and *Fgfr* mutant eyes of E11.5 embryos. Only rare retinal progenitors in the entire retina except the dorsal-most region in the control eyes are positive for TUNEL labeling, while 13% of the OF progenitors are TUNEL-positive, indicating that there exists limited cell death in the OF progenitors and the retinal progenitors in the dorsal-most region (Fig. 2-4C). In the E11.5 *Fgfr* mutant eyes, there is no detectable cell death in the retinal progenitors except in the dorsal-most region, while there is no dramatic increase in the number of TUNEL-positive OF progenitors, suggesting that FGF

signaling does not primarily control the survival of retinal and OF progenitors in the developing E11.5 retina (Fig. 2-4D). Along with the data from BrdU labeling experiments, we conclude that FGF signaling controls the proliferation of retinal and OF progenitors in the developing retina.

The Fate of the OF Progenitors in the *Fgfr* Mutant Eye is Established Initially but Fails to be Maintained

FGF signaling has been shown to regulate specification and/or maintenance of different cell fates in different tissue types (Ciruna and Rossant 2001; Kudoh et al. 2004; Serls et al. 2005). One of the obvious possibilities is that FGF signaling is required for either establishment or maintenance of the OF progenitor cell fate, and thereby for controlling the OF closure. To investigate this possibility, we first studied the expression of the molecular markers for OF progenitors, retinal progenitors and RPE cells in the developing retina of the E10.5-E11.5 control and *Fgfr* mutant eyes. Pax2 is a molecular marker for OF progenitors (Otteson et al. 1998), while Pax6 is expressed in both the neural retina and the RPE (Hitchcock et al. 1996). Chx10 is a molecular marker for all the neural retinal progenitors except the OF progenitors (Burmeister et al. 1996), while Mitf is expressed in the developing RPE cells (Amae et al. 1998). At E10.5, the wild-type control and mutant *Fgfr* eyes show normal Pax2, Pax6, Chx10 and Mitf expression pattern: Pax2 is expressed similarly in the developing OF progenitors in both the mutant and control eyes, while Chx10 and Mitf

were also normally expressed in the neural retina and the RPE of the control and mutant eyes, respectively (Fig. 2-5A, A' and 2-5B, B'). The retinas of the *Fgfr* mutant E10.5 eyes appear to be thinner than the ones in the littermate control eyes, indicating that the function of FGF signaling in the retina has been disrupted by removing *Fgfr1* and *Fgfr2* at E10.5 (Fig. 2-5A' and 2-5B'). These results suggest that FGF signaling is dispensable for the establishment of different retinal cell fates, including OF progenitors, but is required for the proliferation of these early progenitor cells.

At E11.5, Pax6 remains expressed in the neural retina and the RPE but not in the OF progenitors of the control eyes, while Pax2 is expressed in the OF progenitors only (Fig. 2-5C). In contrast, Pax6 starts to be expressed in the OF progenitors of the *Fgfr* mutant eyes, while Pax2 expression in these mutant progenitors declines dramatically (Fig. 2-5C'). This result is consistent with the idea that Pax6 and Pax2 reciprocally repress each other in the developing embryonic retina (Schwarz et al. 2000). Since *Pax2* is required for maintaining the OF progenitor cell fate (Sanyanusin et al. 1995), this result also suggests that the *fgfr* mutant fissure progenitors gradually lose their identity in the absence of FGF signaling. Since both Pax6 and Mitf are highly expressed in the developing RPE cells of the embryonic eye to promote RPE differentiation, Pax6 upregulation in the *Fgfr* mutant fissure progenitors may lead to the cell fate switch from the fissure progenitors to RPE cells. Then, we determined expression patterns of Chx10 and Mitf, which are expressed in the neural progenitor cells and the developing RPE cells, respectively. In the wild-type E11.5 eye, Chx10 is

expressed in the retinal layer of the OF progenitors, switching those progenitors to neural progenitors, while Mitf also gains its expression in the RPE layer of the OF progenitors, converting those progenitors to RPE cells (Fig. 2-5D and 2-5E). In the E11.5 *Fgfr* mutant eyes, however, Mitf expression is not only expressed in the OF progenitors in the RPE layer but is also dramatically upregulated in the OF progenitors in the neural retina layer, which normally develop into the neural retina (Fig. 2-5D' and 2-5E'). Chx10 and Mitf are known to mutually repress each other in the boundary between the neural retina and the RPE (Rowan et al. 2004; Horsford et al. 2005). Consistently, Chx10 should be normally expressed in the OF progenitors in the retinal layer of the wild-type eyes, but is dramatically downregulated in the *Fgfr* mutant eyes (Fig. 2-5D'). Since the Mitf expression in the OF progenitors in the neural retina layer could potentially push their differentiation into the RPE lineage instead of the neural retina, we then examined the expression of a molecular marker for more mature RPE cells, *Dct,* in older *Fgfr* mutant eyes (E13.5). Normally, *Dct* mRNAs are present in mature RPE cells and the presumptive ciliary body progenitors on the periphery of the E13.5 neural retina (Fig. 2-5F). Consistent with the idea that the OF progenitors in the unclosed fissure develop into RPE cells, the cells in the periphery of the unclosed fissure of the E13.5 mutant *Fgfr* eyes express *Dct* mRNAs and have also accumulated the dark pigment (Fig. 2-5F'). Taken together, we conclude that in the absence of FGF signaling, all the OF progenitors adopt the RPE cell fate instead of half of them in the neural retina layer differentiating into the neural retina, and that FGF signaling is required for maintaining the OF progenitor

cell fate and the proper switch of the OF progenitors in the neural retina layer into the neural retina.

Dynamic Cell Movements and Morphological Changes of the OF Progenitors During the OF Closing Process

It has been widely established that coordinated cytoskeletal changes are critical for regulating cell movements and tissue integration of different cell types in a variety of organisms (Marx 2003). In order to gain a better understanding of the OF closing process, we carefully examined the dynamic cytoskeletal changes during the normal OF development in great detail. At E10.5, the two lips of the OF start to become close to each other, still leaving a gap between them through which mesenchymal cells continue to migrate (Fig. 2-6A). The OF progenitors in both the neural retina and RPR layers exhibit obvious apical-basal polarity, which is evidenced by the accumulation of F-actin filaments on their apical side and the continuous layer of the basal membrane (Fig. 2-6A). The previous studies have indicated that the OF closure initiates in the middle of the proximal-distal axis and proceeds in both directions to the most proximal and distal points (Hero 1990). Indeed, at E11.5, the gap between the OF lips is getting narrower and narrower from the most distal to the middle point, and it completely disappears in the middle point of the OF (Fig. 2-6B-D). At this time point, the basal membranes on both sides of the OF lips remain intact, however, the F-actin filaments start to heavily accumulate on both the basal

and apical sides of the neural retina (Fig. 2-6B-D). Interestingly, the OF progenitors in the neural retina layer continue to elongate from the most distal to the middle point, while the OF progenitors in the RPE layer become more flat when approaching the middle point (Fig. 2-6B-D). At E11.75, while the same trend observed at E11.5 continues (Fig. 2-6E-H), in the middle point of the OF at this developmental stage, the basal membranes on both sides of the OF lips start to fuse and become one (Fig. 2-6G). At E11.875, in more area of the OF, the basal membranes on both sides of the OF lips fuse together (Fig. 2-6I), and the F-actin accumulation on both the basal and apical sides is strong. In the locations more close to or at the middle point of the OF, the basal membranes completely dissolve in the RPE layer, and begin to partially disappear in the retinal layer, indicating that the RPE layer of the OF has already fused together, while the retinal layer is in the process of fusing together (Fig 2-6J-K). At E12.0, the retinal layer of the OF has also finished the fusion, and interestingly, the part of the neural retina originated from the OF progenitors proliferates and bulges in, while the RPE cells originated from the OF progenitors become the part of the continuous RPE layer (Fig. 2-6L). At E12.5, the bulged part of the retina has also flattened and becomes part of the continuous neural retina layer, which directly contacts the RPE layer (data not shown). These observations represent a detailed description, for the first time to our knowledge, of the closing process of the OF.

The *Fgfr1 Fgfr2* Mutant OF Progenitors Fail to Undergo Proper Morphological Changes in the Process of the Fissure Closing

Having discovered how the OF closes at the cellular level in the wild-type eye, we then determined why the *Fgfr* mutant OFs fail to close. *N-cadherin* has been implicated in coloboma formation in zebrafish since its mutations lead to defects in the closing of the fissure (Masai et al., 2003), while FGF signaling has been shown to regulate mesoderm cell fate specification and morphogenetic movements at the primitive streak of the mouse embryo (Ciruna and Rossant 2001). Thus, we examined the expression and cellular localization of N-cadherin and F-actin filaments at both the control and *Fgfr* mutant E10.5 and E11.875 eyes. At E10.5, the two lips of the OF in the middle point of the A-P axis, where the OF is going to fuse first, in both the control and *Fgfr* mutant eyes have moved close to each other for the preparation of the closing (Fig. 2-7A-A' and 2-7B-B'). In this stage, in both the control and mutant eyes, the OF progenitors express comparably high levels of N-cadherin (Fig. 2-7A-A' and 2-7B-B') and F-actin (not shown) in their apical side but relatively lower levels on their lateral sides and the basal side, indicating that the OF closure defects in the Fgfr mutant eyes must take place after E10.5.

Since the OF closing process proceeds from the middle point of the fissure to the periphery (toward both the most distal and proximal points of the OF), instead of studying different developmental time points, we could simply examine different positions of the OF at E11.875, when the OF has already started to fuse in the middle

point, to obtain the essential information of different stages of the OF closing process. In the E11.875 control eyes, at the distal point of the OF, the two lips of the fissure have moved close to each other like those at the middle point at E10.5 (Fig. 2-7C). On both the apical and basal sides of the OF progenitors, F-actin accumulation remains at high levels, but N-cadherin expression has already reduced on their apical side (Fig. 2-7C'). In the sections closer to the middle point of the fissure at the A-P axis, the RPE layer and the retinal layer of the fissure express lower levels of N-cadherin and F-actin on their apical side (Fig. 2-7 D and D'). In the middle point of the A-P axis, the RPE layers at both sides of the fissure have begun to fuse with each other and generate a continuous RPE layer, where N-cadherin and F-actin are the lowest (Fig. 2-7E and E'). In contrast, in the *Fgfr* mutant E11.875 mutant eyes, from the more distal point of the A-P axis to the middle point, the two lips of the fissure still leave a gap where some mesenchymal cells continue to migrate in, and F-actin and N-cadherin remain expressed at high levels, particularly on the lateral sides (Fig. 2-7G-J'). Interestingly, in the *Fgfr* mutant retina, the lip on the nasal side is frequently positioned up and away from the lip on the temporal side, which could be the reason why the fissure fails to close. These results suggest that the failure in the downregulation of N-cadherin and F-actin, which is caused by defective FGF signaling, may directly contribute to coloboma formation.

We have also noticed dramatic differences in the thickness of the RPE layer and the retinal layer of the fissure between the control and *Fgfr* mutant eyes. In the OF area of the *Fgfr* mutant eyes, the RPE layer is thicker, while the retinal layer is

much thinner than those in the control eyes (compare the red and yellow lines in Fig. 2-7C-E with those in 2-7G-I). The differences are more pronounced at the middle point since the RPE layer becomes more flat there (compare Fig. 2-7E with 2-7I). Prior to the closing of the fissure, the RPE layer begins to flatten by stretching out, while the retinal layer starts to thicken by elongating the length of the OF progenitors in the retinal layer. Such anticipated morphological changes fail to take place in the *Fgfr* mutant eyes, and as a result, the progenitors in the RPE and retinal layers of the OF are longer or shorter than those in the control eyes, respectively (compare outlined cells in Fig. 2-7F and 2-7J). This failure can be attributed to the failure in the down-regulation of N-cadherin expression in the mutant eye (Fig, 2-7F' and 2-7J'). Taken together, these observations suggest that N-cadherin downregulation in the OF progenitors regulated by FGF signaling may lead to morphological changes and thus the OF closure, and further suggest that morphological defects of the OF progenitors, which might be caused by higher levels of N-cadherin expression in the *Fgfr* mutant eyes, lead to coloboma formation.

The Optic Disc Fails to be Specified and the Optic Stalks Degenerates in the *Fgfr* Mutant Eyes

Pax2 is also essential for the development of the optic stalk since its mutation leads to transdifferentiation of the optic stalk into the neural retina (Torres et al. 1996; Schwarz et al. 2000). As shown earlier, in the *Fgfr* mutant eyes, the OF progenitors

fail to maintain *Pax2* expression though its initial expression is established. To determine whether the optic stalk progenitors are ever specified in the *Fgfr* mutant eyes, we analyzed expression patterns of *Pax2* in the mutant and control eyes at different developmental stages. At E11.5, in addition to its expression in the OF progenitors, Pax2 is also expressed in the optic stalk of both the mutant (Fig. 2-8B and 2-8F) and control (Fig. 2-8A and 2-8E) eyes, indicating that the optic stalk cell fate is established in the *Fgfr* mutant eyes. However, the optic disc does not form in the *Fgfr* mutant E11.5 retina (Fig. 2-8D). At E13.5, when the OF has already completely closed in the control eyes, Pax2 expression disappears from the ventral retina, and is restricted to the optic stalk and the optic disc, a ring-shaped structure located in the center of the neural retina that wraps the projecting axons of the retina ganglion cells (Fig. 2-8G). In contrast, Pax2 expression in the *Fgfr* mutant retina completely disappears, and the Pax2 positive mutant optic stalk cells are dramatically reduced (Fig. 2-8H). At E15.5, Pax2 is still expressed in the optic disc and astrocytes of the optic nerve of the control eyes (Fig. 2-8I), but completely disappears from *Fgfr* mutant eyes (Fig. 2-8J). These observations indicate that the optic stalk is properly specified but fails to be maintained in the *Fgfr* mutant eyes.

As we showed earlier, FGF signaling is important for proliferation of retinal progenitor cells. Thus, we also applied the BrdU incorporation assay to determine the proliferation rates of the optic stalk progenitors in both the control and mutant eyes. There are no obvious differences in the BrdU-positive optic stalk progenitors between the control and mutant E11.5 eyes (Fig. 2-8A and 2-8B). Since the loss of the optic

stalk in the mutant eyes may be due to apoptosis, we used the TUNEL assay on the frontal sections of E11.5 embryonic eye and quantified the Pax2-positive cells that are also TUNEL-positive. In the control eyes, 35.5% of Pax2-positive optic stalk progenitors are positive for TUNEL labeling, while in the *Fgfr* mutant eyes, 50.0% of the optic stalk progenitors are BrdU-positive, indicating that there is an increase in apoptosis in the optic stalk progenitors in the absence of *Fgfr1* and *Fgfr2* (Fig. 2-8E and 2-8F). Therefore, our results show that the *Fgfr* mutant optic stalk progenitors are lost due to apoptosis, and further suggest that FGF signaling is important for their survival.

The degeneration of the OS destroys the prerequisite for the formation of the optic nerve, which explains why we could not find optic nerves in newborn and P15 mutant eyes. Since the optic nerve is the exclusive route for the axons of the retina ganglion cells to travel to the brain, we next examine where the ganglion cell axons go in these 'optic-nerveless' mutant eyes. First, we determine if the retinal ganglion cells form in the *Fgfr* mutant eyes by using mRNA in situ hybridization to examine expression of the genes that are known to be important for specification and development of retinal ganglion cells, *Fgf15*, *Math5* and *Brn3b*. *Fgf15*, *Math5* and *Brn3b* are expressed in the developing retinas of the E13.5 control and *Fgfr* mutant eyes, indicating that RGCs form in the absence of *Fgfr1* and *Fgfr2* (Fig. 2-9) To determine if the RGC axons in the mutant eyes form and where they project, we then used βIII-tubulin staining to detect the RGC axons. The RGC axons are normally routed to the optic stalk though the optic disc in the E15.5 control eyes (Fig 2-8K). In

contrast, the RGC axons are targeted to the subretinal space and the unclosed fissure in the E15.5 *Fgfr* mutant eyes (Fig. 2-8L), which is likely due to the lack of the optic disc since it has been shown previously that the lack of the optic disc leads to misrouting of the RGC axons to the subretinal space (Chang et al. 2006). Therefore, these observations can explain why the *Fgfr* mutant eyes lack the optic nerve.

IV. Discussion

Although a number of genes have been implicated in coloboma formation, it remains largely unclear what cellular defects cause the failure of the OF closure and what major signaling pathways control the OF closing process. FGF signaling is known to regulate cell proliferation, fate determination, movement and morphological changes from flies to mammals, but its role(s) in the retina has not been carefully determined. In this study, we have documented, for the first time, the detailed cellular events during the OF closing process, particularly the changes in cell morphology, cytoskeleton organization, proliferation and apoptosis. By conditionally knocking out *Fgfr1* and *Fgfr2* from the developing eye, we show that FGF signaling is involved in controlling the proliferation of neural retinal progenitors, the maintenance of the OF progenitor fate, the transformation of the OF progenitors into both the neural retina and the RPE progenitors, and morphological changes of the OF progenitors during the closure. Based on the detailed analysis of the coloboma phenotype in the *Fgfr* mutant eyes, we propose that coordinated cell proliferation, cell

morphological changes and cell fate switches collectively contribute to the OF closure, and that defective FGF signaling in the retina leads to coloboma formation by disrupting such highly orchestrated cellular events. Finally, FGF signaling is required for the formation of the optic disc and the maintenance of the optic stalk. Therefore, this study has provided significant insight into how the OF closure is regulated and how the defective OF closure leads to coloboma formation, and has also revealed important roles of FGF signaling in the specification of the optic disc and the maintenance of the optic stalk.

The OF Closure is Accompanied by Series of Highly Coordinated Cellular Events

The OF closure process begins from the middle of the anterior-posterior axis of the OF and proceeds rapidly in both directions. In this study, we have carefully examined the OF closing process in developing embryonic eyes, and have further revealed several interesting aspects of the OF closure. First, the nasal and temporal lips of the OF juxtapose to each other with their basal membranes closely touching, preventing further migration of mesenchymal cells into the eye. Such juxtaposition of the two OF lips is likely achieved by balanced proliferation in both the nasal and temporal sides of the retina since we have observed that the neural retinal progenitors in both sides indeed proliferate at similar rates, which is evidenced by BrdU labeling. Then, the two basal membranes between the two lips start to dissolve, and the fusion of the OF proceeds from the RPE layer to the neural retinal layer. The basal

membrane is the last barrier for the OF fusion, and is likely removed by a secreted protease(s) to allow the two adjacent new cells to establish new junctions and other physical interactions. Therefore, the identification of the proteases and the revelation of their regulation will be important for gaining a better understanding of how the OF closing process is initiated.

Second, the OF progenitor cells in the RPE and retinal layers undergo distinct but apparently coordinated cell morphological changes just before they begin to fuse. The OF progenitors in the RPE layer, which are close to the initial fusion point of the OF, flatten their height and thereby increase their width, while those corresponding OF progenitors in the neural retinal layer increase their length (height) and reduce their width. Such morphological changes of the OF progenitors in both the layers take place simultaneously, suggesting that such coordinated morphological changes are critical for ensuing fusion. In addition, we have also observed that F-actin and *N-cadherin* accumulation on the apical and basal sides undergo dynamic changes just before the OF closure. Since dynamic changes in cadherin-mediated adhesion and F-actin accumulation have been implicated in cell morphological changes and movements of different cell types, our results in this study suggest that N-cadherin and F-action are also involved in the regulation of the morphological changes and movements of the OF progenitors. Conceivably, the morphological changes in both layers of the OF could potentially help correctly position the two lips for facilitating the fusion, and also ensure that the newly transformed OF progenitors in the fused fissure have identical morphologies to the neural retinal or RPE progenitors following

the fusion.

Third, programmed cell death or apoptosis may also be involved in the reorganization of the OF area during the fusion to eliminate the wrongly positioned cells. Apoptosis is known to participate in many developmental processes including morphogenetic movements. In the OF area, a significant number of the progenitor cells undergo apoptosis before and during the OF closing process, but there is very little apoptosis detected in the rest of the retina except the small dorsal-most region, suggesting that the apoptosis in the OF area may serve important purposes, such as removal of extra or incorrectly positioned cells for the OF fusion. Alternatively, the dying cells in the OF area may generate signals for stimulating morphological changes and/or other biological functions.

Fourth, the transformation of the OF progenitors into RPE and neural retinal cells also proceeds prior to the OF fusion. At E10.5, when the OF has not begun to fuse, the OF progenitors express Pax2, while the neural retina expresses Chx10 and Pax6 and the RPE expresses Pax6 and Mitf. Then, at E11.5, when the OF starts to fuse at the middle point, the OF progenitors in the neural retinal layer lose their Pax2 expression and gain Chx10 and Pax6 expression, while those in the RPE layer also stop Pax2 expression and start to express Pax6 and Mitf, indicating that the OF progenitors gradually differentiate into neural retinal progenitors and RPE cells. Such cell fate switches for the OF progenitors must be one of the requirements for the OF progenitors to integrate into the neural retinal and RPE layers during the OF fusion process. Since these cellular events take place at the same time as the OF closing

process, it remains unclear if these events are simply well-orchestrated cellular events prior to the OF fusion or one of the cellular events triggers the other ones. In any case, the revelation of such highly orchestrated cellular events during the OF closure would greatly help gain a deeper understanding of how the OF closure and coloboma formation is controlled at the molecular and cellular level.

FGF Signaling Controls Proliferation, Cell Fate Switches and Morphological Changes of the OF Progenitors

FGF signaling has been shown to promote proliferation of different progenitor cells of developing embryos, adult and embryonic stem cells (Sun et al. 2002; Israsena et al. 2004; Schmahl et al. 2004; Lavine et al. 2005; Levenstein et al. 2006; White et al. 2006; Zaragosi et al. 2006; Wang et al. 2007). In this study, we show that FGF signaling controls multiple cellular events that accompany the OF closing process, including the proliferation, cell fate switches and morphological changes of the OF progenitors. First, FGF signaling regulates the proliferation of the neural retinal and OF progenitors. In the *Fgfr* mutant eyes, the OF and neural retinal progenitors proliferate much slower based on BrdU labeling, and their retinas are much thinner than those in control eyes. Since there is no obvious change in apoptosis, these results indicate that FGF signaling controls the proliferation of the neural retinal and OF progenitors. In addition, in the *Fgfr* mutant eyes, the two OF lips are misaligned with each other and are never in direct contact, and there is reduced and uneven proliferation on the nasal and temporal sides, suggesting that

uneven proliferation rates of the retinal and OF progenitors on both the temporal and nasal sides may contribute to the misaligned fissure lips and thus the OF closure defect. The idea that highly regulated proliferation is required for the OF closure is further supported by a recent study on *Phactr4* mutant mice (Kim et al. 2007). A mutation in *Phactr4*, which encodes a phosphatase that inhibits cell-cycle progression, leads to coloboma formation, which is shown to be caused by dramatically increased cell proliferation (Kim et al. 2007). Therefore, the decreased and uneven proliferation on both the nasal and temporal sides of the *Fgfr* mutant retina likely prevents the correct alignment of the two OF lips, contributing to coloboma formation. Although it remains unclear how FGF signaling controls the proliferation of the OF and neural retinal progenitors in the developing eye, FGF signaling controls the proliferation of human embryonic stem cells and adipose-derived stem cells by regulating the ERK signaling cascade (Zaragosi et al. 2006; Greber et al. 2007). The ERK signaling promotes the G1-S transition of the cell cycle through multiple mechanisms, such as upregulation of Cyclin Ds and stabilization of c-Myc (Meloche and Pouyssegur 2007). This study also suggests that FGF signaling regulates the ERK cascade in the developing retina. In the future, it will be of great interest to decipher how the FGF-ERK pathway regulates the proliferation of the OF and neural retinal progenitors.

Second, FGF signaling regulates cell fate maintenance and switch of the OF progenitors during the fissure closing process. In the *Fgfr* mutant eyes, the OF progenitors are specified normally at E10.5, but lose their cell fate prematurely by

turning off Pax2 expression and turning on Mitf expression at E11.5, indicating that FGF signaling is required for maintaining the cell fate of the OF progenitors. To further support the idea that in the absence of FGF signaling the OF progenitors differentiate into the RPE cells, all the mutant OF progenitor-derived cells at E13.5 express a mature RPE cell marker, Dct, demonstrating that FGF signaling is required for the cell fate switch from the OF progenitors to the neural retinal progenitors. Mitf and Chx10 mutually repress each other's expression, and FGF signaling is required for maintaining Chx10 expression in the development of the optic cup (Nguyen and Arnheiter 2000a; Rowan et al. 2004; Horsford et al. 2005). Our results in this study indicate that the FGF-Chx10-Mtif signaling circuitry is also re-used in the OF progenitors for the cell fate switch during the fissure closing.

Third, FGF signaling also regulates morphological changes of the OF progenitors, which is likely one of the key cellular events for the OF closing process. As mentioned earlier, the wild-type OF progenitors undergo dynamic changes in the localization and expression of N-cadherin and F-actin. In the *Fgfr* mutant OF progenitors, N-cadherin expression and actin accumulation on the apical side fail to be down-regulated, and extensive cell morphological changes before the fissure closing fail to take place. During *Drosophila* gastrulation, FGF signaling is required for mesoderm invagination by controlling cell shape changes through regulating actin organization and apical localization of adherens junctions (Barrett et al. 1997; Gryzik and Muller 2004; Nikolaidou and Barrett 2004; Smallhorn et al. 2004; Stathopoulos et al. 2004; Kolsch et al. 2007). FGF signaling is also required for migrations of

mesoderm from the primitive streak by regulating expression of *E-cadherin* (Ciruna and Rossant 2001). Likely, in the *Fgfr* mutant eyes, the morphological defects of the OF are caused by the defects in N-cadherin expression and F-actin localization. Thus, FGF signaling controls the OF closure by regulating expression of adhesion molecules, actin reorganization and thereby cell morphological changes. In the future, it will be important to figure out how FGF signaling modulates N-cadherin expression and actin organization.

Taken together, we show in this study that FGF signaling regulates the proliferation, cell fate switch and shape changes of the OF progenitors. Since these events are interconnected and coordinately regulated during the OF closing process, we propose that FGF signaling controls the OF closure by orchestrating multiple events such as cell proliferation, cell fate switch, adhesion and cell shape changes.

FGF Signaling Plays Important Roles in the Formation of the Optic Disc and the Maintenance of the Optic Stalk

In the early optic cup stage, the optic stalk and the OF are two continuous structures highlighted by their shared *Pax2* expression (Morcillo et al. 2006). After the OF closes, *Pax2* remains expressed in the optic stalk and the optic disc but disappears from the ventral retina. In the *Fgfr* mutant eyes, the optic disc fails to form due to premature loss of the *Pax2*-positive cells, and the unclosed fissure further prevents the formation of the ring-like optic disc at the posterior end of the OF. Consistent with the observation that the optic disc does not form in the *Fgfr* mutant

eyes, the RGC axons are misrouted to the subretinal space and the unclosed fissure area. Since *Pax2* expression is initiated normally in the E10.5 *Fgfr* mutant eyes, premature loss of *Pax2*-positive cells in *Fgfr* mutant eyes indicates that FGF signaling is important for maintaining *Pax2* expression and thus the fate of the optic disc progenitors. Our results also show that FGF signaling is required for the maintenance of the optic stalk. Although the failure in the exit of the RGC axons could result in aplasia of the optic stalk (Wallace and Raff 1999; Dakubo et al. 2003), the *Fgfr* mutant optic stalk already shows dramatically increased cell death at E11.5, when the RGC cells have not been generated, indicating that FGF signaling is directly required by the optic stalk to survive. Consistent with the direct role of FGF signaling, pERK is also expressed in the developing optic stalk of the E11.5 embryo. Therefore, FGF signaling is required for the formation of the optic disc and the maintenance of the optic stalk.

Figure 2-1. Retina-specific conditional knockout of *Fgfr1* and *Fgfr2* leads to coloboma formation.

(A) A side view of an E13.5 control embryonic head, in which the eye (A') has a normal RPE epithelium. (B) A side view of a *Six3-Cre; Fgfr1*$^{fx/fx}$*; Fgfr2*$^{fx/fx}$ E13.5 embryonic head, in which the size of the eye (B') is reduced and there is a gap in the RPE epithelium on the ventral side (black arrowhead). (C) A section of a control neonatal (P0) eye showing the optic disc and the optic nerve (green arrowhead). (D) A section of a P0 retina-specific *Fgfr1* and *Fgfr2* double mutant eye showing the unclosed retinal fissure (green arrowhead). (E) A P15 control eye with an intact optic nerve cord (white arrowhead). (F) A P15 retina-specific *Fgfr1* and *Fgfr2* double mutant eye showing coloboma (white arrowhead) and absence of the optic nerve. All the wild-type and mutant pairs are shown at the same scale.

Six3-cre;
Fgfr1fx/fx; Fgfr2fx/fx
Control
E13.5
P0
P15
A
B
A'
B'
C
D
E
F

Figure 2-2. The retina-specific *Fgfr1* and *Fgfr2* double mutant eyes exhibit normal dorsal-ventral polarity.

A and C or B and D are shown in the same magnification with the dorsal side on the top and the ventral side on the bottom. (A and C) *Vax2* mRNA wholemount in situ hybridization on the E10.5 control (A) and the *Fgfr* double mutant (C) embryonic eyes. (B and D) *Raldh3* mRNA in situ hybridization on the sagittal sections of E11.875 control (B) and the *Fgfr* double mutant (D) embryonic eyes. *Vax2* and *Raldh3* are expressed normally in both the control and *Fgfr* mutant eyes.

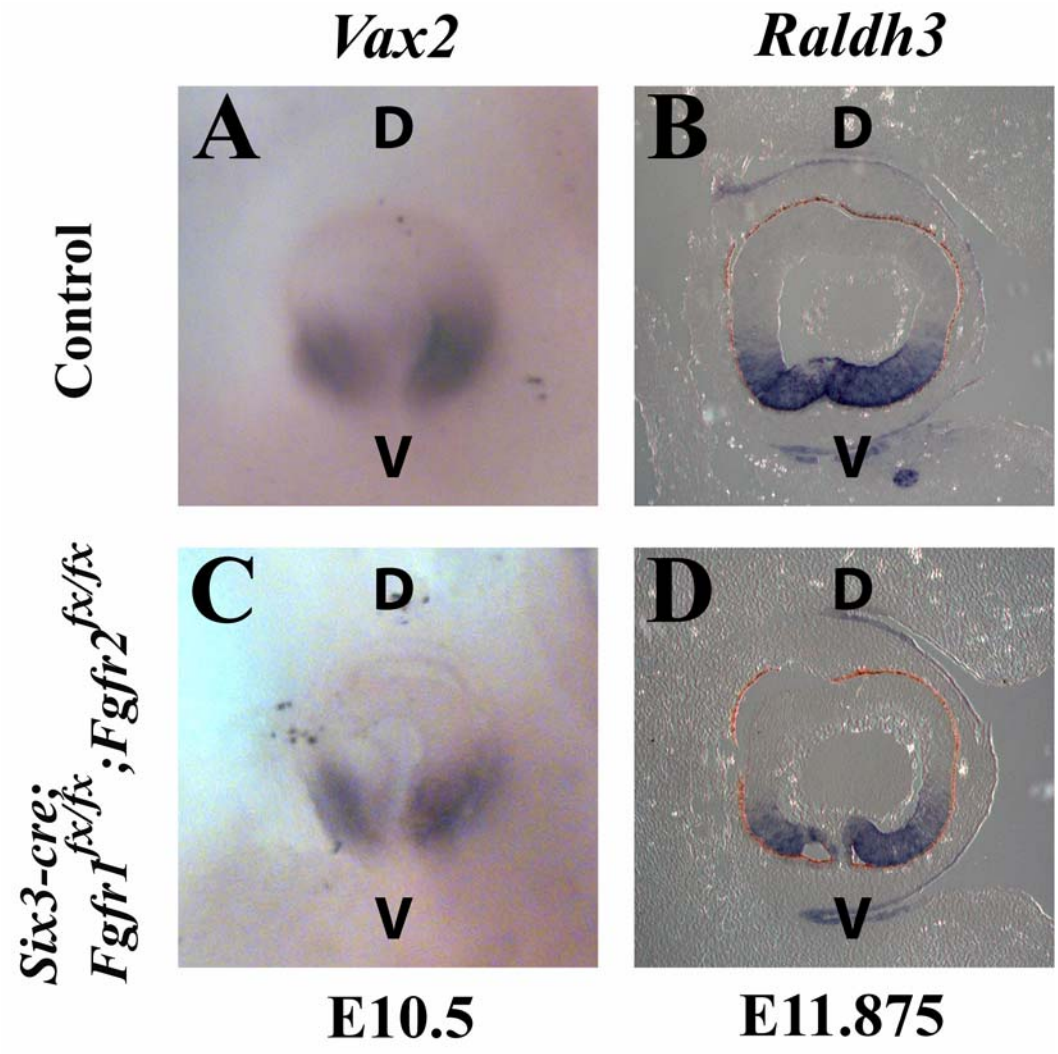

Vax2
Raldh3
A
D
V
B
D
V
Control
C
D
V
D
D
V
Six3-cre;
Fgfr1fx/fx;Fgfr2fx/fx
E10.5
E11.875

Figure 2-3. FGF signaling activity is dramatically down-regulated in the fissure progenitors that are mutant for *Fgfr1* and *Fgfr2*.

All the images represent sagittal sections of control (A-D) and mutant (E, F) embryonic E11.5 eyes. (A, B) *Fgfr1* (A) and *Fgfr2* (B) mRNA in situ hybridization showing that they both are expressed in the retina including fissure progenitors (brackets). (C) pERK, an indicator of FGF signaling activity, is expressed in the control retina including fissure progenitors (bracket). (D) An E11.5 *six3-Cre; Fgfr1*$^{fx/+}$*; Fgfr2*$^{fx/+}$*; Z/EG* eye sagittal section shows that *Six3-Cre* can efficiently carry out LoxP recombination in the fissure progenitors (bracket). (E) pERK expression is dramatically reduced in the fissure progenitors that lack both *Fgfr1* and *Fgfr2* (bracket). (F) In *Six3-Cre; Fgfr1*$^{fx/fx}$*; Fgfr2*$^{fx/fx}$*; ZEG* mutant eyes, the GFP-marked *Fgfr* mutant fissure progenitors persist, and the GFP-positive mutant neural retinal progenitor cells are primarily localized to the temporal side but are absent from the nasal side.

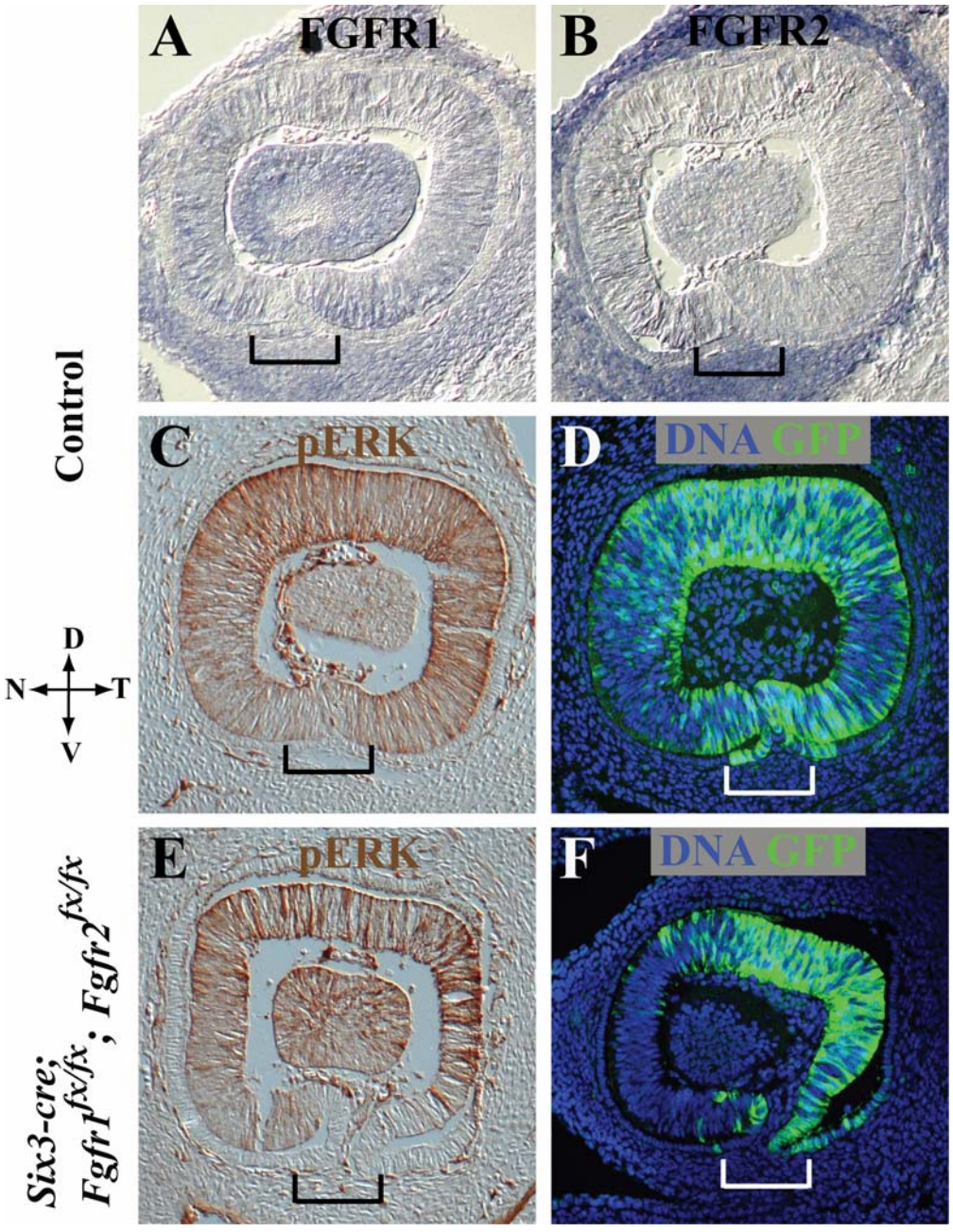

A
FGFR1
B
FGFR2
Control
C
pERK
D
DNA GFP
D
N
T
V
E
pERK
F
DNA GFP
Six3-cre; Fgfr1fx/fx; Fgfr2fx/fx

Figure 2-4. Fgf signaling is required for the proliferation of fissure and retinal progenitors.

The images in A-D represent the same scale of sagittal sections of E11.5 embryonic eyes, while the ones in A' and B' show the fissure areas (brackets) of A and B at higher magnifications. In the *Fgfr* mutant eyes (B and B'), GFP-positive retinal and fissure progenitor cells proliferate much slower than those in the control eyes (A and A'). (C, D) Control (C) and mutant (D) retinal and fissure progenitor cells have similar but very limited apoptosis activity. The apoptotic cells are primarily limited to the fissure area and the dorsal-most area of the neural retina.

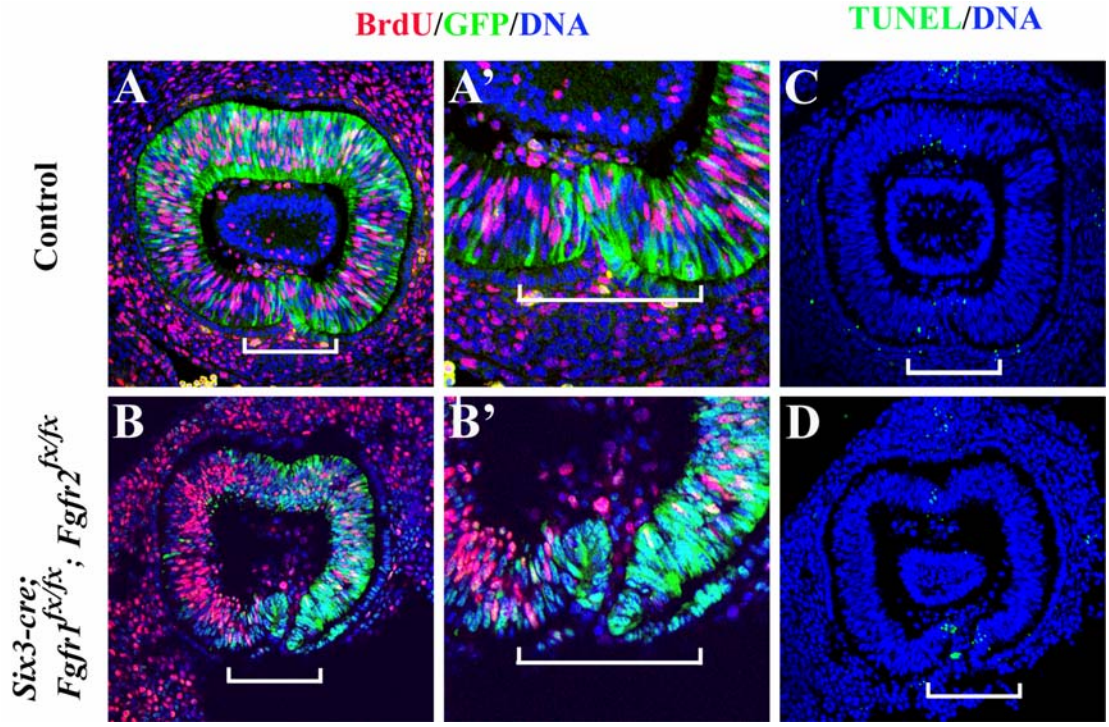

BrdU/GFP/DNA
TUNEL/DNA
Control
Six3-cre; Fgfr1fx/fx; Fgfr2fx/fx
A
A'
C
B
B'
D

Figure 2-5. FGF signaling is required for the maintenance and later cell fate switches of fissure progenitors.

The images in A-F represent the sections from the control eyes, while the ones in A'-F' are the sections from the *Fgfr* mutant eyes. E and E' only show the fissure areas at higher magnifications, and the fissures in A-E and A'-E' are indicated by arrowheads. (A, A') Pax2 and Pax6 are expressed at similar levels in the fissure progenitors of the control (A) and *Fgfr* mutant (A') E10.5 embryonic eyes. (B, B') Mitf and Chx10 are expressed at similar levels in the fissure progenitors of the control (B) and *Fgfr* mutant (B') E10.5 embryonic eyes. (C, C') Pax2 expression almost completely disappears but Pax6 is fully expressed in the *Fgfr* mutant fissure progenitors. (D, E) The fissure progenitors in the retinal layer of the control eyes have been switched to the retinal progenitors and express Chx10, while those in the RPE layer express Mitf. (D', E') In the *Fgfr* mutant eyes, the fissure progenitors in both the retinal and RPE layers have been switched to RPE cells expressing Mitf. (F, F') *Dct* mRNAs are expressed in the RPE cells of both the control and *Fgfr* mutant eyes, and are also present in the unclosed mutant fissure (black arrowhead in F').

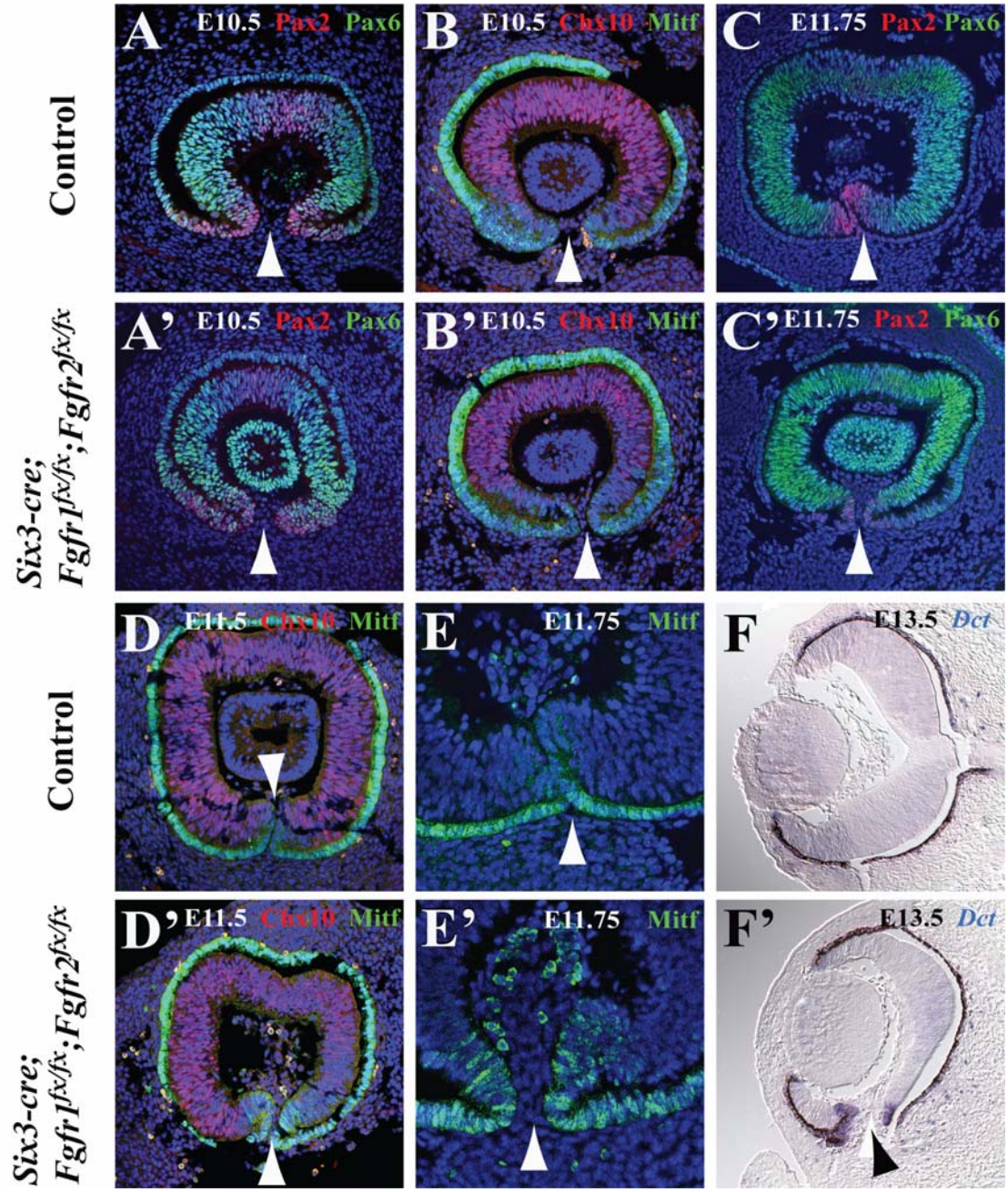

Control
A
E10.5 Pax2 Pax6
B
E10.5 Chx10 Mitf
C
E11.75 Pax2 Pax6
Six3-cre; Fgfr1fx/fx;Fgfr2fx/fx
A'
E10.5 Pax2 Pax6
B'
E10.5 Chx10 Mitf
C'
E11.75 Pax2 Pax6
Control
D
E11.5 Chx10 Mitf
E
E11.75 Mitf
F
E13.5 Dct
Six3-cre; Fgfr1fx/fx;Fgfr2fx/fx
D'
E11.5 Chx10 Mitf
E'
E11.75 Mitf
F'
E13.5 Dct

Figure 2-6. Multiple coordinated cellular events are accompanied during the fissure closure.

All the images represent the fissure area at the different stages of the OF closing process from the sagittal sections. (A) At E10.5, the two OF lips remain untouched, leaving an open space (arrow) for mesenchymal cells to migrate in. The basal membranes (red lines indicated by arrowheads) on both the nasal and temporal sides indicate the distance between the two OF lips. (B-D) At E11.5, the two lips at the more distal point of the OF still leave a gap (arrow, B), while they touch each other in the positions close to the middle point of the OF but there are some untouched areas (arrows, C and D). In addition, their basal membranes remain intact. (E-H) At E11.75, the two lips in the position close to the most distal point of the OF still leave a gap (arrow, E), while they completely juxtapose each other at the middle point of the OF (arrow, G), but there are some untouched areas in other positions (arrows, F and H). Again, their basal membranes remain intact at this stage. (I-K) At E11.875, the two lips at the more distal point of the OF juxtapose each other (arrow, I), while at the positions close to the middle point of the OF, their basal membranes start to dissolve, leaving a gap in the basal membranes (arrows, J and K). In addition, the OF progenitors in the RPE layer have fused. (L) At E12.0 the RPE has become a continuous epithelial sheath, while the neural retinal layer is also fused and bulges in, which may be due to local proliferation.

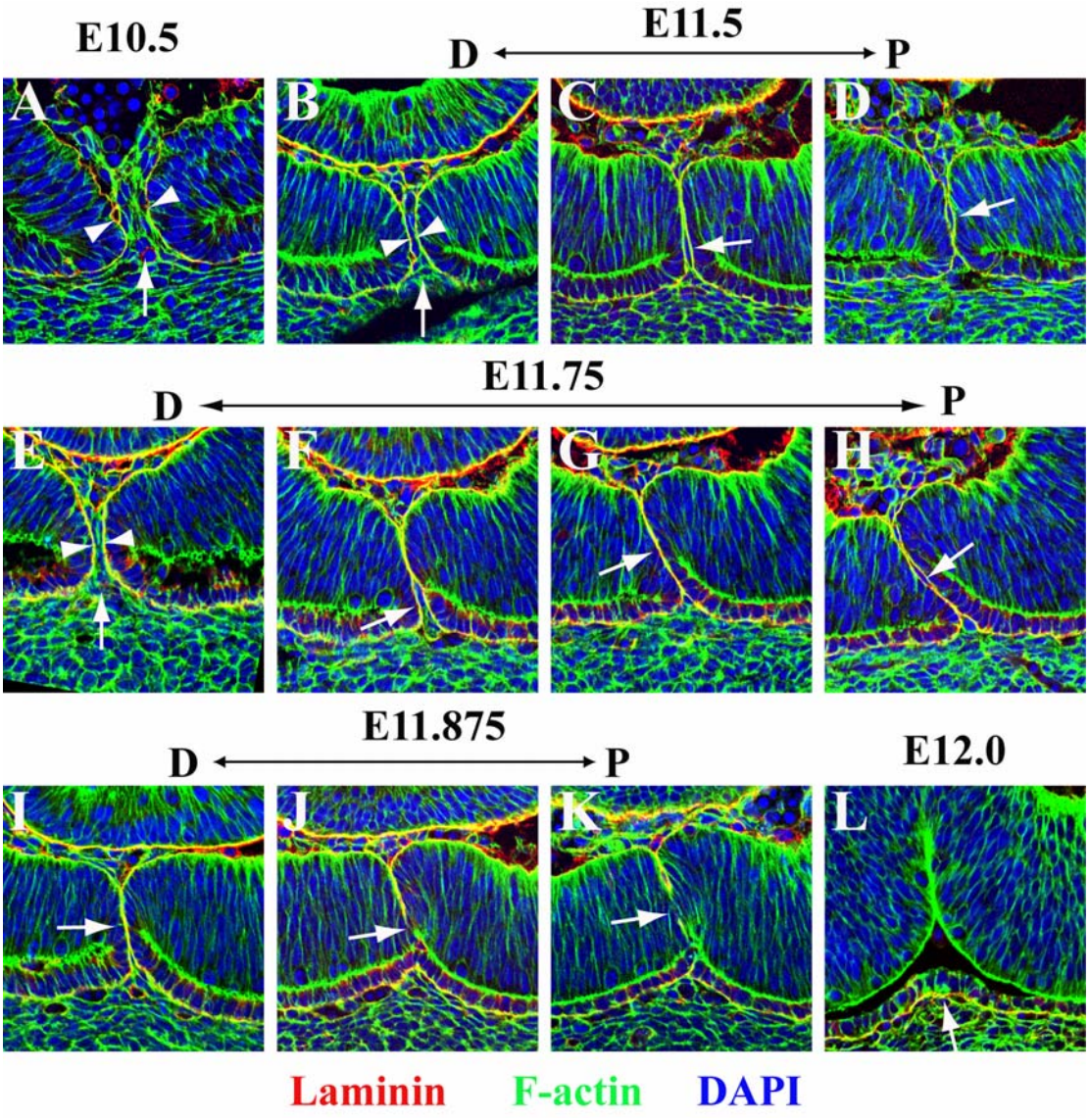

E10.5
E11.5
D
P
A
B
C
D
E11.75
D
P
E
F
G
H
E11.875
D
P
E12.0
I
J
K
L
Laminin
F-actin
DAPI

Figure 2-7. FGF signaling is required for morphological changes of fissure progenitors during the fissure closing.

(A, B) At E10.5, the fissure progenitors in both the control and *Fgfr* mutant eyes express comparable levels of N-cadherin on their apical side. (C-E) at E11.875, actin accumulation on the apical side of the fissure progenitors declines along the distal (C,D) to the mid-point (E) of the fissure. (C'-E') At E11.875, N-cadherin expression in fissure progenitors is dramatically down-regulated. (F, F') Just before the fusion of the RPE layer, the fissure progenitors in the retinal and RPE layers undergo elongation and shortening (one prospective neural retinal cell and two prospective RPE cells are outlined), respectively, while the levels of N-cadherin expression in the fissure progenitors are very low (F'). (G-I) At E11.875, actin accumulation on the apical side of the fissure progenitors stays at similar levels along the distal (G) to the mid-point (I) of the *Fgfr* mutant fissure. (G'-I') At E11.875, N-cadherin expression remains high in the mutant fissure progenitor cells. The two lips (arrows) of the fissure on the temporal and nasal sides fail to meet. At the mid-point of the mutant fissure, the cell length (a red line in I) in the retinal layer is shorter than that (a red line in E) in the control fissure, while the cell length (a yellow line in I) in the RPE layer is longer than that (a yellow line in E) in the control fissure. (J, J') At the mid-point of the mutant fissure, the fissure progenitors in the retinal and RPE layers fail to elongate and shorten (one prospective neural retinal cell and two prospective RPE cells are outlined), respectively, while the levels of N-cadherin expression in the fissure progenitors remain high (J').

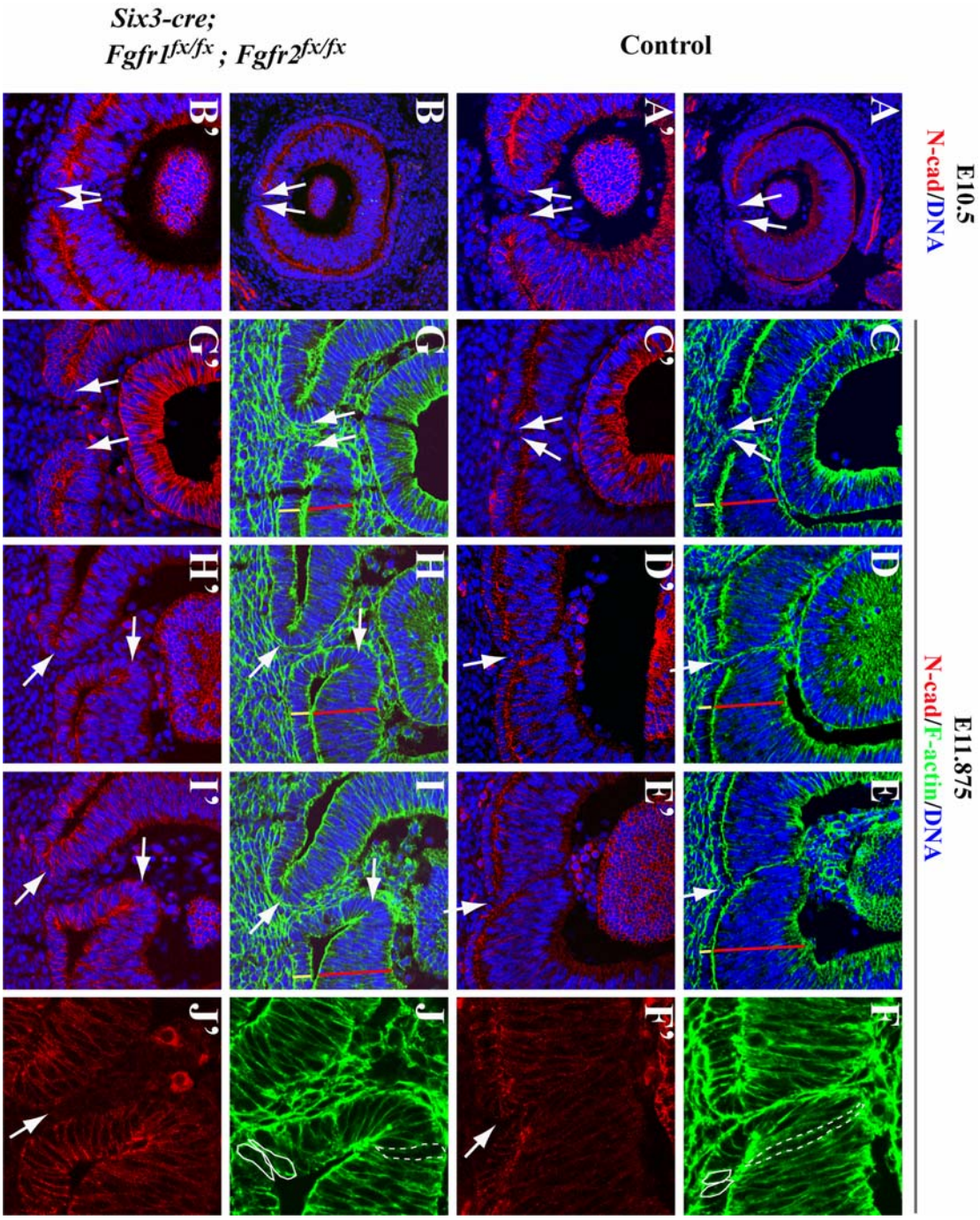

Six3-cre;
Fgfr1fx/fx ; Fgfr2fx/fx
Control
E10.5
N-cad/DNA
E11.875
N-cad/F-actin/DNA
A
A'
B
B'
C
C'
D
D'
E
E'
F
F'
G
G'
H
H'
I
I'
J
J'

Figure 2-8. FGF signaling is required for the formation of the optic disc and the maintenance of the optic stalk.

All the images except C and D represent frontal sections, while C and D are sagittal sections. (A, B) At E11.5, Pax2 expression and proliferation remain similar in the optic stalk progenitors on the dorsal (arrowhead) and ventral (arrow) sides of the control (A) and *Fgfr* (B) mutant eyes. (C, D) At E11.5, Pax2 expression has already decreased in the mutant optic disc progenitor cells (arrow, D) in comparison with that in the control ones (arrow, C). (E, F) At E11.5, there are more dying optic stalk progenitors (arrowheads) in the mutant eye (F) than in the control eye (E). (G, H) Pax2 remains expressed in the optic disc (arrowheads, G) and the optic stalk of the control eye, while no optic disc but an unclosed fissure opening (arrowhead, H) and the Pax2-positive remnant (arrow) of the degenerating optic stalk are present in the mutant eye. (I, J) The optic disc (arrowheads, I) is present in the E15.5 control eye, but only a unclosed fissure opening (arrowhead, J) is present in the control eye. (K,L) β III tubulin stains the axons of RGCs. The optic nerve (arrowhead, K) is present in the control eye but is absent in the mutant eye. The axons (arrowheads, L) of the RGCs are mistargeted to the unclosed fissure and the subretinal space.

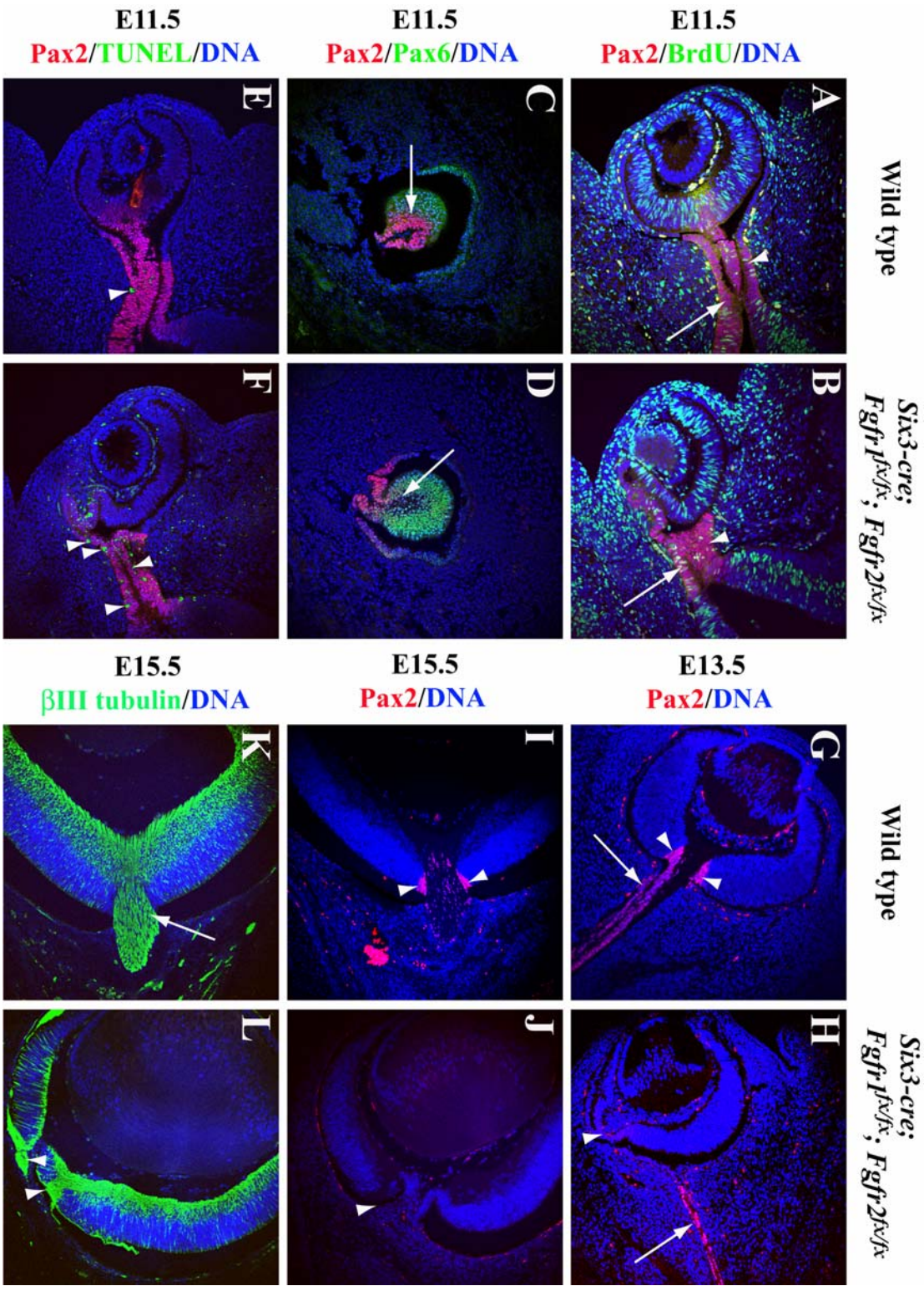
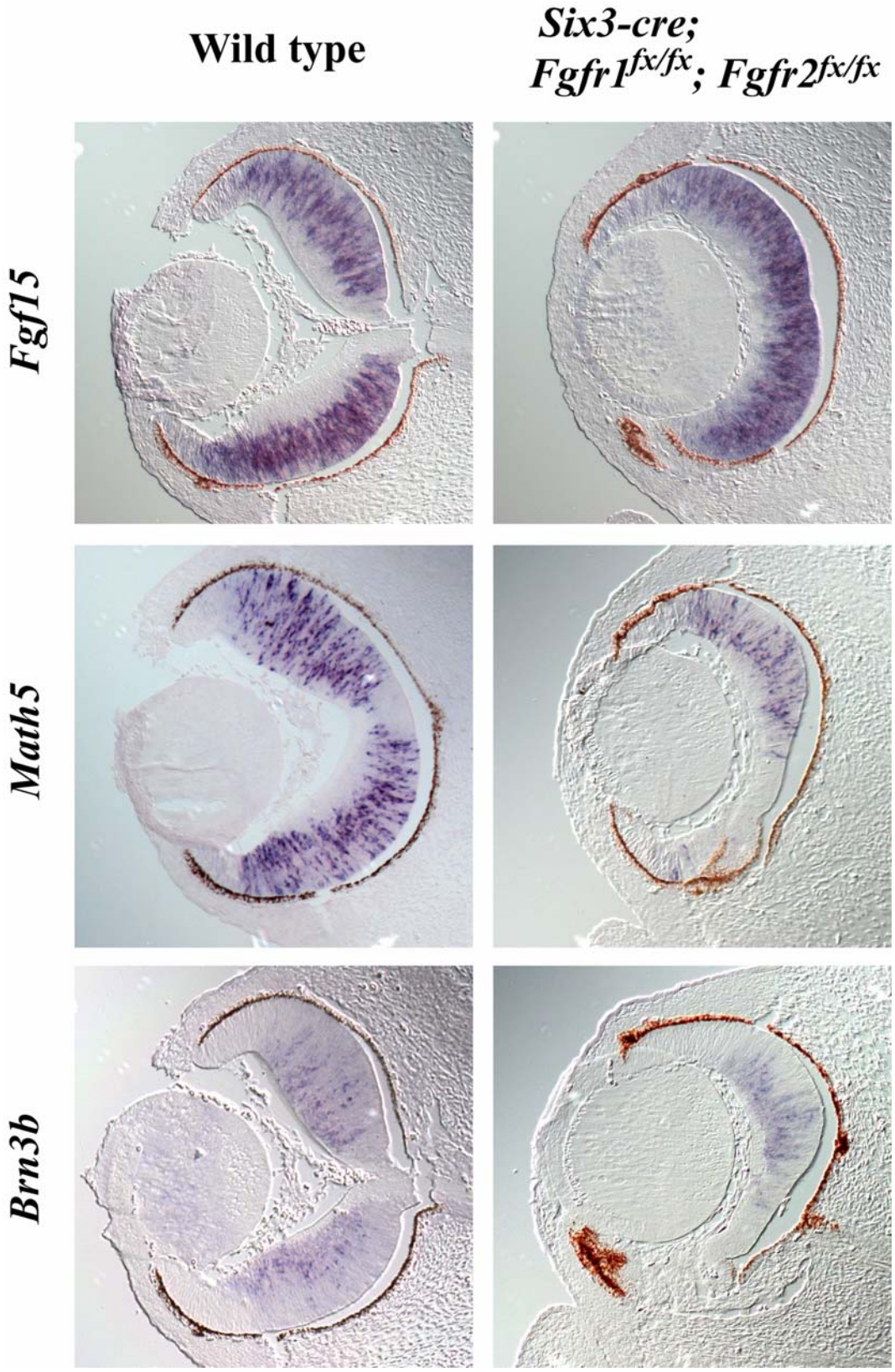

E11.5
Pax2/TUNEL/DNA
E11.5
Pax2/Pax6/DNA
E11.5
Pax2/BrdU/DNA
E
C
A
Wild type
F
D
B
Six3-cre;
Fgfr1fx/fx; Fgfr2fx/fx
E15.5
βIII tubulin/DNA
E15.5
Pax2/DNA
E13.5
Pax2/DNA
K
I
G
Wild type
L
J
H
Six3-cre;
Fgfr1fx/fx; Fgfr2fx/fx

Figure 2-9 Retinal ganglion cells are generated in Fgfr mutant retinae.

mRNA in situ hybridization for *Brn3b*, *Math5* and *Fgf15* of E13.5 control and mutant eyes are shown.


Wild type
Six3-cre;
Fgfr1fx/fx; Fgfr2fx/fx
Fgf15
Math5
Brn3b

CHAPTER THREE

CONCLUSIONS AND FUTURE DIRECTIONS

I. Conclusions

The main conclusions of my dissertation, based on the experimental results, are the following:

1. **The OF closure is a sequential event that involves cell movement, tissue fusion and cell fate switch**.

   First, the nasal and temporal lips of the OF move close and directly juxtapose to each other with only basal membranes separating them. Then, the two lips fuse at the middle point of the OF along the proximal-distal axis, with the basal membranes removed from the OF progenitors in both the lips of the fissure that are about to fuse. Simultaneously, the progenitors at the fusion point also undergo extensive morphological changes and switch their cell fate to either RPE cells or neural retinal cells. Finally, after reorganization, the fused area in both the RPE and neural retinal layers is morphologically identical to the rest of the retina.

2. **FGF signaling is required for the OF closure by orchestrating cell proliferation, cell fate switches and cell shape changes.**

   Fgfr1 and *Fgfr2* double conditional knockout eyes show slow and asymmetric

proliferation of the neural retinal progenitors in the nasal and temporal sides, the incorrect cell fate switch of OF progenitor cells into RPE cells and abnormal cell shape changes, which may collectively cause the misalignment of the two fissure lips and thus the failure in the fissure closure.

3. FGF signaling is required for the formation of the optic disc and the maintenance of the optic stalk.

Fgfr1 and *Fgfr2* double conditional knockout eyes fail to specify the optic disc due to the premature loss of the *Pax2*-positive OF progenitor cells and the gradual loss of the optic stalk. The optic nerve in the *Fgfr* mutant eyes fails to form due to lack of the optic disc and/or the degeneration of the optic stalk.

II. Future directions

1. To study the molecular mechanisms of the OF closure

One essential process that often takes place during embryogenesis is the drawing together and fusion of two epithelial sheets. The typical examples include the dorsal closure in *Drosophila*, the ventral enclosure in *Caenorhabditis elegans*, the neural tube closure and eyelid closure in vertebrates. The studies on these model systems have revealed some general signaling pathways and cytoskeleton machineries that control the epithelia fusion (Martin and Wood 2002; Martin and Parkhurst 2004). In this study, we have carefully examined the closing process of the OF, showing that it generally follows the common steps that the other epithelium fusion normally takes: two epithelial sheets are first drawn close to each other, and the epithelial fronts are then well-knitted together to form a continuous epithelial sheet. However, the closing of the OF involves fusions of two layers, the neural retinal and RPE layers. In this fusion, the two layers in each side of the OF have to break up from each other and then fuse with their counterparts on the other side of the fissure. Therefore, the knowledge gained from studying the fusion of the OF not only helps understand how two epithelial cell sheets fuse with each other but also provies a better understanding of how the two layers are coordinated in the fusion process.

Although the vertebrate eye is a well-studied system, the OF closure has drawn little attention. With the combination of the rich knowledge on eye development and a large reservoir of genomic, genetic and molecular tools available for mice, the mouse OF closure will be another good model system for understanding how epithelial closure is regulated and the defective fusion-associated human diseases.

To investigate what genes and signaling pathways are involved in the regulation of the OF closure, the microarray and in situ hybridization approaches can be used to discover the genes that are specifically expressed in the OF area. The OF progenitor cells and their neighboring neural retinal and RPE cells can be isolated through microdissection, and their gene expression profiles can be compared using microarray. Then the genes that are identified by microarrays as being expressed specifically in the OF progenitors can be further confirmed by mRNA in situ hybridization. Since *Pax2* and *Vax2* are two genes known to be expressed in the OF progenitor cells, they can be used to verify our microarray results. The results from these experiments will help to reveal the unique property of the OF progenitor cells that distinguish them from other retinal progenitor cells, which may help further define the molecular mechanisms underlying the OF closure.

In the normal developing eye, the nasal lip and the temporal lip of the OF appear to behave differently: the temporal lip is always pushed upward, while the nasal lip remains under the temporal one, suggesting that the nasal lip may behave more actively than the temporal one and push it upwards. To test this idea, the nasal and temporal lips can be isolated, and their gene expression profiles can be carefully

compared using microarrays. If the nasal lip of the OF is more active in movement, the progenitors on the nasal side may highly express the genes that are important for controlling cytoskeletal dynamics, such as small GTPases like Rho. In addition, the genes that are important for the regulation of the nasal-temporal polarity can be also identified.

To further understand how these newly identified OF-specific genes are involved in the regulation of the OF closure and the nasal-temporal polarity, the functions of these genes will be further investigated using shRNA-mediated gene knockdown and overexpression and standard genetics in mice. Although these genes could be specific to the OF or the progenitors on either the temporal or nasal side in the eye, they may be not only expressed in the eye. To quickly screen through these genes for their potential functions in the OF closure, *in vitro* embryonic eye explant cultures can be used to overexpress or knockdown gene functions to see if the manipulation of the function of a particular gene has any effect on the OF closure. Alternatively, chick embryonic eyes can also be used to test the functions of these genes in the OF closure, since the mechanisms underlying eye development are highly conserved in vertebrates (Chow and Lang 2001). The expression patterns of these genes in developing chick eyes will be confirmed using mRNA in situ hybridization. Then, these genes can be overexpressed in the chicken eyes by electroporation, and their expression can be downregulated by RNAi or morpholino oligonucleotides. These findings can be further verified in mice using standard and conditional gene knockout technologies.

2. To further understand how FGF signaling controls the fissure closure

Although we show that FGF signaling controls the fissure closure by orchestrating multiple coordinated cellular events, the detailed molecular mechanisms underlying the cellular events have not been revealed. The requirement of FGF signaling is likely mediated by the ERK kinase cascade in the OF progenitors since p-ERK, the active form of ERK, is dramatically downregulated in the *Fgfr* mutant OF progenitor cells. The E11.5 fissure progenitor cells from the wild-type and *Fgfr* mutant eyes will be isolated, and their gene expression profiles will be obtained using microarray analysis. In this study, I have shown that expression of Pax2, Pax6, Mitf and Chx10 is altered in the *Fgfr* mutant OF progenitors. I expect that expression of *Fgfr1* and *Fgfr2* is also downregulated in the mutant eyes. The expression changes of these genes can be used to verify our microarray results. From these gene expression profiles, I should be able to obtain useful information about the downstream targets of the FGF-pERK pathway that are specifically involved in the OF closure. I would anticipate finding the genes that are known to be involved in the regulation of cell cycle progression and morphological changes. I can also utilize the strategies outlined earlier to further exploit how the FGF-pERK pathway regulates the closing process of the OF by molecularly manipulating their downstream targets in mice or chick.

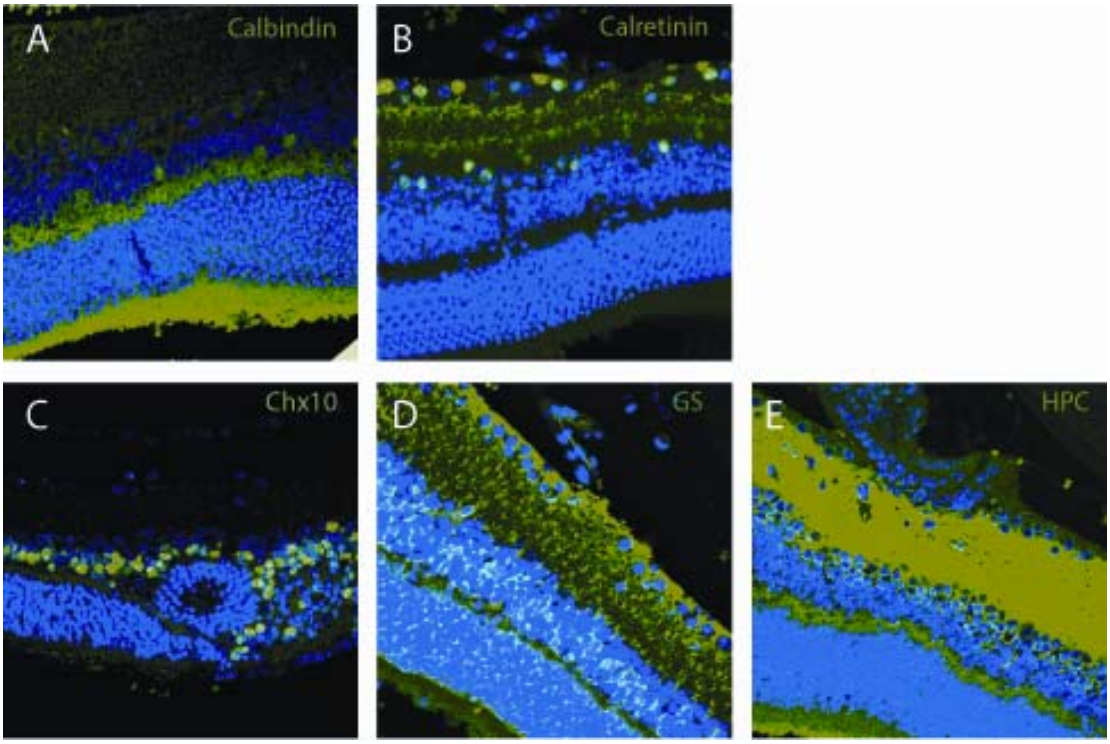
3. FGF signaling in retinal cell differentiation and retinal stem cell establishment and function

Although defective FGF signaling affects the proliferation of the neural retinal progenitors, the differentiated retina tissues still develop in the *Fgfr* mutant eyes. By checking molecular markers for different major retinal cell types, all the major retinal cell types (RGC, amacrine cell, bipolar cell, horizontal cell, Müller cell and photoreceptor cell) are generated (Fig. 3-1). However, since the Cre activity is mosaic (see results section), it is possible that the remaining retina cells are the progeny of wild type retinal progenitor cells which escape the Cre recombinase activity. Furthermore, each type of retinal neurons is composed of several subtypes; it is unclear whether different subtypes of retinal neurons are generated. In the future, it is important to use GFP-marked *Fgfr* mutant retinal progenitors to determine if the *Fgfr* mutant retinal progenitor cells can contribute to the mature retinal tissues. By comparing the ratios of GFP-positive and -negative cells in each of the retinal cell types as well as subtypes, we could obtain information about the requirement of FGF signaling in the differentiation of retinal cells.

FGF signaling can induce the neural retinal fate *in vivo* (Guillemot and Cepko 1992; Pittack et al. 1997; Hyer et al. 1998; Nguyen and Arnheiter 2000), and promote the proliferation and maintenance of retinal stem cells (RSCs) *in vitro* (Tropepe et al. 2000; unpublished data from the Xie laboratory). It would be interesting to investigate whether FGF signaling is also involved in the establishment of the RSCs. Our lab has established an *in vitro* culture system to isolate and culture RSCs from the adult mouse retina. In the future, the system described in this dissertation can also

be used to test whether the *Fgfr* mutant adult retina still maintains RSCs. Even if we could establish RSC cell lines from the *Fgfr* mutant adult retina, it is possible that these RSC cell lines are derived from wild-type RSCs due to mosaic Cre expression. One of the ways to circumvent this problem is to genotype the established cell lines from the mutant retinas. If FGF signaling is required for RSC establishment or maintenance, the RSC cell lines should be wild-type but not *Fgfr1*- and *Fgfr2*-deficient. If FGF signaling is dispensable for RSC establishment or maintenance, *Fgfr1* and *Fgfr2* mutant RSC lines can be established *in vitro*. These mutant RSC lines can be further used to determine if FGF signaling affects retinal differentiation.

Figure 3-1. All the major retinal cell types are generated in *Fgfr* mutant eyes. Confocol images of immunostaining on P24 Fgfr mutant eye sections of (A) Calbindin, for horizontal cells (B) Calretinin, for amacrine cells and ganglion cells (C) Chx10, for bipolar cells. (D) GS, for Müller cells. (E) HPC for amacrine cells


A
Calbindin
B
Calretinin
C
Chx10
D
GS
E
HPC

CHAPER FOUR

Materials and Methods

Standard solutions

PBS, LB, SOC, TAE, Ampicillin, 0.5M EDTA, 1MTris, SSC are supplied by the Media Prep facility of Stowers Institute for Medical Research (SIMR).

1xPBS 4% formaldehyde

16% formaldehyde (TED PELLA, Inc.)---------1ml

1XPBS----------3ml

This fixation solution is made freshly before use.

Davidson's fixative

37% formalin----------50ml

ethanol----------75ml

glacial acetic acid ----------25ml

d.d.H2O ----------75ml

30% sucrose

Sucrose (Sigma) ----------150g

It is dissolved in 1xPBS to make the final volume of 500ml.

10xTBST

TRIS base (Sigma) ----------60.57g

Sodium chloride (Sigma) ----------89-90g

Concentrated Hydrochloric acid (Fisher) -----35ml

Tween20 (EMD) ----------2ml

These chemicals are dissolved in one liter of double distilled (d.d.) H2O, and the pH of the solution is adjusted to 7.8.

Sodium citrate buffer (for the antigen retrieval)

Tri-sodium citrate dehydrate (Sigma) ---------2.94g

d.d. H2O----------1L

The pH is adjusted to 6.0 with a 1N HCl solution

Citrate buffer (for the antigen retrieval for p-ERK staining)

Tri-sodium dehydrate (Sigma) ----------2.94g

d.d. H_2O ----------1L

Its pH is adjusted to 9.0 with a 2N NaOH solution.

DAPI stock solution (1mg/ml):

DAPI (Sigma) ----------10mg

Dimethylformamide (Sigma) ----------10ml

The solution is divided into small aliquots and stored in the dark at -20℃.

50x Stock Base Solution (1.25M NaOH, 10mM EDTA pH12)

5N NaOH ----------250ml

0.5M EDTA ----------20ml

d.d. H_2O ----------1L

Adjust pH to 12.0 with NaOH/HCl

50x Stock Neutralization solution (2M Tris-HCl pH5)

Tris-HCl (Sigma) ----------315.2g

d.d. H_2O ----------1L

Adjust pH to 5.0 with NaOH/HCl

Working solutions

Dilute 50X stock solution to 1X working strength solution with dd. H2O

Working solution should be made freshly every 2 weeks

Hybridization mix:

For 1 litter:

Formamide (EMD) ----------500ml (50%)

20XSSC pH 5.0 ---------- ----------65ml (1.3X)

0.5M EDTA----------10ml (5.0mM)

10mg/ml tRNA (Roche) ----------2.5ml (50ug/ml)

Tween 20 (EMD) ---------- ----------2.0ml (0.2 %)

20% SDS---------- 5.0ml (0.1%)

50mg/ml heparin (Sigma) ---------- 2.0ml (100ug/ml)

Alkaline Phosphatase Buffer:

For 100ml

d.d. H_2O ----------74ml

1M Tris (pH9.5) ----------10ml (100mM)

1M MgCl2 ----------5ml (50mM)

1M NaCl ----------10ml (100mM)

10% Tween-20 ----------1ml (0.1%)

Wash Buffer

For 500ml

20XSSC ----------25ml (1XSSC)

formamide ---------- 250ml (50%)

10% Tween-20 ---------- 5ml (0.1%)

d.d. H_2O ---------- 220ml

Mice

Six3-Cre

Six3-Cre strain was kindly provided by Dr. Furuta (Furuta et al. 2000). In *Six3-Cre* mice, the *Cre* recombinase with the nuclear localization signal of the SV40-T gene was inserted into the first coding exon of a 9-kb genomic clone of the mouse *Six3* locus. The strain is maintained in C57BL/6 background. The *Cre* activity in

Six3-Cre mice starts around E9.5, and functions in the developing OC and OS as well as the ventral forebrain.

Fgfr1 conditional knockout mice

Fgfr1$^{fx/fx}$ strain was kindly provided by Dr. Chu-Xia Deng (Xu et al. 2002). In this strain, exons 8-14 which encode the transmembrane and most of the catalytic kinase domain (Yamaguchi et al. 1994) are flanked by LoxP sites. A recombination between the two *LoxP* sites generates a conditional null allele (*Fgfr1*$^{\Delta/\Delta}$), as homozygous *Fgfr1*$^{\Delta/\Delta}$ mice exhibit same phenotype as those of *Fgfr1* null mutants.

Fgfr2 conditional knockout mice

Fgfr2$^{fx/fx}$ strain was generously provided by Dr. David M. Ornitz (Yu et al. 2003). In *Fgfr2*$^{fx/fx}$ mice, exon 8, 9 and 10, which encode IgIIIb, IgIIIc and transmembrane domain, respectively, are flanked by LoxP sites. A recombination between the two *LoxP* sites generates a conditional null allele (*Fgfr2*$^{\Delta/\Delta}$), as homozygous *Fgfr2*$^{\Delta/\Delta}$ mice die between E10 and E11, and shows the same phenotype as that of *Fgfr2* null mice.

Z/EG: a _Cre-LoxP_ system reporter line

The *Z/EG* strain (Novak et al. 2000) was purchased from the Jackson Laboratory, and maintained in the SIMR Lab Animal Services Core Facility. The *Z/EG* is a Cre-LoxP system reporter line: without *Cre*, *LacZ* is expressed throughout embryonic and adult tissues, while a *Cre*-mediated recombination between the two *LoxP* sites in the *Z/EG* allele removes *LacZ* gene, and activates the expression of EGFP.

Mice maintenance and breeding

In addition to the transgenic strains, time-mated CD1 mice were provided by LASF of SIMR. All the animal work was performed in compliance with protocols approved by the Institutional Animal Care and Use Committee at SIMR.

Six3-Cre and *Z/EG* mice were maintained as heterozygous, while *Fgfr1*^{flox}, *Fgfr2*^{flox} mice were maintained as homozygous. I also generated *Fgfr1*$^{flox/flox}$ *Fgfr2*$^{flox/flox}$ double homozygous and *Fgfr1*$^{flox/flox}$; *Fgfr2*$^{flox/flox}$; *ZEG* strains by standard mouse breeding. To make homozygous conditional null mice for *Fgfr1* and/or *Fgfr2*, I first generated *Six3-Cre*$^{+}$; *Fgfr1*$^{flox/+}$, *Six3-Cre*$^{+}$; *Fgfr2*$^{flox/+}$ and *Six3-Cre*$^{+}$; *Fgfr1*$^{flox/+}$; *Fgfr2*$^{flox/+}$ mice by crossing *Six3-Cre* mice with *Fgfr1*^{flox}, *Fgfr2*^{flox} homozygous or double homozygous. Then, I generated *Six3-Cre*$^{+}$; *Fgfr1*$^{flox/flox}$, *Six3-Cre*$^{+}$; *Fgfr2*$^{flox/flox}$ *Six3-Cre*$^{+}$; *Fgfr1*$^{flox/flox}$; *Fgfr2*$^{flox/flox}$ embryos or mice by crossing *Six3-Cre*$^{+}$; *Fgfr1*$^{flox/+}$; *Fgfr2*$^{flox/+}$ males with *Fgfr1*$^{flox/flox}$; *Fgfr2*$^{flox/flox}$ females. To add the *Z/EG* background into mutant mice, I crossed *Six3-Cre*$^{+}$; *Fgfr1*$^{flox/+}$; *Fgfr2*$^{flox/+}$ males with *Fgfr1*$^{flox/flox}$; *Fgfr2*$^{flox/flox}$; *Z/EG* females.

To obtain embryos at a specific time point, plugs were checked every morning after matings are set up. After the plugs were found, females were separated from males to ensure the age of the embryo is correct. The day that a plug is found is designed as E0.5.

DNA preparation for genotyping

The genomic DNA was extracted from either tail for adult mice or yolk sac

for embryos. To extract DNA from the tails, I used a quick lysis DNA extraction method. The tails were placed in thin-wall PCR tubes with 75ul 1xbase solution, and were incubated at 95℃ in a thermal cycler for 40 minutes. After the tubes were cooled down, 75ul 1x neutralization solution was added to each of the tubes, and the DNA was ready for the next step PCR genotyping.

To extract the genomic DNA from the yolk sac, we used the Nuclei Lysis and Protein Precipitation Solutions purchased from Promega and followed the manufacture's protocol. The yolk sac was placed in an eppendorf tube with 500ul of the Nulear Lysis Solution, 120ul 0.5M EDTA and 17.5ul Proteinase K (Promega). The tubes were then incubated in a thermomixer (Eppendorf) at 55℃ with vigorous shaking for 1-3 hours. Then, after being cooled down to room temperature, the samples were then added 200ul of the Protein Precipitation Solution, vortexed vigorously at high speed for 20 seconds, chilled on ice for 5 minutes, and were centrifuged for 4 minutes at 13,000xg at 2-8 ℃. The supernatant containing the genomic DNA was removed from the tubes and are then transferred to new tubes containing 600ul isopropanol, mixed thoroughly, and were centrifuged for 1 minute at 13,000xg at 2-8℃. After the supernatant was removed, the DNA pellet was washed with 70% ethanol, and was centrifuge for 1 minute at 13,000xg at 2-8℃ again. After the ethanol was removed from the tubes, the DNA pellet was air-dried for 10 minutes, and was dissolved in 200-400ul d.d.water, which is ready for next step PCR genotyping.

PCR-based mouse genotyping

The standard PCR procedure to genotype mice is described as below:

The PCR reaction (20ul)

10X Taq buffer: 2ul

2mM dNTP: 2ul

Primers mix: 2ul

DNA: 4ul

Taq: 0.1ul

H2O: 9.9ul

The PCR cycle

I. 95℃ 4min 1cycle

II. 95℃ 30sec; 60℃ 30sec; 72 ℃ 30sec 35cycle

III. 72 ℃ 5min 1cycle

IV. 4 ℃ hold

The Primers used are as follow:

Six3-Cre: TACCTGGAAAATGCTTCTGT and TGATCTCCGGTATTGAAACT

Fgfr1$^{fx/fx}$: CTGGTATCCTGTGCCTATC and CAATCTGATCCCAAGACCAC for distinguishing *fx* allele with wild type allele.

GTATTGCTGGCCCACTGTTC and CAATCTGATCCCAAGACCAC for recognizing conditional null (Δ) allele.

Fgfr2$^{fx/fx}$: ATAGGAGCAACAGGCGG and TGCAAGAGGCGACCAGTCAG for distinguishing fx allele with wild type allele.

ATAGGAGCAACAGGCGG and CATAGCACAGGCCAGGTTG for recognizing conditional null (Δ) allele.

Z/EG: CGTAAACGGCCACAAGTTCAG and ACTCCAGCAGGACCATGTGATCG

Tissue processing and preparation

Adult mouse eyeballs were enucleated by putting a fine forceps on the bottom of the eyeball and gently pulling out. The eyeballs were then incubated with the Davidson's fixative or 4% formaldehyde at room temperature overnight. On next day, the eyeballs were washed 3 times with 1xPBS, soaked in 75% ethanol, and were sent to the SIMR Histology Facility to be embedded into paraffin. When embedding, the eyeballs were oriented so that the plane crossing the center of the cornea and the optic nerve is parallel to the bottom of the block.

Embryos were harvested following the procedures described in 'Manipulating the Mouse Embryo, A Laboratory Manual third edition' (Nagy et al. 2003). The yolk sacs were put into the Nuclei Lysis Solution for isolating genomic DNA as described above; the embryos were incubated in 4% formaldehyde. For paraffin embedding, the embryos were fixed overnight and washed three times with PBS, and went through a series of graded ethanol and xylene using a SAKURA Tissue-Tek VIP tissue processing machine, and then were embedded into paraffin in either sagittal or frontal section orientation. For cryosection, the embryos were fixed for 4 hours, washed with 1xPBS, cyopreserved by soaking in 30% sucrose overnight, and were

frozen into OCT with either sagittal or frontal orientation. For the whole-mount in situ hybridization, the embryos were fixed in 4% formaldehyde for 3 hours at room temperature, and then proceeded with the hybridization procedure.

The paraffin-embedding samples were sectioned under a Microm HM360 microtome, and the frozen samples were sectioned under a LEICA CM3050S cryostat. For embryonic eye samples, sectioning levels were monitored under a dissection microscope when sectioning, and serial sections were put alternatively onto 4-8 slides depending on the size of the developing eye. The sections were cut at 8um thickness.

Histology and immunohistochemistry

Most immunohistochemistry was performed on paraffin sections. The slides were deparaffinized by using a Leica staining machine. HE staining was performed following the standard protocol using the Leica staining machine. For most of the primary antibodies, immunofluorescent staining was performed as follows: the antigen retrieval was routinely performed for paraffin sections by heating slides in the sodium citrate buffer (pH6.0) at 95℃ for 10 minutes and cooling down for 20 more minutes; after being washed with 1xTBST, the slides were incubated with diluted primary antibodies in a humid chamber overnight at 4℃, washed with 1xTBST 3 times for 5 minutes each time, incubated with diluted secondary antibodies in a humid chamber for 2 hours at room temperature, washed with TBST 3 times for 5 minutes each time, counterstained with DAPI (100ng/ml), washed 2 times with 1xTBST, and were finally mounted using the VECTASHIELD mounting medium (VECTOR).

Phosphorylated ERK staining was performed by closely following the published protocol (Pan et al. 2006) with some minor modifications. Briefly, the staining was done on cryosections: the frozen slides were air-dried for 10 minutes, and were washed with 1xTBST to remove OCT. The slides were then heated in the sodium citrate buffer (pH 9.0) at 80℃ for 30 minutes, and were cooled down for 20 more minutes; to reduce the background activity of endogenous peroxidase, the slides were incubated with 3% H_2O_2 for 10 minutes; after being washed with 1xTBST, the slides were blocked with 5% normal goat serum (Jackson ImmunoResearch) for 1 hour, and were then incubated with rabbit anti-p-ERK antibodies (1:250) in a humid chamber overnight at 4℃. On the following day, after being washed with 1xTBST 3 times, the slides were incubated with poly-HRP conjugated anti-rabbit IgGs (Vision Biosystems) for 2 hours at room temperature. The slides were then washed 3 times with 1xTBST, and were incubated with DAB chromogen (DAKO) until the color was developed. Filamentous actin staining was also done on cryosections by incubating the slides with Alexa 488-conjugated phalloidin (Invitrogen) in a humid chamber at room temperature for 1 hour.

The following antibodies were used in this study: rabbit anti-Pax2 (1:400 Invitrogen), mouse anti-Pax6 (1:5 hybridoma bank), rabbit anti-Pax6 (1:400 Invitrogen), sheep anti-Chx10 (1:400 Chemicon), mouse anti-Mitf (1:100 Lab Vision), rabbit anti-N-cadherin (1:400 Santa Cruz), Rabbit anti- β III-tubulin (1:2000 Covance), mouse anti-Laminin (Sigma), rabbit anti-GFP (1:400 Invitrogen), Rabbit anti-p-ERK (1:250 Cell Signaling), Alexa 488- or Alexa 546- conjugated goat or

donkey secondary antibody (1:600 Invitrogen). The antibodies were diluted with the Antibody Diluent (Invitrogen).

mRNA in situ hybridization

mRNA in situ hybridization was performed on paraffin-embedded sections using digoxigenin-labeled RNA probes. Following deparaffinization, the slides were prehybridized with a hybridization solution at 65℃ for 3 hours in a sealed humid chamber, and were then hybridized with probes at 65℃ in the humid chamber overnight. On the second day, after being washed with the wash buffer at 65℃ 5 times for 20 minutes each time, the slides were blocked in 20% normal sheep serum in 1xTBST (Jackson ImmunoResearch) for 1 hour, and then incubated with an anti-digoxygenin antibody (Roche) (1:2000 dilution in blocking buffer) overnight at 4℃. On the third day, after washes, the color precipitation was developed by incubating the slides in the NBT-BCIP solution. For the whole-mount E10.5 embryo in situ hybridization, the embryos were first digested with 10μg/ml proteinase K (Promega) for 15 minutes, post-fixed with 4% formaldehyde for 20 minutes, rinsed 3 times with 1xTBST, and then followed the same procedure as for paraffin slides.

The RNA probes were synthesized following a standard procedure: probe synthesizing plasmids were linearized with a restriction enzyme for 4 hours, purified with phenol-chloroform-isoamyl alcohol (25:24:1) (Invitrogen), and were then used for *in vitro* transcription:

1ug of linearized template DNA in RNAse free H2O ------9ul

5x buffer ----------4ul

Rnaisin (40U/ul) (Promega) ----------1ul

100mM DTT (Promega) ----------2ul

NTP mix with 3.5mM Dig-11-UTP----------2ul

T3,T7 or SP6 RNA polymerase (Promega) ----------2ul

The *in vitro* transcription was carried out by incubating the reaction system at 37℃ for 2 hours and was followed by 1 hour Dnase I (Invitrogen) digestion to remove the template DNA. The probes were purified by ethanol precipitation, dissolved in 100ul hybridization solution, aliquoted and were stored in -20℃ freezer for later use.

Vax1 and *Vax2* probe synthesizing plasmids were purchased from ATCC, *Dct* from Open Biosystem, *Fgf15* from Riken. *Math5*, *Brn3b* probe synthesizing plasmids were generous gifts from Dr. Gan Lin. *Raldh3*, *Fgfr1*, *Fgfr2* probe synthesizing plasmids were generated by T-cloning of RT-PCR products from an E14.5 mouse head cDNA library into pGEM-T easy vector (Promega). Primers used for RT-PCR are:

Raldh3:ATGCACTGAGCAGAGGCCAGTT and TGCTGTGAGTCCATAGTCGGT

Fgfr1: CCGCAGCCTCACATTCAGTG and CGCTCTGGTGTGTGTAGATCC

Fgfr2: GAGTTGCAGTGCATGTTGAAAG and CTCGGAGACCCCTGCTAGCAT

BrdU incorporation assay and TUNEL assay

For the BrdU incorporation assay, time-mated mice were IP-injected with BrdU (20mg/ml Sigma) at 0.1mg/g body weight 2 hours before sacrifice. Proliferating cells that have incorporated BrdU during the two hours interval were revealed by BrdU immunofluorescent staining following the standard protocol for

immunostaining on paraffin sections as described above. The primary antibodies used here are mouse anti-BrdU (Amersham) and rabbit anti-BrdU antibodies (Megabase Research Products). The secondary antibody used are Alexa 488- or Alexa 546- conjugated goat anti-mouse or anti-rabbit antibodies (Invitrogen).

For the cell death analysis, we used TUNEL- (Terminal deoxynucleotidyl transferase UTP Nick End Labeling) based ApopTag Fluorescein Direct *In Situ* Apoptosis Dectection Kit from Chemicon to label the dying cells following the manufacturer's protocol. Briefly, the paraffin-embedded slides were deparaffinized, heated in a 10mM sodium citrate buffer (pH6.0) at 95℃ for 10 minutes, cooled down for 20 minutes, rinsed with 1xTBST twice, equilibrated with the Equilibration buffer for about 1 minute, and were incubated with a working strength TdT Enzyme mixture in a humid chamber at 37℃ for 1 hour; the TUNEL reaction was stopped by incubating slides with a working strength Stop/Wash Buffer for 10 minutes. The TUNEL labeled sections were then double stained with other antibodies following the standard procedures.

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