FGF SIGNALING IN XENOPUS LAEVIS LENS REGENERATION

BY

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DISSEbATION

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Abstract

The larvae of the frog *Xenopus laevis* is capable of regenerating lenses. In this regenerative process, the corneal tissue is capable of forming a lens after removal of the original lens, but the molecular mechanisms for this process is unknown. This dissertation examines the relationship between FGF signaling and lens regeneration, exploring the hypothesis that the FGF signaling plays a key role in triggering lens regeneration. First, the mRNA expression levels of FGFs and FGFRs in the cornea and retina were observed, as the key ligand from the retina travels to the cornea to interact with its receptor and trigger lens regeneration. The described experiments found that *FGF1*, *FGF8*, and *FGF9* mRNA are more expressed in the retina than the cornea, indicating that they may be the signal for inducing lens regeneration. Three receptor mRNAs (*FGFR1*, *FGFR2*, and *FGFR3*) were found to be expressed in the cornea. Second, the necessity of FGFR signaling was investigated *in vitro* using a small molecule inhibitor (SU5402) and dominant negative FGFR1. Both experiments demonstrated that lens regeneration is inhibited upon FGFR signaling inhibition, indicating the necessity of FGFR signaling in lens regeneration. Finally, the sufficiency of various factors for lens regeneration was tested using an *in vitro* cornea culture assay. FGF1 strongly induced lens formation, and FGF8 also appeared to weakly induce lens formation, whereas FGF2 and FGF9 did not induce lens formation. In addition, insulin and FBS also appeared to induce lens formation in cornea cultures. Overall, this dissertation illuminates the roles that FGF signaling play in *Xenopus laevis* lens regeneration. In the future, this work may guide subsequent research in treating eye diseases.
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Introduction

The vertebrate lens and cornea

The lens and cornea play important roles for vision in animals with camera eyes. In the vertebrate eye, the lens represents a transparent ellipsoid mass suspended between the anterior chamber just below the cornea and vitreous chamber of the eye cup. The transparency of the lens and minimizing the amount of light scatter within the lens are important factors for conducting light to the retina, which is essential for normal vision (Bassnett et al., 2011; Dahm et al., 2011). The cornea, located on the outer exposed surface of the eye, is also transparent. For terrestrial vertebrates, the cornea makes the largest contribution to the overall refractive index of the eye, necessary for focusing light onto the retina (reviewed in Land, 2012). The lens itself has a refractive index gradient, required for minimizing spherical aberration and decreasing the focal length required between the surface of the eye and the retina (Brown et al., 1999; Augusteyn, 2008). Unlike the cornea, the lens can either change shape or position to focus an image on the retina, but both of these tissues are required for proper focus of light onto the retina.

Lens development

The lens is derived from head ectoderm, specifically from one of the cranial placodes. These placodal regions surround the neural plate region during late gastrulation and neurulation stages in vertebrates (reviewed in Schlosser, 2010). In mammals, the lens placode invaginates to form a lens pit, which then becomes the primitive lens vesicle that detaches from the head epithelium (McAvoy et al., 1999; Gwon, 2006; Cvekl and Duncan, 2007). However, in the case of *Xenopus* and zebrafish, a morphologically identifiable hollow lens vesicle does not form, but the lens placode thickens and forms a lens rudiment consisting of a solid mass of cells void of a central lumen (Mcdevitt and Brahma, 1973; Dahm et al., 2011; ...
2007). This mass of cells then develops and differentiates into the lens epithelium and lens fibers. In both cases, the lens detaches from the head epithelium and forms a rounded structure consisting of an anterior epithelial cell layer and a posterior elongated fiber cell mass.

Vertebrate lens development occurs as a result of a complex suite of induction processes. Through sequential inductive interactions, the future lens can be localized to a disc of tissue in the region of the head ectoderm. Lens-forming competence is acquired early in development, most prominently in gastrula stage ectoderm (stage 11-12) in *Xenopus laevis* (Servetnick and Grainger, 1991). The developing tissue then gains an increasingly stronger lens-forming bias before the approach of the optic vesicle at the neurula stage, around stage 19 in *Xenopus* (Henry and Grainger, 1987, 1990; Grainger et al., 1997; Walter et al., 2004; Jin et al., 2012). Signaling from the optic vesicle then represents the late phase of lens development which pinpoints the site of lens development and directs the polarity of the developing lens.

**Signaling during lens development**

The early phase of lens induction is characterized by the expression of a number of transcription factors, including otx2 (Bovolenta et al., 1997; Zygar et al., 1998), pax6 (Hirsch and Harris, 1997; Li et al., 1997; Zygar et al., 1998), foxe3 (also known as Xlens1; Kenyon et al., 1999; Blixt et al., 2000; Brownell et al., 2000), six3 (Oliver et al., 1995; Bovolenta et al., 1998; Seo et al., 1998; Zhou et al., 2000), and sox2/sox3 (Kamachi et al., 1995; Penzel et al., 1997; Kamachi et al., 1998; Zygar et al., 1998). Of these transcription factors, Pax6 has been called the “master control gene” (Halder et al., 1995) for eye development, and is necessary and sufficient for inducing eye development in vertebrate embryonic tissues (Chow et al., 1999; Ashery-Padan et al., 2000; Onuma et al., 2002). As for signaling during the early phase of lens induction, the levels of FGF8 (discussed later) and BMPs are important for developing competence and bias (reviewed by Gunhaga, 2011; Ogino et al., 2012). BMP7 null mice are anophthalmic or microphthalmic and the embryos do not exhibit *Pax6* expression in the presumptive lens ectoderm prior to lens placode.
formation (Dudley et al., 1995; Wawersik et al., 1999). Similarly, knocking out BMP4, normally expressed in the optic vesicle, result in mice that do not have lens placodes (Furuta and Hogan, 1998).

Later lens development and differentiation involves additional signaling molecules, along with those mentioned above. During this later phase when the lens epithelium is under the influence of the optic vesicle and retina, the lens placode is fully formed, the lens detaches from the cornea, and the lens cells differentiate to form a mature lens in a directed and specific manner. Transcription factors are involved in lens fiber differentiation, including: prox1 (Oliver et al., 1993; Tomarev et al., 1996; Glasgow and Tomarev, 1998), mafb (Ishibashi and Yasuda, 2001; Coolen et al., 2005), sox1 (Kamachi et al., 1998; Nishiguchi et al., 1998; Nitta et al., 2006), c-maf (Yoshida et al., 1997; Kim et al., 1999; Ishibashi and Yasuda, 2001), and l-maf (Ogino and Yasuda, 1998; Ishibashi and Yasuda, 2001). There are a variety of signaling molecules important for the later phase of lens development, including various FGFs (discussed later), BMPs, retinoic acid, and Wnts (reviewed by Lovicu and McAvoy, 2005; Lovicu et al., 2011; Henry et al., 2013). Retinoic acid signaling is necessary for the invagination of the lens placode in mice, and a precise level of retinoid acid signaling is required for proper lens fiber morphology (Cvekl and Wang, 2009). BMP activity in the lens equatorial region is necessary for primary lens fiber formation in mice (Belecky-Adams et al., 2002; Faber et al., 2002) and FGF-induced lens fiber formation in culture (Boswell et al., 2008). Although Wnt signaling is inhibited early during lens induction (Smith et al., 2005), Wnt activity becomes necessary later for cytoskeletal reorganization in lens fiber cell elongation (Chen et al., 2008). Other growth factors (IGF, EGF, and PDGF) also appear to enhance lens fiber differentiation in culture when combined with FGF (Wang et al., 2010).

The vertebrate cornea

The cornea is a thin, transparent membrane in the anterior portion of the eye. Like the lens, the cornea is also derived from head ectoderm during early development. In mammals, the early developing cornea contains an epithelial layer and a endothelial layer, and
Figure 1.1 – Anatomy of the larval *Xenopus* eye

The anatomy of a *Xenopus laevis* larval eye is shown. At the larval stages, the cornea epithelium and cornea endothelium form two distinct layers of tissue. In *Xenopus* lens regeneration, the cornea epithelium and the pericorneal epidermis are capable of regenerating lens tissue upon exposure to factors from the neural retina. Head ectoderm is not normally competent to differentiate into lens. In some newts and salamanders, the iris pigmented epithelium can transdifferentiate into lens through Wolffian lens regeneration.

These cellular layers sandwich a stromal layer (Ozanics et al., 1976; Hay, 1980). The adult mammalian cornea contains an epithelial layer, Bowman membrane, a thick stromal layer, Descemet membrane and an endothelial layer (reviewed in DelMonte and Kim, 2011). In the *Xenopus* larval eye, there are two morphologically separate corneal layers, similar to the epithelial and endothelial layers of the developing mammalian cornea (Figure 1.1). The outer cornea has two epithelial layers and a deeper fibrillar layer, and is continuous with the head epithelium, whereas the inner cornea consists of a single layer of mesenchymal cells and is continuous with the sclera of the eye (Freeman, 1963; Perry et al., 2013). In later development, the outer and inner corneas fuse to form a single cornea. The adult *Xenopus* cornea has a similar structure to the mammalian cornea: there is an epithelial layer, a Bowman-like layer composed of loose collagen fibrils, a stromal layer composed of lamellarly packed collagen fibrils, the Descemet membrane, and an endothelial layer (Hu et al., 2013).
Lens regeneration

Among vertebrates, the process of lens regeneration after complete lens removal is a relatively rare phenomenon that occurs for a small subset of species. The earliest reports of lens regeneration were for salamanders that undergo Wolffian regeneration, named after one of its early scholars (Colucci, 1891; Wolff, 1895, 1901). In this process, the dorsal iris undergoes dedifferentiation and subsequently transdifferentiates into a lens. In contrast, the anuran *Xenopus laevis* has the ability to regenerate a lens from the inner basal layer of the outer cornea (Freeman, 1963).

Wolffian lens regeneration

Wolffian regeneration occurs through transdifferentiation of a portion of the dorsal pigmented iris into lens in a process triggered after the removal of the endogenous lens (Reyer, 1954). This method of regeneration is limited to selected species of newts and salamanders (Henry, 2003), and differs from lens development. More specifically, the origin of the iris and lens tissues are very different, as the embryonic dorsal iris develops from neuronal tissue (optic cup) (Davis-Silberman and Ashery-Padan, 2008) whereas the lens arises from placodal ectodermal tissue. From a transcriptome analysis, genes generally involved in proliferation, cytoskeletal changes, altered gene regulation, and responsiveness to injury were found to be enriched during lens regeneration (Sousounis et al., 2013). Several signaling pathways appear to play a role in Wolffian lens regeneration (reviewed by Grogg et al., 2006; Henry and Tsonis, 2010; Barbosa-Sabanero et al., 2012). Specific signaling molecules observed during regeneration include FGFs (discussed on page 17), retinoic acid (Tsonis et al., 2000), Wnts (Hayashi et al., 2006, 2008), Shh (Tsonis et al., 2004b), and Ihh (Tsonis et al., 2004b). Inhibition of BMP signaling also appears to be necessary for lens regeneration (Grogg et al., 2005), although BMP pathway members were identified to be expressed in a cDNA library constructed from transdifferentiating iris mRNA (Maki et al., 2010).
Lens regeneration in *Xenopus laevis*

Unlike Wolffian regeneration, the lens is regenerated from the cornea epithelium in *Xenopus*. There are five stages of lens regeneration in *X. laevis* classified by Freeman (1963), and cell proliferation has been noted from stage 2 through stage 5 (Waggoner and Reyer, 1975). Morphologically, this regeneration starts with the columnization of the inner layer of corneal epithelial cells (stage 1), followed by thickening of this layer of epithelium into a multilayered clump of cells (stage 2; Freeman, 1963; Campbell, 1965; Overton, 1965; Waggoner, 1973). This is similar to the case in lens development where the lens arises from the inner sensorial layer of the cornea ectoderm (Chanturishvili, 1958). The thickened “placode” then grows into a spherical mass of cells (stage 3). The regenerating lens then detaches from the cornea (late stage 3), starts developing primary lens fibers (stage 4), and eventually forms a spherical lens with both primary and secondary lens fibers (stage 5; reviewed in Henry, 2003; Henry et al., 2008; Henry and Tsonis, 2010). Lens regeneration from the cornea has been observed for *X. laevis* (Freeman, 1963), *X. tropicalis* (Henry and Elkins, 2001), and *X. borealis* (Filoni et al., 2006).

*Xenopus* lens regeneration resembles the late phase of lens development, as lens formation from the cornea is triggered by factors that emanate from the neural retina. Similar to development, proximity to an embryonic optic cup is sufficient for larval corneas to form a lens (Cannata et al., 2008), and conversely, placement of embryonic head ectoderm in the larval eye cup is sufficient for lens formation (Henry and Mittleman, 1995). Lens regeneration from corneas occur in cases when the cornea is in a location that is accessible to retinal factors, such as when corneas are implanted into the vitreous chamber (Reeve and Wild, 1978; Bosco et al., 1993c; Filoni et al., 1997) or after implantation of retinal tissue between the cornea epithelium and endothelium (Reeve and Wild, 1981). Removal of the eyecup or neural retina, i.e. removing any retinal factors, inhibits lens regeneration (Freeman and Overton, 1961; Freeman, 1963; Filoni et al., 1978, 1982). Also, the communication of retinal factors between the neural retina and cornea epithelium is necessary for in vivo lens regeneration (Bosco et al., 1979, 1980; Cioni et al., 1982). Lens regeneration is inhibited if this communication is obscured by the inner cornea (Bosco et al., 1979), the
original lens (Bosco et al., 1980), or a disc of Millipore filter paper (Cioni et al., 1982). Although the culture of an isolated cornea \textit{in vitro} does not result in lens formation, corneas cultured in neural retina-conditioned culture medium are able to differentiate to lens cells (Bosco et al., 1997a). Some experiments suggest that the retinal factor(s) may also be common to developing or regenerating limbs, as implanting a cornea into these limbs also results in lens regeneration (Waggoner, 1973; Filoni et al., 1991; Cannata et al., 1996). Non-corneal ectoderm can also develop competence to regenerate a lens (revealed by later implantation into the vitreous chamber) if an eye is transplanted beneath that ectodermal tissue early in development, suggesting a priming effect of unknown factors from the eye (Cannata et al., 2003).

**Signaling in \textit{Xenopus} lens regeneration**

\textit{Xenopus} lens regeneration involves expression of a similar array of genes as lens development, with some differences. Through large-scale gene expression screens, a large number of genes have been identified to be upregulated during lens regeneration (Henry et al., 2002; Malloch et al., 2009; Day and Beck, 2011). Similar to early expression of Pax6 during \textit{Xenopus} development (Hirsch and Harris, 1997; Li et al., 1997; Schaefer et al., 1999), Pax6 can be detected as early as the first day of lens regeneration (Mizuno et al., 1999b; Cannata et al., 2003). Other early transcription factors (otx2 and SOX3) have also been detected early in lens regeneration (Schaefer et al., 1999). Prox1, a transcription factor that appears after lens placode formation (Oliver et al., 1993; Tomarev et al., 1996; Glasgow and Tomarev, 1998), has been detected at stage 3 of lens regeneration or approximately 4 days after lens removal (Mizuno et al., 1999b; Schaefer et al., 1999).

The sequence of crystallin gene expression appears to be slightly different between lens regeneration and development. Although the \(\alpha\)- and \(\beta\)-crystallins are expressed around the same time as \(\gamma\)-crystallin during development, the appearance of \(\gamma\)-crystallin seems to occur later during lens regeneration than the other crystallins (Brahma and Mcdevitt, 1974; Mizuno et al., 1999a; Schaefer et al., 1999).
Lens cell regeneration in mammals

Mammals have a limited capacity for regenerating lens cells. Though not a true case of lens regeneration, per se, early studies have shown that if the lens is removed from a rabbit while keeping the lens capsule intact, any remaining lens epithelial cells on the capsule may proliferate to regrow portions of the removed lens (Cocteau and Leroy-D’Etiolle, 1827; Middlemore, 1832). More recently, this phenomenon has been shown to occur in cats (Gwon et al., 1993), rats (Lois et al., 2003), and mice (Call et al., 2004). These abnormal forms of lens epithelial cell proliferation lead to the formation of so-called “secondary cataracts” in human patients following cataract surgery, which must be subsequently treated (Wormstone et al., 2009).

Fibroblast growth factor pathway

Fibroblast growth factors

One pathway known to regulate lens development is the fibroblast growth factor (FGF) signaling pathway. The FGF proteins, historically known by their affinity for heparin (Burgess and Maciag, 1989), bind and activate fibroblast growth factor receptors (FGFRs) (Itoh and Ornitz, 2004). The FGF family can be subdivided into seven evolutionarily conserved subfamilies (Itoh, 2007; Itoh and Ornitz, 2011). There are 22 FGFs consisting of FGF1-23, with FGF15/19 considered to be a single gene whose orthologs were initially considered to be separate between mice (FGF15; McWhirter et al., 1997) and humans (FGF19; Nishimura et al., 1999). In addition, one subfamily (FGF11-14) has functions so distinct that it is increasingly considered to be separate from the FGF family, decreasing the number of FGFs to 18 actual family members within six subfamilies (Olsen et al., 2003; Goldfarb, 2005). FGFs share a core β-trefoil motif consisting of 12 anti-parallel β-strands (Eriksson et al., 1991; Zhang et al., 1991; Zhu et al., 1991). FGFs bind to FGF receptors (FGFRs), which are then activated through autophosphorylation, and signaling is initiated through three main downstream pathways: the phospholipase C-gamma
(PLCγ), phosphoinositide 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK) pathways (reviewed in Dailey et al., 2005; Mason, 2007; Dorey and Amaya, 2010).

FGF subfamilies have distinct characteristics that are unique for each subfamily. Typically, FGFs are secreted and act in a paracrine manner, binding their receptor in conjunction with heparin sulfate. However, the FGF1 subfamily members (FGF1 and FGF2) do not have a N-terminal signal sequence for secretion, and are secreted directly across the plasma membrane in a golgi-independent manner (Jackson et al., 1995; Schäfer et al., 2004; Prudovsky et al., 2008; Nickel, 2010). Another interesting subfamily is the FGF15/19 subfamily (FGF15/19, FGF21, FGF23), with members that do not bind heparan sulfate as readily as other FGFs and act in an endocrine manner, as opposed to the usual paracrine action of FGFs (Harmer et al., 2004b; Goetz et al., 2007; Itoh, 2010). Instead of heparin, they require Klotho as a co-receptor to bind FGFRs (Urakawa et al., 2006; Goetz and Mohammadi, 2013). The most distinct FGF subfamily may be the FGF11 subfamily. Members of this subfamily (FGF11-14, also known as the fibroblast growth factor homologous factors, FHF1-4) were identified by sequence similarity to known FGFs, and were predicted to have an intracellular function (Smallwood et al., 1996). FGF11 subfamily members are mainly expressed in neuronal tissues. However, this FGF11 subfamily was soon discovered to not interact with FGFRs, and have a different mode of action than other FGFs (Schoorlemmer and Goldfarb, 2001; Olsen et al., 2003). Instead of binding FGFRs, these FGFs bind voltage gated sodium channels and modulate their function in myocytes and neurons (Liu et al., 2001, 2003; Goldfarb et al., 2007; Laezza et al., 2007).

**FGF receptors**

There are four FGF receptors (FGFRs) with multiple isoforms that can each interact with different subsets of FGFs (Table 1.1). The typical FGFR consists of three extracellular immunoglobulin domains, a transmembrane domain, and two intracellular tyrosine kinase domains (reviewed by Eswarkumar et al., 2005; Mason, 2007). Upon binding to FGF ligands, FGFRs dimerize and undergo sequential autophosphorylation in the
<table>
<thead>
<tr>
<th>Subfamily</th>
<th>FGF</th>
<th>FGFR specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF1</td>
<td>FGF1</td>
<td>1b, 1c, 2b, 2c, 3b, 3c, 4</td>
</tr>
<tr>
<td></td>
<td>FGF2</td>
<td>1b, 1c, 2c, 3c, 4</td>
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<td>FGF4</td>
<td>FGF4</td>
<td>1c, 2c, 3c, 4</td>
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</tr>
<tr>
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<td>FGF3</td>
<td>1b, 2b</td>
</tr>
<tr>
<td></td>
<td>FGF7</td>
<td>2b, 4</td>
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<tr>
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<td>FGF10</td>
<td>1b, 2b</td>
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<td></td>
<td>FGF22</td>
<td>1b, 2b</td>
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<td>FGF8</td>
<td>1c, 2c, 3b, 3c, 4</td>
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<td></td>
<td>FGF16</td>
<td>2c, 3c</td>
</tr>
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<td></td>
<td>FGF20</td>
<td>1c, 2c, 3b, 3c, 4</td>
</tr>
<tr>
<td>FGF11</td>
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<td>No FGFRs</td>
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<tr>
<td></td>
<td>FGF12</td>
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<tr>
<td></td>
<td>FGF14</td>
<td>No FGFRs</td>
</tr>
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<td>1b, 1c, 2b, 2c, 3b, 3c, 4</td>
</tr>
<tr>
<td></td>
<td>FGF23</td>
<td>1c, 2b, 3b, 3c, 4</td>
</tr>
</tbody>
</table>

Table 1.1 – List of known vertebrate FGF family members and corresponding FGFR interactions
Vertebrate FGF family members are listed by subfamily, and interacting FGFR isoforms (i.e. b, c) for each FGF are listed as determined by Ornitz et al. (1996) and Zhang et al. (2006). Members of the intracellular FGF subfamily do not activate any known FGFR. FGFs highlighted in bold indicate those that have been identified in *X. laevis* and that have been investigated in Chapter 2.
cytoplasmic domains, which then enable phosphorylation and activation of downstream
pathway members (Mohammadi et al., 1996; Furdui et al., 2006). Within the general
structure of FGFRs, there are a multitude of different FGFR isoforms expressed in
vertebrates that do not have some of the domains (reviewed by Johnson and Williams,
1993; Mohammadi et al., 2005). For instance, some FGFRs have isoforms in which the
first N-terminal immunoglobulin domain is spliced out, resulting in a receptor with only
two extracellular immunoglobulin domains (Johnson et al., 1990; Mansukhani et al., 1990;
Reid et al., 1990; Miki et al., 1991; Champion-Arnaud et al., 1991). The presence of the
N-terminal immunoglobulin domain decreases FGFR activation, as this domain and its
adjacent acid box region causes FGFR autoinhibition by folding over and interacting with
the heparin-binding and FGF-binding domains (Xu et al., 1992; Wang et al., 1995; Olsen
et al., 2004; Kalinina et al., 2012).

More importantly, in the case of FGFR1, 2, and 3, the receptors each have two
major isoforms (IIIb and IIIc), distinguished by alternative splicing of exons in the third
extracellular immunoglobulin domain (Champion-Arnaud et al., 1991; Werner et al., 1992;
Avivi et al., 1993; Chellaiah et al., 1994). This spliced region is within the FGF-binding
domain, giving these FGFR isoforms affinities for vastly different groups of FGF family
members (Table 1.1; Ornitz et al., 1996; Zhang et al., 2006). The different IIIb and IIIc
isoforms are localized to different tissues, with IIIb isoforms expressed prominently in
epithelial cells and IIIc isoforms expressed more in mesenchymal cells (Miki et al., 1991;
Orr-Urtreger et al., 1993; Wuechner et al., 1996). There is also an additional FGFR-
like protein (FGFRL1, or alternatively, FGFR5) that has sequence similarity with the
extracellular region of FGFRs, but is lacking the intracellular tyrosine kinase domains
(Wiedemann and Trueb, 2000; Kim et al., 2001; Sleeman et al., 2001). Like FGFRs, it
is capable of binding many FGFs, and its role appears to be inhibition of FGF signaling,
possibly as a decoy receptor or in a dominant negative manner through dimerization with
FGFRs (reviewed by Trueb, 2011).
Heparan sulfate facilitates FGF-FGFR binding

Heparan sulfate is an important factor for FGF-FGFR binding (Yayon et al., 1991; Rapraeger et al., 1991). Heparan sulfate consists of a chain of repeating disaccharide units (uronic acid and a glucosamine derivative), with varying amounts of sulfation on each unit (Esko and Selleck, 2002). For FGFs, all of the subfamilies with a paracrine mode of action have a high affinity for heparin (Asada et al., 2009). An exception are the members of the FGF15/19 subfamily that have little to no affinity for heparin, and act in an endocrine manner. Within the paracrine FGF subfamilies, each subfamily shares a slightly different heparin binding site, leading to differing affinities between FGF subfamilies for various heparan sulfate molecular structures (Xu et al., 2012). Furthermore, all of the FGFRs have extracellular heparin binding sites (Kan et al., 1993; Hecht et al., 1995; LaRochelle et al., 1999; Loo et al., 2001). Structurally, a 2:2:2 arrangement of FGF, FGFR, and heparan sulfate binding can lead to FGFR activation (Schlessinger et al., 2000; Mohammadi et al., 2005; Goetz and Mohammadi, 2013), or possibly a 2:2:1 arrangement in which only one heparan sulfate is present in the complex (Pellegrini et al., 2000; Harmer et al., 2004a; Harmer, 2007).

FGF downstream pathways

There are three main downstream pathways for FGF signaling. Of these, the MAPK and PI3K pathways share a common upstream mechanism involving binding of FGFR with FRS2α, which then binds Shp2 and Grb2 (Hadari et al., 1998; Ong et al., 2001). The third downstream pathway is the PLCγ/calcium pathway (Carpenter and Ji, 1999). To activate the MAPK pathway, Grb2 binds and activates SOS to activate Ras, eventually leading to a series of downstream phosphorylation events that promote cell proliferation (Kouhara et al., 1997; Hadari et al., 2001). The MAPK pathway is inhibited by Sef, a protein expressed upon FGF signaling activation (Fürthauer et al., 2002; Tsang et al., 2002; Ron et al., 2008). Sprouty is also a potent inhibitor of the MAPK downstream pathway, potentially through an interaction with Grb2 (Cabrita and Christofori, 2008). For the PI3K pathway, Grb2 binds Gab1 with then recruits PI3K and activates downstream Akt.
signaling for cell survival (Ong et al., 2001; Lamothe et al., 2004). PLCγ is activated by FGFR at a site different from FRS2α (Mohammadi et al., 1991; Peters et al., 1992), and the associated pathway leads to intracellular signaling via PKC activation and calcium release (Carpenter and Ji, 1999).

Role of FGF pathway in lens development

In vertebrates, the FGF pathway plays important roles in embryonic lens development (reviewed in Donner et al., 2006; Robinson, 2006; Gunhaga, 2011). Historically, FGF activity on lens cells was identified early, as FGFs were formerly called “eye-derived growth factors” (Barritault et al., 1981; Arruti et al., 1985) before they were recognized to be identical to FGFs from other tissues (Baird et al., 1985b; Courty et al., 1985; Schreiber et al., 1985). FGF pathways are active both in the early and late phases of lens development, encompassing early placode formation through lens fiber differentiation (Robinson, 2006; Gunhaga, 2011).

FGF function in lens development

In the early phase of lens development, lens cell fate is determined within a subset of the cranial placodes including the presumptive lens ectoderm. FGF expression, in combination with BMP and Wnt antagonists, is necessary for cranial placode formation (Litsiou et al., 2005). In early rostral tissues, FGF8 expression is required for determination of placodal fates (Sjödal et al., 2007). More specifically, inhibition of FGF signaling in chick embryos at gastrulation caused epidermal cell formation instead of placodal formation, and placodal cell generation was rescued by FGF8 application. In the mouse model, chemical inhibition of FGFR signaling using SU9597 inhibited Pax6 expression and led to the later formation of a smaller lens pit (Faber et al., 2001). However, an excess of FGF pathway activation in a cranial placode leads to a non-lens fate (Bailey et al., 2006). Upon placode formation, the mouse lens placode has high levels of FGFR1 and FGFR2 expression, as determined by microarray and RT-PCR analyses (Garcia et al., 2011). Dominant negative inhibition of FGFR signaling by conditionally expressing truncated FGFR1 (specific to the
lens via the pax6 promoter) generated transgenic mice with a smaller lens placode, and later developing smaller lenses throughout embryogenesis (Faber et al., 2001). Similarly, in a mouse conditional knockout of FGFR2 in a lens placode specific manner (using a pax6 promoter), there were defects in eyelid formation, small lenses, and increased apoptosis among lens fiber cells (Garcia et al., 2005). If both FGFR1 and FGFR2 were conditionally knocked out, there was increased apoptosis within the lens placode and a thinner lens placode was present. These mouse embryos typically did not have detectable lens tissue at birth (Garcia et al., 2011).

During later eye development, an FGF concentration gradient between the vitreous and aqueous humors plays an important role in determining lens polarity. There is a greater concentration of FGF in the vitreous humor than the aqueous humor (Caruelle et al., 1989; Schulz et al., 1993) affecting the FGF concentrations in the lens capsule surrounding the lens (de Iongh and McAvoy, 1992; Lovicu and McAvoy, 1993), helping determine lens polarity (Chamberlain and McAvoy, 1997). More specifically, increasing FGF concentrations induce cultured rat lens epithelial cells to proliferate (150 pg/mL), migrate (3 ng/mL), and then elongate into lens fiber cells (40 ng/mL) expressing α- and β-crystallin (Chamberlain and McAvoy, 1989; Lovicu and McAvoy, 1989; McAvoy and Chamberlain, 1989). Similarly, chick lens epithelial cultures also differentiate into lens fiber cells upon FGF stimulation (Le and Musil, 2001) although not as rapidly as treatment with vitreous humor (Huang et al., 2003), and FGF8 application in the developing chick embryo results in increased lens fiber differentiation (Vogel-Höpker et al., 2000). Some FGF9 knockout mice have delayed lens fiber differentiation (Zhao et al., 2001). Lens-specific overexpression of FGF3, 4, 7, 8, or 9 or a secreted FGF1 in the mouse using an αA-crystallin promoter resulted in premature differentiation of lens epithelial cells into lens fiber cells (Robinson et al., 1995b; Lovicu and Overbeek, 1998; Robinson et al., 1998; Govindarajan and Overbeek, 2001). These experiments emphasize the importance of the precise FGF concentration gradient in lens development. The FGF gradient is also important for lens fiber cell orientation, as lens-specific overexpression of FGFs in mice disrupts lens organization (Stolen et al., 1997; Lovicu and Overbeek, 1998; Robinson
et al., 1998). In an interesting study, Yang et al. (2010) devised a protocol to induce
differentiation of human embryonic stem cells into lens cells and found that FGF signaling
was essential in the later stages of differentiation. More specifically, the later application
of FGF2 (day 6 to day 35) was part of the optimal treatment to form lentoids in culture
(Yang et al., 2010). This FGF requirement in stem cell differentiation appears to span the
period of forming lens progenitor cells through early lens fiber differentiation. In a striking
illustration of FGF necessity, a FGF9 mutation (Y162C) in the mouse model gives rise to
a lens-specific phenotype in an otherwise normally developing mouse, consisting of smaller
lenses and reduced vision due to defective lens fiber differentiation (Puk et al., 2011).

**FGFR function in lens development**

As for the receptors, an in situ study noted the presence of murine *FGFR3* in the
embryonic E14.5 lens but not any other *FGFRs* (Yaylaoglu et al., 2005). Early transgen-
esis studies in mice indicated that inhibiting FGFR function in general using a truncated
dominant negative FGFR1 (capable of heterodimerizing and inhibiting multiple FGFRs;
Ueno et al., 1992) disrupted lens development (Chow et al., 1995; Robinson et al., 1995a).
Disruption of FGFR3 function in transgenic mice specific to the lens region also leads
to a lens with a larger amount of epithelial cells and fewer fiber cells (Govindarajan and
Overbeek, 2001). This was accomplished by expressing a soluble extracellular portion of
FGFR3 with an αA-crystallin promoter. Interestingly, the equivalent transgenic mouse
with FGFR1 disruption did not exhibit these abnormalities, with lenses that resembled
those of nontransgenic mice. A similar experiment using a Cre/flox system to conditionally
delete FGFR1 expression in lenses also did not result in lens defects, nor did using null
FGFR1 embryonic stem cells in chimeric mice (Zhao et al., 2006). In addition, the double
mutant with conditional deletions of both FGFR1 and FGFR2 using an α-crystallin
promoter also was developmentally normal (Zhao et al., 2008). On the other hand, the
triple mutant mouse with conditional deletion of FGFR1, 2, and 3 in lens cells had a
severe lens defect with decreased lens fiber elongation and no γ-crystallin expression (Zhao
et al., 2008; Newitt et al., 2010). Only a single allele of FGFR2 or FGFR3 expressed in the
lens was required to rescue the FGFR deficient lens back to a normally developing lens, suggesting redundancy in function between FGFRs (Zhao et al., 2008). These deletion studies suggest that FGFR function is necessary, but no single FGFR plays a defining role in mouse lens development. Supporting the notion of FGFR necessity, deletion of Ndst1, an enzyme required for heparan sulfate synthesis, causes diminished FGF-FGFR binding and also gives rise to lens defects (Pan et al., 2006; Qu et al., 2011). In particular, Qu et al. (2011) found that FGF3-FGFR3b binding on lens cell surfaces was almost completely inhibited (as opposed to somewhat weaker FGF3-FGFR1b binding and intact FGF3-FGFR2b binding) after Ndst1 deletion. This suggests that FGFR3 function plays an important role in lens fiber differentiation, possibly in conjunction with the FGF gradient (Govindarajan and Overbeek, 2001).

**FGF and FGFR expression in *Xenopus* lens development**

Members of the FGF pathway have been identified to be expressed in *Xenopus* lens and cornea tissues. FGF1, FGF3, FGF13, FGF14, and FGF20 were found in *Xenopus* eye tissues in an in situ study of embryos across a variety of stages (Lea et al., 2009). Furthermore, all four FGFRs were found to be expressed in various *Xenopus* eye tissues. FGFR3 was especially found to be expressed in the lens (Lea et al., 2009), similar to the case in mouse (Peters et al., 1993), newt (Shi et al., 1994), rat (de Iongh et al., 1997) and chick (Walshe and Mason, 2000) lenses. FGFR2 was found in the cornea of the *Xenopus* stage 35 and stage 40 eyes, after the formation of a lens (Lea et al., 2009).

**Sef/Spry in lens development**

Sef and Spry have also been observed to have a role in lens development. Spry1 mRNA is expressed in the embryonic rat lens vesicle and later in the developing lens epithelium, and Spry2 is expressed ubiquitously in all embryonic developing rat lens tissues and then lost in the most mature lens fiber cells postnatally (Boros et al., 2006). Spry2 expression was confirmed by immunofluorescence, with expression most prominent in the lens epithelium and early lens fiber cells. A mouse conditional deletion of Spry1 or Spry2 in the lens using the Pax6 promoter resulted in a failure of the lens vesicle to detach from the
cornea, and premature lens fiber differentiation (Kuracha et al., 2011). This phenotype was present for both individual \textit{Spry1} and \textit{Spry2} deletions and also for the double-null (\textit{Spry1} and \textit{Spry2}) lenses. A preliminary survey of \textit{Spred2} expression in mice included slightly positive expression in the developing lens vesicle and later lens (Tuduce et al., 2010). As for Sef, similar to the \textit{Spry1} expression pattern, Boros et al. (2006) found that Sef protein is strongly expressed in the lens epithelium in a variety of species (mouse, rat, chicken, cow, and human). Overexpression of Sef in the mouse lens led to inhibition of lens fiber cell elongation, similar to the case in FGFR null mice (Newitt et al., 2010).

**Role of FGF pathway in lens regeneration**

**FGF pathway in Wolffian lens regeneration**

Wolffian regeneration studies in the newt have shown that FGF and FGFR family members are expressed in the dorsal iris as it is transdifferentiating into a lens. FGF1 and FGF2 expression are both correlated with lens regeneration. An in situ study noted that \textit{FGF1} was expressed in the dedifferentiating dorsal iris and regenerating lens after lens removal (Del Rio-Tsonis et al., 1997). \textit{FGF2} was also identified by RT-PCR to be upregulated in both the ventral and dorsal iris after lens removal (Hayashi et al., 2004). In a recent transcriptome analysis comparing expression between ventral and dorsal iris after lentectomy, \textit{FGF10} expression was enriched in the dorsal iris relative to the ventral iris both 4- and 8- days post lentectomy and was confirmed by qRT-PCR (Sousounis et al., 2013). FGFR1, 2, and 3 are expressed in the iris tissues during lens regeneration. In contrast, the normal newt iris expresses \textit{FGFR2} and \textit{FGFR3} mRNA but not \textit{FGFR1} (Del Rio-Tsonis et al., 1997, 1998). FGFR1 protein is expressed in the dorsal lens-forming iris and the lens epithelium of the regenerated lens, but not in the ventral iris, although a detectable amount of \textit{FGFR1} mRNA by in situ is present in the ventral iris (Del Rio-Tsonis et al., 1998). FGFR2 expression is seen in both the ventral iris and dorsal iris during regeneration, and was also observed in the regenerating lens (Del Rio-Tsonis et al., 1997). FGFR3 is also expressed more in the dorsal iris than the ventral iris, especially 9 days after lens removal (McDevitt et al., 1997).
FGF pathway activation has been shown to be necessary for Wolffian lens regeneration. In a study that applied SU5402, a small molecule inhibitor of FGFR tyrosine kinase activity, lens regeneration was inhibited, suggesting that FGFR activation is necessary for lens regeneration (Del Rio-Tsonis et al., 1998). In a more recent study, Hayashi et al. (2004) used a daily injection of a soluble competitive recombinant FGFR2 IIIc (FGFR2/Fc) into the eye to study the effects of FGFR inhibition. FGFR2/Fc injection resulted in inhibition of lens regeneration, whereas the injection of a similar soluble recombinant FGFR2 (IIIb) did not inhibit lens regeneration. Thus, the specific FGFR IIIc isoform may be required for lens regeneration (Hayashi et al., 2004). In addition, like development, FGF pathway activation plays a role in lens structure and lens fiber differentiation. Application of exogenous recombinant FGF1 or FGF4 during the lens regeneration process perturbs the structure of the regenerated lens, and can result in multiple lens formation (Del Rio-Tsonis et al., 1997; Yang et al., 2005). These experiments demonstrate the the precise gradient of FGF is necessary for proper lens structure in the later stages of lens regeneration.

FGF pathway activation by specific FGF ligands appears to be sufficient to induce Wolffian lens regeneration in newt tissues. Hayashi et al. (2002) examined the effect of adding FGF2, FGF4, FGF8, FGF10, EGF, IGF1, and VEGF to cultured newt dorsal iris epithelial cells. Of these ligands, FGF2 and FGF4 were sufficient to induce in vitro lens development in these cultures, in a similar manner as Wolffian lens regeneration in vivo. Furthermore, a FGFR inhibitor (SU5402) was able to inhibit this effect, strengthening the argument that FGF2 and FGF4 may be key ligands for specific FGF receptors in this transdifferentiation process (Hayashi et al., 2002). Interestingly, an earlier in vitro culture study of embryonic chick retinal pigmented epithelial cells also noted that FGF2 addition to the culture medium resulted in formation of lentoid bodies, mimicking Wolffian lens regeneration (Hyuga et al., 1993). In a later in vivo study, Hayashi et al. (2004) examined the effect of injecting FGF1, 2, 4, 7, 8, 9, and 10 into the anterior or posterior chamber of the eye without lens removal, as well as EGF, IGF, and VEGF. Of these growth factors, only FGF2 was sufficient to induce the formation of a second lens from the dorsal iris tissue. This suggests that FGF2 signaling is a key factor for Wolffian lens formation. Taken
together, these Wolffian regeneration studies suggest that FGF2 activating FGFR2 IIIc, leading to activation of downstream pathways, is important for transdifferentiation from iris to lens. The nature of these activated downstream pathways is not fully understood.

**FGF pathway in *Xenopus* lens regeneration**

Expression of members of the FGF pathway has been observed in the regenerating *Xenopus* cornea and retina. Day and Beck (2011) performed a microarray analysis that compared transcript expression between regenerating *Xenopus* corneas, lenses, and sham operated corneas (an operation where the cornea endothelium and lens were allowed to remain intact, thus not inducing regeneration). In this analysis, *FGF8b* was identified to be upregulated in regenerating corneas. Similarly, in our lab, a subtracted cDNA library was analyzed to detect differences in gene expression between control corneas and corneas undergoing lens regeneration (Henry et al., 2002). In an analysis of this data, *FGFR3* was identified to be up-regulated in regenerating corneas (Malloch et al., 2009). This subtracted library also identified other downstream members of the FGF pathways. Specifically, *mek2* (MAPK pathway), *grb2* (upstream of the MAPK and PI3K pathways), and *spry2* (negative regulator of FGFRs) were identified to be upregulated in the regenerating cornea (Malloch et al., 2009). Using immunohistochemistry, Arresta et al. (2005) correlated FGFR2 (also known as Bek) protein expression with regions of ectoderm tissue competent to form a lens (i.e. the cornea and pericorneal ectoderm). FGFR2 was not present in flank epidermis, which is not competent to form a lens at the stages examined (Arresta et al., 2005). The authors also found that autotransplantation of an eye underneath head epidermal tissue, in regions that are not normally competent to regenerate a lens, increases the competence of that epidermal tissue to regenerate a lens, as shown previously by Cannata et al. (2003). They demonstrated that this operation leads to an increase in FGFR2 expression in treated head epidermal tissue, relative to normal head epidermis (Arresta et al., 2005). There is some evidence for the sufficiency of FGF pathway activation in cornea lens regeneration: the application of a relatively high concentration of FGF1 (500 ng/mL) was found to be sufficient for lens cell formation.
(though not complete lenses) in cultured *Xenopus* corneas (Bosco et al., 1994, 1997b). This dissertation will expand upon the knowledge of the correlation between the FGF pathway and *Xenopus* lens regeneration in Chapters 2 and 3.

**Role of insulin and IGF in lens development and regeneration**

Insulin and IGF may play important roles in lens development. Both insulin receptors and IGF receptors are present in various vertebrate lens epithelial and fiber cells (Hollenberg, 1975; Bassas et al., 1987; Bassnett and Beebe, 1990; Sinha et al., 1990; Jacobs et al., 1992; Ibaraki et al., 1996; Naeser, 1997). IGF may play a necessary role in chick lens fiber differentiation, as lens fiber differentiation following addition of vitreous humor in chick lens epithelial cultures was attenuated by treatment with IGF1 antibody (Beebe et al., 1987). As for sufficiency, addition of insulin or IGF both induce cultured chick lens epithelial cells to proliferate and initiate fiber differentiation, increasing δ-crystallin expression levels (Milstone and Piatigorsky, 1977; Le and Musil, 2001). Similarly, addition of insulin or IGF to rabbit lens epithelial cell cultures induced cell proliferation in a dose-dependent manner (Reddan et al., 1975; Reddan and Wilson-Dziedzic, 1983). On the other hand, in the mouse system, overexpression of IGF1 in lens cells did not appear to induce early lens fiber differentiation (Shirke et al., 2001). Insulin or IGF treatment in the rat lens epithelial explant system has only a modest effect on increasing lens fiber differentiation, as measured by crystallin expression, but the combination of FGF and insulin or IGF1 greatly enhanced crystallin expression relative to adding each factor alone (Chamberlain et al., 1991; Richardson et al., 1993; Civil et al., 2000). Transgenic overexpression of IGF1 (Shirke et al., 2001) or the receptors for insulin or IGF (Xie et al., 2007) in the mouse lens expands the transitional zone of the lens epithelium and shifts the location of the germinative zone resulting in defective lens morphology, suggesting that these factors help specify the location of the germinative (equatorial) zone during development.

There is conflicting evidence on the correlation between Wolffian lens regeneration and insulin or IGF. There is a single early abstract by Williams and McGlinn (1979) claiming that insulin supplementation in newt iris cultures induced lens fiber formation.
However, another study found that although insulin in newt iris cultures may enhance depigmentation, insulin treatment did not lead to lens formation (Connelly, 1980). As for IGFs, cultured newt irido-corneal complexes *in vitro* responded to IGF1 treatment with lens fiber formation (Connelly and Green, 1987). On the other hand, addition of IGF1 to cultured newt iris cell reaggregates did not promote lens formation (Hayashi et al., 2002). IGF1 injection into the anterior chamber of the eye in the newt also did not induce lens regeneration (Hayashi et al., 2004). To date, no studies have investigated the role of IGF or insulin in *Xenopus* lens regeneration. This dissertation will examine the correlation between insulin and *Xenopus* lens regeneration in Chapter 3.
Chapter 2

FGF signaling is Required for Lens Regeneration in *Xenopus laevis*

Introduction

The fibroblast growth factors (FGFs), formerly known as “heparin-binding growth factors,” are a family of growth factors with high affinity for heparin sulfate proteoglycans (reviewed in Ornitz and Itoh, 2001). In mammalian systems, there are a total of 22 FGFs, numbered FGF1-23, as FGF15/19 represents a single FGF initially discovered in different species (mouse FGF15 and human FGF19; Ornitz and Itoh, 2001; Robinson, 2006). These FGFs have been divided into seven subfamilies, each classified by sequence homology (Itoh and Ornitz, 2004; Itoh, 2007; see Table 1.1). Among the members of the FGF family, significant differences among FGFs concerning localization and function have been found between various tissues (Xu et al., 1999; Ford-Perriss et al., 2001; Bottcher and Niehrs, 2005; Dailey et al., 2005; Thisse and Thisse, 2005; Robinson, 2006; Itoh, 2007; Lea et al., 2009). However, the members of specific FGF subfamilies generally have common FGFR receptor specificities, suggesting similar downstream effects may be elicited by members of each subfamily (Ornitz et al., 1996; Zhang et al., 2006). There are a total of four FGF receptors (FGFRs), and each has multiple isoforms. The most commonly made distinction in FGFR isoforms are the IIIb and IIIc isoforms, differing by alternative splicing of a pair of exons, and possessing different FGF affinities (Groth and Lardelli, 2002). Known FGF/FGFR interactions are summarized in Table 1.1.

It is well established that some vertebrates are able to regenerate parts of the eye including the lens (Henry, 2003; Tsonis and Del Rio-Tsonis, 2004; Filoni, 2008; Henry et al., 2008; Henry and Tsonis, 2010). Lens regeneration is restricted among vertebrates, generally limited to some urodeles and anurans of the genus *Xenopus* (Henry, 2003; Tsonis
et al., 2004a; Henry et al., 2008; Henry and Tsonis, 2010). The latter include *X. laevis* (Freeman, 1963), *X. tropicalis* (Henry and Elkins, 2001), and *X. borealis* (Filoni et al., 2006). In *Xenopus* lens regeneration, the outer cornea epithelium forms a thickening and subsequently a lens vesicle that develops into a mature lens (Freeman, 1963). Thus, in *Xenopus*, both the embryonic lens and the regenerated lens originate from head ectodermal tissues. There has been some evidence correlating FGF pathway function with *Xenopus* lens regeneration. In one study, it was shown that the addition of FGF1 protein (formerly referred to as “aFGF” or “acidic FGF”) to isolated cultured corneas would trigger lens cell differentiation (Bosco et al., 1997b). Specifically, Bosco et al. (1997b) showed that the addition of FGF1 enabled cultured outer corneas to undergo transdifferentiation into lentoids containing lens fibers, whereas these cultures in media alone do not transdifferentiate. In addition, a later study demonstrated a correlation between FGFR2 protein expression and lens regeneration competent ectoderm (Arresta et al., 2005). In this study, Arresta et al. (2005) established that only those ectodermal tissues known to be competent to transdifferentiate into lenses were labeled by an antibody specific to FGFR2 IIIc protein, also known as the *bek* isoform of FGFR2, suggesting that FGFR2 may play a role in *Xenopus* lens regeneration.

Currently, we do not know exactly which FGFs and FGFRs are expressed in *Xenopus* larval eye tissues, and the requirement of FGFR signaling has not been shown in the context of cornea-lens transdifferentiation in the larval eye. To examine these questions, we have characterized the expression of FGFs and FGFRs during lens regeneration, and further, using a pharmacological inhibitor of FGFRs (SU5402), our experiments suggest the necessity of FGFR function in lens regeneration in *X. laevis*.

**Results**

**FGFs are expressed in cornea and retinal tissues**

RT-PCR experiments were performed to assess the expression of FGFs in the cornea epithelium and neural retina during various time points prior to and during lens regeneration. mRNA expression within cornea and retinal tissues was evaluated in both control
Expression of FGFs as determined by RT-PCR assays. Total RNA was collected from corneas and retinas of both non-regenerating control and lens-regenerating larvae. Regenerating corneas and retinas were collected at four timepoints (1, 3, 5, and 7 days after lens removal). Note that the bands for FGF1, FGF8, and FGF9 are uniformly less intense in the cornea when compared to the retina, as determined from replicate RT-PCR reactions. 0d denotes non-regenerating control eye tissues; + denotes positive control cDNA derived from mRNA of whole embryos (st. 37-38); - denotes the negative control without addition of template cDNA.

non-regenerating and regenerating eye tissues during four timepoints (1, 3, 5, and 7 days) following lens removal. The presence of mRNA was assessed for the presence of all Xenopus laevis FGF sequences included in the NCBI database (i.e. FGF1, 2, 3, 4, 7, 8, 10, 12, 13, and 20). In addition, RT-PCR primers were designed from available genomic Xenopus tropicalis FGF sequences in the JGI genome database for those not available in the NCBI database. Primers were successfully designed for six additional FGFs in X. laevis (FGF5, 9, 11, 14, 15/19, and 16; Table 5; amplicon GenBank accession numbers are JF433082, JF433083, JF433084, JF433085, JF433086, and JF433087 respectively). PCR products were verified by sequence analysis. Eleven FGFs (FGF1, 2, 5, 7, 8, 9, 10, 11, 12, 13, 14, and 16; see Fig. 2.1) were detected in both control cornea tissues and corneas undergoing the process of lens regeneration. Twelve FGFs (FGF1, 2, 5, 7, 8, 9, 10, 11, 12, 13, 14, and 16; see Fig. 2.1) were detected in retinal tissues throughout these timepoints. Though these assays are not quantitative, some potential differences were observed in the level of the amplified PCR products for certain FGFs and at various time points (Fig. 2.1). Of interest, the expression levels of FGF1, FGF8, and FGF9 mRNA in the cornea were consistently lower than the corresponding levels in the retina.
Figure 2.2 – RT-PCR expression of FGFRs in eye tissues

Expression of FGFRs as determined by RT-PCR assays. Total RNA was collected from corneas and retinas of both non-regenerating control and lens-regenerating larvae. Regenerating corneas and retinas were collected at four timepoints (1, 3, 5, and 7 days after lens removal). 0d denotes non-regenerating control eye tissues; + denotes positive control cDNA derived from mRNA of whole embryos (st. 37-38); - denotes the negative control without addition of template cDNA.

FGFRs are expressed in cornea and retinal tissues

Similarly, RT-PCR experiments were performed to characterize the expression of FGFRs in the cornea and retina during lens regeneration. As shown in Figure 2.2, only FGFR1, 2, and 3 were expressed in the cornea throughout the period of regeneration examined in this study. In contrast, all four FGFRs were detectable in the retina, though more prominent levels of PCR product were consistently observed for FGFR1 and 4.

SU5402 application inhibits lens regeneration

Increasing dosages of SU5402 (2µM, 5µM, 10µM, and 25µM), a FGFR inhibitor, were tested for their ability to inhibit lens regeneration. Lenses were identified in sectioned eye cultures via anti-lens antibody staining (Fig. 2.3). Under control conditions (0.25% DMSO in modified L-15 medium), 82% of cultured eyes regenerated a lens (23 out of 28 cases; Fig. 2.3A-B, 2.4). On the other hand, application of SU5402 resulted in a dose-dependent inhibition of lens regeneration (Fig. 2.3C-L, 2.4). Though there was no inhibition of lens regeneration with 2µM SU5402 (81% regeneration; 13 out of 16 cases examined; p = 0.62), there was almost no regeneration at the higher concentrations tested (10µM and 25µM SU5402). Furthermore, individual cases of lens regeneration at the higher concentrations of SU5402 were small and represented only a preliminary stage of lens regeneration (stage 2, as described in Freeman 1963), in which the developing lens appears only as a thickening of the cornea epithelium (data not shown). The lens regeneration rate was halved at 5µM
SU5402 (38% regeneration; 6 out of 16 cases), representing a significant decrease in the lens regeneration rate relative to the control cases \((p = 0.0038)\). There was almost no regeneration upon application of \(10\mu\text{M SU5402}\) (5.9% regeneration; 1 out of 17 cases; \(p = 4.5\text{e-7}\)) and \(25\mu\text{M SU5402}\) (2.5% regeneration; 1 out of 40 cases; \(p = 2.6\text{e-12}\)). From this data, the IC50 for lens regeneration can be determined to be approximately 5\(\mu\text{M SU5402}\). The cultured eye tissues otherwise all appeared to be healthy and of normal morphology at all doses tested (see Fig. 2.3).

**Discussion**

Experimental evidence shows that critical retinal factors trigger *Xenopus* cornea-lens transdifferentiation or “lens regeneration”. Through the removal of various eye tissues after lentectomy, Filoni et al. (1982) found that the presence of the neural retina was key to inducing lens regeneration during larval stages. In *X. laevis*, although lens regeneration ability declines as the tadpole ages (Freeman, 1963), cornea epithelia from later stages including adult frogs inserted into the vitreous chamber are still capable of transdifferentiating into lenses in culture (Filoni et al., 1997). Bosco et al. (1993c) showed that the presence of neural retina was sufficient to induce lens regeneration in cultured corneas. Further, Bosco et al. (1997a) showed that corneas transdifferentiated into lens after exposure to centrifuged, filtered, retina-conditioned culture medium, thus indicating that some diffusible factor was responsible for inducing lens regeneration. From this evidence, one can hypothesize that a retinal signaling ligand is responsible for inducing lens regeneration, though this has yet to be identified.

As the retina represents the source of key signaling factors required to support lens regeneration, it is possible that one or more of the FGFs detected in the present study could play a role in this process. Normally, the cornea is isolated from the signaling factors provided by the retina via the presence of the lens and the inner cornea endothelium (Freeman, 1963). This ensures that supernumerary lenses are not normally formed in the larval eye (Reeve and Wild, 1978; Bosco et al., 1979; Filoni et al., 1980). Although we are describing expression at the level of transcription, one could argue that key signals
Figure 2.3 – Sectioned eyes after SU5402 treatment in culture
Development of control and SU5402 treated in vitro eye cultures. SU5402 inhibits FGFR function by competitively binding to the FGFR kinase domain. The left column shows sections of representative eyes imaged using differential interference contrast. The right column shows each corresponding fluorescent image illustrating α-lens antibody staining. SU5402 concentrations: (A-B) 0 µM control; (C-D) 2 µM; (E-F) 5 µM; (G-J) 10 µM; (K-L) 25 µM. The single case of lens regeneration with 10 µM SU5402 is shown in G-H. The typical results of non-regenerating cases are shown for 10 µM SU5402 (H-I) and 25 µM SU5402 (K-L). Arrows point to regenerated lenses; scale bar equals 200 µm.
Figure 2.4 – Lens regeneration rates upon application of SU5402
Results of in vitro eye culture experiments treated with SU5402 to inhibit FGFR function. As shown here, the IC50 for inhibiting lens regeneration is close to 5µM SU5402. Numbers of regenerated lenses and eyes examined are located above each bar; y-axis indicates lens regeneration rate; error bars denote Wilson score intervals in which Z=1.

involved in lens regeneration should be expressed only by the retinal tissue and not be expressed in the cornea. Likewise, as the receptor for presumptive FGF signaling, the associated FGFRs should be expressed in the cornea during lens regeneration.

Though the RT-PCR analyses reported here are not quantitative, some potential differences in expression between retina and cornea may exist for FGF1, 8, 9, 11, and 14 based on the rather dramatic differences in the intensity of the PCR products detected (Fig. 2.1). Of these, FGF11 and FGF14 are members of the intracellular FGF subfamily, formerly known as the FGF11 subfamily or the FGF homologous factors, which are expressed in neuronal tissues and do not interact with known FGFRs (Table 1.1; Olsen et al., 2003; Goldfarb, 2005; Itoh and Ornitz, 2008). Hence, FGF1, FGF8, and FGF9 are plausible candidate FGFs that could represent the key retinal signal(s) that trigger lens regeneration. Other FGFs (FGF2, 5, 7, 10, 12, 13, and 16) were also expressed by retinal tissues and could represent key signals involved in lens regeneration. Of course, this study describes expression at the level of transcription, and it will be important to examine expression at the level of translation in future studies.
From compiling studies in various vertebrates, Robinson (2006) noted that certain members of all seven subfamilies of the FGFs and all four FGFRs (1-4) have been observed in the eye during development or in the adults of various vertebrates. In a recent X. tropicalis study, Lea et al. (2009) showed that FGF1, FGF3, FGF13, FGF14, and FGF20 are expressed during embryonic eye development. Here we have found additional FGFs (FGF2, 5, 7, 8, 9, 10, 11, 12, and 16) that are expressed in the eye during the later larval stage of X. laevis (i.e., stage 48-51) and have not been able to detect FGF3 and FGF20. Furthermore, during embryonic lens development in X. tropicalis, FGFR3 is expressed in the developing lens, FGFR2 is prominently expressed in corneal epithelium, and FGFR1 and FGFR4 are expressed in “cells surrounding the lens” in early tadpoles (Lea et al., 2009). This is similar to our finding that FGFR1 and FGFR4 are prominently expressed in the larval retina, whereas FGFR1, FGFR2, and FGFR3 are expressed in the cornea.

As for lens regeneration, previous research using X. laevis corneal explant cultures has shown that addition of FGF1 protein, previously known as acidic or aFGF, to cornea explants induced the transdifferentiation of these cells into lens cells (Bosco et al., 1997b). In these experiments, the authors established this using in vitro cultured outer cornea epithelium. The corneas transdifferentiated only when FGF1 was added to the serum supplemented L-15 media. Our results agree with the notion that FGF1 may be a signaling ligand for inducing cornea-lens transdifferentiation. In the newt, as opposed to other FGFs (e.g., 1, 4, and 7-10) and various growth factors (i.e., EGF, IGF, and VEGF), FGF2 has the unique ability to trigger the generation of a new lens after injection into the eye chamber (Hayashi et al., 2004). Significantly, we have found that FGF2 is expressed both in the cornea and the retina in X. laevis (Fig. 2.1). In another study, FGF2 and FGFR3 (also named PFR3, or Pleurodeles homolog of FGF receptor 3) expression specifically seemed to be correlated with Wolffian lens regeneration (McDevitt et al., 1997).

As described above, the 22 known members of the FGF family belong to seven subfamilies based on sequence homology (Itoh and Ornitz, 2004). It is interesting to note that FGF1, FGF8, and FGF9 identified in our study as possible signaling candidates involved in lens regeneration are members of three different FGF subfamilies (Itoh and
Ornitz, 2004). Two of the three candidates, *FGF8* and *FGF9*, are functionally thought to be ancestral members of their respective subfamilies in the mouse system (Itoh and Ornitz, 2008). FGF9 has been shown to interact with only FGFR2 and FGFR3, whereas FGF1 and FGF8 can activate all four FGFRs (Table 1.1; Ornitz et al., 1996). This may indicate that a combination of FGFRs could be activated during lens regeneration. Here, we have observed the expression of *FGFR1*, *2*, and *3* in the cornea, so activation of these FGFRs could be involved in triggering *X. laevis* lens regeneration.

As for the role of FGFR in lens regeneration, the *bek* isoform of FGFR2 was shown to be present in *Xenopus* epidermis only in regions where the epidermis was capable of transdifferentiating into lenses (Arresta et al., 2005). Specifically, these regions include the cornea epidermis overlying the eye and the pericorneal epidermis immediately surrounding the eye. In that study, the authors utilized an experimental approach to impart lens-forming competence on epidermis not normally competent to undergo transdifferentiation. This consists of implanting eye tissues beneath the target epidermis, based on the protocol of Cannata et al. (2003). If an eye was implanted beneath head epidermis distant from the eye (at stage 46), the head epidermis became competent to transdifferentiate into lenses if challenged later (at stage 53) by implanting this tissue into the vitreous chamber. Arresta et al. (2005) found that this head epidermis also expressed FGFR2 IIIc after exposure to the implanted eye. However, the authors only established a correlation between lens-forming competence and expression of FGFR2, and did not perform any direct tests to see if FGFR2 expression is responsible for establishing this regeneration capability.

There is evidence linking FGF pathway activation with lens development in other vertebrates (reviewed by Robinson, 2006). In particular, regarding FGFR function, expression of the dominant negative FGFR1 in the developing mouse lens placode (using a *Pax6* promoter expressing a dominant negative truncated form of *FGFR1*) inhibited lens cell proliferation and differentiation, thus demonstrating that FGFR activation is necessary for lens development (Faber et al., 2001). Similarly, a conditional knockout of FGFR2 in the mouse lens placode produced very small or absent lenses (Garcia et al., 2005).
The small molecule SU5402 has been shown to inhibit FGFR autophosphorylation by competitively binding the tyrosine kinase domain of FGFR1 (Mohammadi et al., 1997). Due to sequence conservation between the tyrosine kinase domains of FGFRs, SU5402 can inhibit the function of all four FGFRs (Delaune et al., 2005; Grand et al., 2004; Mansukhani et al., 2005). However, SU5402 inhibitory activity is not completely specific to FGFRs, as it has also been shown to inhibit Vascular Endothelial Growth Factor Receptor (VEGFR) tyrosine kinase activity in NIH 3T3 cells, and to a lesser extent, Platelet-Derived Growth Factor Receptor (PDGFR) tyrosine kinase activity (Sun et al., 1999). Past lens regeneration studies in the newt model have investigated the FGFR pathway using SU5402. In the case of Wolffian lens regeneration, Del Rio-Tsonis et al. (1998) found that inhibiting FGFR function by using SU5402 led to inhibition of lens regeneration in that system. More recently, Hayashi et al. (2002) used an in vitro method of culturing newt dorsal iris cell reaggregates and established that FGF2 and FGF4 were able to induce lens formation in these cultures. They then showed that FGFR function was necessary for this phenomenon by inhibiting lens formation by adding SU5402. In our investigation of Xenopus cornea-lens transdifferentiation, SU5402 almost completely inhibited lens regeneration. The observed IC$_{50}$ for lens regeneration was at 5µM, less than the published IC$_{50}$ of 10-20µM for FGFR autophosphorylation in NIH 3T3 cells (Mohammadi et al., 1997). This concentration is less than the 20µM concentration found to be effective in the newt by Del Rio-Tsonis et al. (1998) and the 10µM concentration used by Hayashi et al. (2002), mentioned above. Taken together, the evidence suggests that FGFR activation is both necessary (as established in the present study) and sufficient (as established by Bosco et al., 1997b, see also Arresta et al., 2005) for lens regeneration in Xenopus.
Chapter 3

*Xenopus* lens regeneration requires FGFR function and is induced by FGF1, insulin, and components of FBS

Introduction

The necessity for FGFR signaling during lens regeneration has been demonstrated by SU5402 small molecule inhibitor experiments in both Wolffian lens regeneration (Del Rio-Tsonis et al., 1998) and in *Xenopus* lens regeneration (see Chapter 2, Fukui and Henry, 2011). However, SU5402 also is a potent inhibitor of VEGFRs (Sun et al., 1999) and potentially of other tyrosine kinase receptors, so an alternative explanation could be that these earlier experiments may have demonstrated the importance of VEGF signaling. On the other hand, FGF treatment studies suggested that FGFs may be sufficient to trigger lens regeneration. In *Xenopus* isolated corneal tissue explants, treatment with FGF1 appears to induce lens cell formation in these cultures (Bosco et al., 1997b; Bosco, 1998). However, the amount of FGF1 used was very high (500 ng/ml), and investigation of potentially lower physiological concentrations of FGF (typically less than 50 ng/ml in the intraocular space; Baird et al., 1985a; Caruelle et al., 1989) are necessary before establishing its sufficiency in lens regeneration. In Chapter 2 (Fukui and Henry, 2011), evidence was presented suggesting that FGF1, FGF8, and FGF9 could play roles in *Xenopus* lens regeneration, yet FGF8 and FGF9 have not been previously tested for their potential to induce lens formation in corneal tissue.

As insulin and IGFs appear to play a role in lens development, they may also play a role in lens regeneration. More specifically, insulin receptors and IGF receptors have been identified to be expressed in the developing lens in various species (Hollenberg, 1975; Bassas et al., 1987; Bassnett and Beebe, 1990; Sinha et al., 1990; Jacobs et al., 1992; Ibaraki et al., 1996; Naeser, 1997). IGF1 (also known as lentropin) is necessary for lens fiber differentiation in lens epithelial cultures (Beebe et al., 1980, 1987). Insulin and IGF also appear to act upon cultured lens epithelial cells to induce differentiation.
into lens fiber cells (Reddan et al., 1975; Milstone and Piatigorsky, 1977; Reddan and Wilson-Dziedzic, 1983). Furthermore, insulin and FGF may have a synergistic effect on lens formation. For instance, combining insulin and FGF treatments appears to greatly enhance lens fiber formation in lens epithelial cell cultures (Chamberlain et al., 1991). A similar synergistic effect was also noted for IGFs enhancing FGF-induced lens fiber differentiation (Richardson et al., 1993; Liu et al., 1996). This evidence suggests that investigating the correlation between insulin/IGF and lens regeneration may be useful, especially in combination with FGF signaling.

This chapter investigates the specific necessity of FGFR signaling for lens regeneration by conditional expression of a dominant negative FGFR (XFD) and the sufficiency of various FGFs and other factors such as insulin or FBS in cultures of cornea tissues in vitro. We successfully generated transgenic F0 larvae expressing XFD upon heat shock, and then use the transgenic larval tissues in an in vitro culture system to show that FGFR function in the corneal epithelium is necessary for Xenopus laevis lens regeneration. We developed a novel method that enables the corneal tissue to maintain a flattened morphology and more efficiently attach and spread in culture wells. We also use this culture method to investigate the individual sufficiency of FGF1, FGF2, FGF8, FGF9, insulin, and FBS on in vitro lens regeneration. Together, these results provide further evidence that the FGF and insulin/IGF pathways play key roles in lens regeneration.

Results

Transgenic larvae express XFD upon heat shock

In order to test the necessity of FGFR function in lens regeneration, a dominant negative FGFR1 (XFD) lacking the intracellular kinase domains was used to inhibit FGF downstream pathway activation (Amaya et al., 1991). A transgenesis construct was made so this dominant negative FGFR could be inducibly expressed by heat shock (Figure 3.1; Lin and Slack, 2008). An HA tag was added to the C-terminal end of XFD for later verification of expression. To help identify those F0 transgenic animals that have genomic integration of the introduced DNA, this construct also contains a separate
Figure 3.1 – Transgenesis construct for XFD expression

Diagrams of transgenesis plasmids used to explore the effect of FGFR inhibition on lens regeneration. (A) The experimental transgenesis construct containing the dominant negative FGFR1 gene (XFD) expressed using the heat shock promoter and a gamma-crystallin promoter (γ-Cry) expressing GFP. (B) The control plasmid contains a heat shock promoter (HSP70) expressing only the HA tag. GFP: sequence encoding green fluorescent protein, HA: hemagglutinin tag, MCS: multiple cloning site, polyA: poly(A) tail. HGEM transgenesis cassette based on that of Fu et al. (2002) and Beck et al. (2003).

gamma-crystallin promoter that drives expression of GFP within the lens (based on the “heat shock green-eyed monster” construct; Fu et al., 2002; Beck et al., 2003). With this second reporter gene, transgenic animals can be readily identified by the presence of green fluorescent lenses. As a control, a similar construct was made to express only the HA tag upon heat shock without XFD, but was otherwise identical to the experimental transgenesis construct (Figure 3.1B).

Transgenic larvae were generated as described in Chapter 5 (see page 57). Successful transgenic animals were identified by their green fluorescent lenses, indicative of GFP transgene expression (Figure 3.2A-C). In the case of older larvae (stage 47 or later) used in the experiments below, the fluorescence was typically bright enough to observe green color in the eye using only ambient light (Figure 3.2A) but all cases were verified by using fluorescence illumination. XFD expression was verified by Western blot using transgenic larval tails collected after isolation of corneal tissues. For each transgenic tail, one half was heat shocked (40 minutes at 34°C) and one half was left at room temperature for an equivalent time as control. Larval tails were flash frozen using a dry ice/ethanol bath at approximately three hours after heat shock and stored at -80°C until later homogenization for protein expression analysis. As shown in Figure 3.2D, XFD was expressed only in those transgenic tail halves that were heat shocked.
Figure 3.2 – Transgenic larvae

Transgenic larvae expressing GFP in lens tissue and XFD protein upon heat shock activation. (A) An example of an transgenic larval eye under bright field illumination. (B) Corresponding fluorescence image of transgenic larval eye. (C) Overlay of fluorescence and visible images shown in A and B. (D) Expression of the transgene was confirmed by Western blot analysis shown here for 3 specimens. Expression of the XFD protein containing a C-terminal HA tag was verified by anti-HA antibody, and anti-tubulin antibody was used as a protein loading control. The scale bar in C indicates 500 µm.

XFD expression inhibits lens regeneration

The effect of FGF pathway inhibition upon lens regeneration was assayed using an *in vitro* culture method with transgenic larval eye tissue. To start these cultures, eye tissues were assembled by combining wild type retinas (eye cups) with transgenic corneas in modified L-15 culture medium (Figure 5.4). These eyes were then cultured in individual wells (in 24-well plates) with 1 ml of culture medium per well. As shown previously, combining a neural retina with a cornea in culture is sufficient to recapitulate lens regeneration in *Xenopus* (Bosco et al., 1993c; Fukui and Henry, 2011). As we were investigating the necessity of FGF pathway activation in the cornea due to presumptive FGF signaling from the neural retina, we only needed to inhibit the FGF receptor in corneal tissue. To this end, the use of a wild type retina excluded the possibility of transgene expression in the cultured retina, removing the possibility/effects of FGF pathway inhibition in that tissue, which could also affect its production of ligands that trigger this process. To express the XFD (dominant negative FGFR1) transgene, eye cultures were heat shocked daily at 34°C for 40 minutes each. As controls, some cultures were not heat shocked and some corneal
tissue was derived from larvae that were generated with the control transgenesis construct, which upon heat shock expressed only the HA tag.

Using this culture method, eyes were analyzed for lens regeneration after 14 days in culture (Figure 3.3, Figure 3.4). Only those eyes that had corneas derived from XFD transgenic animals, and were heat shocked, expressed XFD during the culture period. To assay for lens formation, we used the fact that regenerated lenses will express lens proteins, including gamma-crystallins (Henry and Grainger, 1990). Therefore, since the transgenic construct includes a gamma-crystallin promoter expressing GFP, regenerated lenses could be readily identified by green fluorescence (Figure 3.3B, E, H, K). Due to the use of wild type retinal tissue, the presence of GFP expression only revealed lens cell formation specific to the transgenic corneal tissue, removing any possibility of contamination related to the retinal tissues and potentially any other contaminating tissues.

Lens regeneration was inhibited upon XFD expression (Figure 3.4). With the control conditions, 1). 22/47 eyes (47%) with the control transgene and no heat shock regenerated lenses, 2). 18/52 control non-transgenic eyes (35%) regenerated lenses with daily heat shock, and 3). 28/65 eyes (43%) regenerated lenses using corneas containing the XFD transgene but without heat shock. Between the three control conditions, there is no significant difference in lens regeneration rates: all pairwise comparisons between these regeneration rates have p > 0.1 (smallest pairwise comparison p value is 0.11). On the other hand, only 14/66 eyes (21%) regenerated lenses under the experimental condition (with XFD transgene and daily heat shock; rightmost bar in Figure 3.4). There is a very significant difference between the control conditions without heat shock and the experimental condition, for both controls without the XFD transgene (p = 0.0019, leftmost bar in Figure 3.4) and with the XFD transgene (p = 0.0029, third bar in Figure 3.4). The decrease in lens regeneration rates between the two conditions with heat shock, the control condition without XFD in the transgenesis construct and the experimental condition with XFD in the transgenesis construct, is also close to the threshold for significance (p = 0.053). Based on these regeneration rates, although heat shock itself may slightly inhibit
Figure 3.3 – Regenerated transgenic lenses
Partially transgenic “eyes” cultured for 14 days at room temperature. (A-C) Cultured eye with corneal tissue containing the control transgenic construct without XFD and without heat shock. (D-F) Cultured eye using transgenic corneal tissue containing the control transgenic construct treated with daily heat shock. (G-I) Cultured eye with XFD transgenic corneal tissue with no heat shock. (J-O) Cultured eye with transgenic cornea expressing XFD with daily application of heat shock. (A, D, G, J, M) Cultured eyes under bright light. (B, E, H, K, N) Corresponding fluorescence images of cultured eyes. (C, F, I, L, O) Overlays of fluorescence and visible images of eyes. Regenerated lenses were identified by their green fluorescence (B, E, H, K). An example of an eye that did not regenerate a lens is also shown (M-O). The scale bar indicates 200 µm. White arrowheads point to lenses.
Figure 3.4 – Rates of lens regeneration in transgenic eye cultures
Quantification of lens regeneration rates between different transgenic constructs with or without XFD and either the presence or absence of heat shocks. Lens regeneration rates of cultured eyes were assayed after 14 days in culture. The rightmost bar represents the experimental condition in which XFD was expressed in the cornea of the cultured eye. Numbers above each bar indicate the number of regenerated lenses observed for each condition and the number of eyes cultured for each condition. The Y axis represents the regeneration rate for each condition. Error bars denote Wilson score confidence intervals with Z=1.

lens regeneration, XFD expression appears to more significantly inhibit this process, indicating an important role in FGFR signaling in lens regeneration.

FGF signaling is sufficient for lens regeneration in cultured *Xenopus laevis* corneas

Although various cornea culture methods have been designed for mammals, *Xenopus* cornea culture methods have not been thoroughly developed. The few instances of culturing larval cornea tissues appear to leave the corneas free-floating in culture medium, resulting in the formation of small vesicular structures (Campbell and Jones, 1968; Bosco et al., 1993c, 1994, 1997b; Bosco, 1998). We have developed a novel *in vitro* cornea culture method to assay the sufficiency of various factors for *Xenopus* lens regeneration that enables the corneas to attach and spread on the bottom surface of gelatin-coated plastic tissue culture wells, which promotes a more natural flattened morphology. In this method, the intact larval outer cornea epithelium is isolated, oriented so the inner basal layer faces the bottom of the culture well, and is pressed (“pinned”) onto the bottom of a gelatin-coated culture well using the highly sharpened tips of #5 Dumont forceps (Figure 5.5). Corneas cultured using this method will adhere to the bottom of the well and start spreading within two days (Figure 5.5D, E). The neural retina, the natural source of lens
regeneration factors, is omitted in these cultures to allow for assaying specific factors that may be sufficient to induce lens regeneration.

In Chapter 2, we established that FGF1, 8, and 9 are differentially expressed being much more highly expressed in the retina. Therefore, we investigated the sufficiency of these FGF proteins, along with FGF2, on lens regeneration (Fukui and Henry, 2011). For FGF1, the one previously reported experiment testing its sufficiency in *Xenopus* lens regeneration used 500 ng/ml FGF1, which is a physiologically high concentration for this ligand (Bosco et al., 1997b). Here we observed the effect of various concentrations of FGF1 on *in vitro* cultured corneas (Figure 3.5, Figure 3.6). More specifically, cornea cultures were treated with a range of concentrations from 10 to 1000 ng/ml FGF1 in minimal medium (67% L-15 medium without added serum). Control cultures containing no added FGF1 protein were also evaluated. As shown in Figure 3.5, cultured corneas quickly attached and spread on the bottom of their respective wells. After 9 days in culture, corneas were evaluated for regenerated lenses using anti-lens antibody staining (Henry and Grainger, 1990), and lens formation was observed in cases following a continuum of FGF1 treatment. In addition to positive anti-lens staining, the small lenses typically had a raised, three-dimensional, rounded structure as opposed to the flattened epithelial appearance of the other cultured corneal tissue.

Tiny lenses were observed upon FGF1 treatment at all concentrations, with a positive correlation between FGF1 concentration and lens formation rate (Figure 3.6). A significant increase in lens formation was observed with a FGF1 concentration as low as 50 ng/ml relative to the control condition with no FGF1 treatment (p = 0.011), with the 20 ng/ml case being close to significant (p = 0.053). At the upper end of the concentrations tested (100 ng/ml FGF1 and above), although the increasing concentrations of FGF1 appear to cause gradual increases in rates of lens formation, the results do not show a significant difference between these concentrations (p = 0.36 between the 100 ng/ml and 1 µg/ml conditions). The EC50 for FGF1 application appears to be between 20 ng/ml (19% regenerated) and 50 ng/ml (26% regenerated), assuming a maximal lens regeneration rate of approximately 50% at the highest FGF1 concentrations. From these observations
Figure 3.5 – Lens regeneration in cultured corneas treatment
Corneas cultured for 9 days at with various levels of FGF1 or FGF8 treatment and then stained using polyclonal anti-lens antibody. Corneas were treated with no FGF (A-D), 10 ng/ml FGF1 (E, F), 20 ng/ml FGF1 (G, H), 50 ng/ml FGF1 (I, J), 100 ng/ml FGF1(K, L), 500 ng/ml FGF1 (M, N), 1 μg/ml FGF1 (O, P), and 500 ng/ml FGF8 (Q, R, S, T). Images were taken under bright light (A, C, E, G, I, K, M, O, Q, S) and fluorescence illumination to detect anti-lens antibody staining (B, D, F, H, J, L, N, P, R, T). Insets in C-R contain higher-magnitude images of corresponding lenses in each panel. Panels S and T are magnified images of the lens in panels Q and R, respectively. Depressions in the culture wells (black arrowheads in A) indicate points at which the cornea was pressed into the bottom of the well using sharpened forceps. White arrowheads in C-P point to regenerated lenses. The scale bar indicates 100 μm for the insets in C-R, 200 μm for all panels A-R, and 25 μm for panel S and T.
Figure 3.6 – Lens regeneration rates in cultured corneas upon FGF1 treatment

Quantification of lens formation rates for FGF1 treated in vitro cornea cultures. Cornea cultures were treated for 9 days with various concentrations of FGF1 and then evaluated for lens regeneration. The leftmost bar indicates the control condition in which no FGF1 was applied. Numbers of corneas with lens regeneration and total corneas observed for each condition are indicated above each bar. The Y-axis indicates the regeneration rate for each condition. Error bars show Wilson score confidence intervals with Z=1.

As of regeneration rates, we can conclude that FGF1 application is sufficient to trigger lens formation, and effective at concentrations as low as 20 ng/ml FGF1.

In the case of FGF8, similar to the FGF1 treatments above, cornea cultures were treated with 10, 20, 50, 100, or 500 ng/ml FGF8 in minimal medium for 9 days. Lenses were detected using anti-lens antibody staining, as shown in Figure 3.5. However, as shown in Figure 3.7, there were fewer cases of positive anti-lens staining observed. In particular, there was a small amount of regeneration observed at the higher concentrations of 100 ng/ml (1/15 regenerated) and 500 ng/ml FGF8 (3/16 regenerated, p = 0.12). No cases of regeneration was observed at the lower concentrations of FGF8 tested. This may indicate that FGF8 has a slight potential to induce lens regeneration at those higher concentrations.

As for FGF2 and FGF9 treated cultures, a range of FGF concentrations up to 500 ng/ml were tested for their potential to induce lens regeneration (Table 3.1). Corneas were cultured in minimal medium for 9 days and assayed using anti-lens antibody staining. For corneas treated with these two FGFs, very few cases of lens regeneration was observed, with no condition exhibiting significantly more lens regeneration than the control condition with no FGF treatment. Therefore, FGF2 and FGF9 treatments appear not to be suffi-
Figure 3.7 – Lens regeneration rates in cultured corneas upon FGF8 treatment

Quantification of lens formation rates for FGF8 treated in vitro cornea cultures. Various concentrations of FGF8 were used to treat cornea cultures. Cultures were evaluated for lens regeneration after 9 days. The leftmost condition is the control in which no FGF8 was applied. The number of corneas with positive anti-lens staining and the number of corneas observed are indicated above each bar for each condition. The Y-axis shows the regeneration rate and the error bars show Wilson score confidence intervals with Z=1.

<table>
<thead>
<tr>
<th>FGF</th>
<th>concentration</th>
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<th>total corneas</th>
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<tr>
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<td>16</td>
</tr>
<tr>
<td>FGF2</td>
<td>10 ng/ml</td>
<td>0</td>
<td>16</td>
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<tr>
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<td>20 ng/ml</td>
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<td>16</td>
</tr>
<tr>
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<td>50 ng/ml</td>
<td>1</td>
<td>14</td>
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<tr>
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<td>16</td>
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<tr>
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<td>0</td>
<td>16</td>
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<tr>
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Table 3.1 – Lens regeneration rates in cultured corneas upon FGF2 or FGF9 treatment

Lens formation rates were quantified for in vitro cornea cultures treated with FGF2 or FGF9. Various concentrations of FGFs were used as indicated above. The number of cases with lens regeneration was determined using anti-lens antibody staining after FGF treatment for 9 days in culture. The total number of corneas assayed for each condition are also listed.
cient for inducing lens regeneration in the \textit{in vitro} cornea culture assay. Together, these FGF treatment studies suggest that FGF1 is a potent inducer of lens regeneration, whereas FGF8 only slightly induces lens regeneration and the two other FGFs tested (FGF2 and FGF9) do not induce lens regeneration.

**Insulin, FGF1, and FBS are sufficient for lens regeneration in cultured corneas**

We further investigated the effects of FGF1, insulin, and fetal bovine serum (FBS) treatments on lens regeneration in \textit{in vitro} cultured corneas. All combinations of treatments with and without insulin (5 $\mu$g/ml), FGF1 (500 ng/ml), and FBS (10% v/v) were tested for their potential to induce lens regeneration. Corneas were cultured for 2, 5, 7, and 9 days under each treatment condition. The presence of any regenerated lenses were detected by anti-lens antibody staining. Interestingly, all three factors were individually sufficient to trigger lens regeneration in cornea cultures (Figure 3.8, Figure 3.9). Under almost all conditions except for the control untreated corneas with no factors added, lenses were first detected after 7 days in culture. Across all experimental conditions, lens regeneration rates increased with a longer culture period of 9 days. Notably, the treatment condition with both FGF and FBS supplementation lead to lens formation earlier than other conditions, first observed after 5 days in culture (Figure 3.9). In contrast, the highest overall regeneration rates occurred when insulin was included.

Since insulin and FBS have never been correlated with \textit{Xenopus} lens regeneration, we sought to confirm lens regeneration using a second mode of detection. To this end, we examined lens formation in insulin and FBS treated 9-day cornea cultures by detecting gamma-crystallin mRNA expression via in situ hybridization to detect initial transcript expression. gamma-crystallin is expressed in lenses during the later stages of lens regeneration (Brahma and Mcdevitt, 1974; Mizuno et al., 1999a; Schaefer et al., 1999). We were able to detect gamma-crystallin mRNA in 9-day cornea cultures localized to small circular regions, suggestive of lens formation (Figure 3.10). This experiment also demonstrates that lens cells are forming in these cultures.
Figure 3.8 – Cultured corneas with regenerated lenses after insulin, FGF1, and FBS treatment

Corneas were cultured under various conditions and then stained with polyclonal anti-lens antibody. Treatment conditions include combinations of 5 μg/ml insulin (INS), 500 ng/ml FGF1 (FGF), and 10% v/v fetal bovine serum (FBS). (A, B) Cornea culture under control condition with no treatments and no lens regeneration. (C, D) Cornea treated with FBS only that regenerated two lenses. (E, F) Cornea treated with only FGF1. (G, H) Cornea treated with FGF1 and FBS. (I, J) Cornea treated with insulin only. (K, L) Cornea treated with insulin and FBS. (M, N) Cornea treated with insulin and FGF1 that regenerated two lenses. (O, P) Cornea treated with all three factors. (A, C, E, G, I, K, M, O) Brightfield images of each cornea. (B, D, F, H, J, L, N, P) Corresponding fluorescence images of each cornea showing lens staining. Sets of 4 obvious depressions in the brightfield images indicate points where the cornea was pressed into the bottom of the well. Arrowheads point to regenerated lenses. The scale bar indicates 500 μm.

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Figure 3.9 – Lens regeneration rates in cornea culture with insulin, FGF1, and FBS treatment

Lens regeneration rates in corneas cultured for 2, 5, 7, or 9 days under different conditions. Treatments applied include insulin (5 µg/ml), FGF1 (500 ng/ml), and fetal bovine serum (FBS, 10% v/v). Each numerator indicates the number of corneas observed with regenerated lenses, while the denominator indicates the total number of corneas cultured for each condition. Conditions with lens regeneration are shaded with increasing intensity for clarity to correlate with increasing rates of lens regeneration.

Figure 3.10 – gamma-crystallin expression in cultured corneas

In situ staining for gamma-crystallin was performed on cultured corneas. (A) Cultured cornea treated with 5 µg/ml insulin for 9 days. (B) Cultured cornea treated with insulin and 10% v/v FBS. Positive in situ staining is indicated by dark purple coloring, in contrast to brown coloration exhibited by occasional pigmented epithelial cells included in these cultures. Arrowheads point to positive staining regions, and the scale bar indicates 200 µm.
Discussion

FGFR signaling is necessary for lens regeneration

The *Xenopus laevis* dominant negative FGFR1 transgene (*XFD*) was initially developed to study *Xenopus* mesoderm development in early embryogenesis (Amaya et al., 1991, 1993). *XFD* has since been inserted into a transgenesis construct to observe tissue-specific effects (Kroll and Amaya, 1996) and more recently for studying tail regeneration (Lin and Slack, 2008). *XFD* acts by heterodimerizing with endogenous FGFRs to inhibit FGFR downstream activation (Amaya et al., 1991). Our experiments using this dominant negative FGFR1 transgene have shown that inhibition of FGFR function in cornea tissue leads to inhibition of cornea-lens regeneration in *Xenopus laevis* as assayed in an *in vitro* system. This is in agreement with our previous experiments using the small molecule FGFR inhibitor SU5402 (see Chapter 2; Fukui and Henry, 2011). However, in those earlier experiments there is always the possibility that the small molecule inhibitor may not have specifically altered the FGF pathway, as SU5402 is also known to readily inhibit VEGFR function and potentially other tyrosine kinase receptors (Sun et al., 1999). The newer experiments reported here narrow down inhibition to the FGF pathway by expressing a dominant negative FGFR (Amaya et al., 1991). At this point, we cannot claim that FGFR1 is necessary as opposed to other FGFRs because the dominant negative FGFR1 can heterodimerize and inhibit the functions of FGFR1, FGFR2, and FGFR3 (Ueno et al., 1992). In fact, we have shown that all three of these receptors (but not FGFR4) are expressed in *Xenopus* cornea epithelial tissue (Chapter 2; Fukui and Henry, 2011). On the other hand, as there is some evidence that expression of different dominant negative FGFRs have slightly different developmental effects (Hongo et al., 1999; Ota et al., 2009), future experiments could investigate the effects of other dominant negative FGFRs on lens regeneration.

The corneal tissue that differentiates to form a lens during lens regeneration must have specific receptors receiving the key signals to form lenses. It is long known that the regenerated lens originates from the inner layer of the outer cornea in larval *Xenopus*
*laevis* (Freeman, 1963). Past studies have strongly suggested that some key signal from the neural retina is required to reach the cornea to trigger lens regeneration in *Xenopus* (Filoni et al., 1981; Reeve and Wild, 1981; Filoni et al., 1982, 1983; Bosco and Filoni, 1992; Bosco et al., 1993c, 1997a). We have shown that FGFR1, 2, and 3 are expressed in *Xenopus* cornea tissue (Chapter 2; Fukui and Henry, 2011). By examining co-cultured transgenic corneas with non-transgenic eye cups, we have found in this study that FGFR function specifically in corneal tissue is important for lens regeneration. This study demonstrates a more specific necessity than the small molecule (SU5402) experiment that inhibited FGFR function in all the cultured eye tissues (Chapter 2; Fukui and Henry, 2011). This evidence supports the hypothesis that a key signal for regeneration is represented by FGFs that are transmitted to the outer cornea and detected via corneal FGFRs. As FGFR function in the transdifferentiating tissue itself appears necessary for lens regeneration, the key signal for lens regeneration may be FGFs from the neural retina. These observations also support the results from Chapter 2 that suggests specific FGFs (FGF1, FGF8, or FGF9) could represent the key signal for lens regeneration, acting upon FGFR1, 2, or 3 in the cornea to trigger lens formation (Fukui and Henry, 2011). Our results are also in line with a previous correlation observed between FGFR2 expression and competence to form a lens in *Xenopus* (Arresta et al., 2005).

Although inhibition of FGFR function inhibited lens regeneration, not all lens regeneration was inhibited upon XFD expression. F0 animals generated using the REMI transgenesis method are known to have different copy numbers of the transgene inserted into the genome (Kroll and Amaya, 1996; Sparrow et al., 2000). As we are using F0 larval tissue, there may be a similar difference in copy numbers of the transgene for each larval eye used, affecting the strength of FGFR inhibition. More specifically, transgenic tissue with lower copy numbers of the transgene may not have expressed enough XFD upon heat shock to sufficiently abolish downstream FGF signaling. In addition, some mosaicism in expression within individual larvae has been observed using the transgenesis method (Sparrow et al., 2000), which raises the possibility of larvae with transgenic lenses that do not contain the transgene in all corneal cells, which leads to a lack of XFD expression.
Figure 3.11 – Interactions between candidate FGFs and FGFRs

The interactions between the candidate FGFs tested and isoforms of potential key FGFRs (b and c) are shown. A line between a FGF-FGFR pair indicates an interaction, as determined by Ornitz et al. (1996). Candidate FGFRs were determined in Chapter 2 (Fukui and Henry, 2011).

in some cells. Nevertheless, our experiment still shows a substantial decrease in lens regeneration rates upon XFD expression.

**FGF1 strongly induces lens formation in the corneal culture system**

We have also shown through the corneal culture assay that FGF1 stimulation of corneal cultures is sufficient for lens formation. This is in agreement with the study by Bosco et al. (1997b) showing FGF1 sufficiency for lens regeneration. We have expanded the range of FGF1 concentrations tested and have used a different approach to culturing *Xenopus* corneas. Lenses were observed as early as 5 days in culture, consistent with Bosco et al. (1997b) who observed lens regeneration starting at 4 days of culture. Through this study of different FGF1 concentrations, we have found that a FGF1 concentration as low as 20 ng/ml or 50 ng/ml is sufficient for inducing lens regeneration. This concentration is an order of magnitude smaller than the previously established concentration of FGF1 shown to trigger lens regeneration (Bosco et al., 1997b; Bosco, 1998). In our experiments, we have also shown that FGF8 may at most be a weak inducer of *Xenopus* lens regeneration, and FGF9 appears not to trigger lens regeneration. One explanation for FGF8 slightly triggering lens formation at high concentrations may be that FGF8 shares affinity for several FGFR isoforms with FGF1 (Figure 3.11; Ornitz et al. 1996), potentially leading
to the same FGFR signaling that triggers lens regeneration as FGF1. On the other hand, FGF2 can activate all FGFR isoforms that FGF8 activates (Figure 3.11; Ornitz et al. 1996) but did not trigger lens formation in our assay. Thus, our results may indicate that FGF8 can weakly trigger lens regeneration. Overall, our experiments establish a much stronger case for FGF1 sufficiency in inducing lens regeneration and suggest FGF8 as a potential weak inducer of lens regeneration.

A notable difference between Wolffian lens regeneration and our system concerns the specific FGF ligand sufficient for lens formation. FGF1, FGF8, and FGF9 were all identified as potential key factors for *Xenopus* lens regeneration in Chapter 2 (Fukui and Henry, 2011). After testing all three of these FGFs, our results indicate that FGF1 is specifically sufficient for inducing lens regeneration. FGF1 has been demonstrated not to be sufficient for triggering Wolffian lens regeneration (Hayashi et al., 2004). In Wolffian lens regeneration, FGF2 appears to be the key factor that triggers lens regeneration (Hayashi et al., 2002, 2004, 2006). Interestingly, in our examination of the effect of FGF2 on *in vitro* cornea cultures, FGF2 did not appear to induce lens regeneration even at a high concentration (500 ng/ml). Therefore, FGF1 and FGF2 appear to have very different roles in these different lens regeneration processes.

Interestingly, the FGF1 concentration sufficient for lens regeneration reflects more similarly with endogenous FGF2 levels as measured in mammals (Baird et al., 1985a,b; Tripathi et al., 1992). For instance, the half-maximal FGF1 concentration for *Xenopus* lens regeneration is similar to the 40 ng/ml FGF2 required for inducing lens fiber differentiation in rat lens epithelial cell cultures (Lovicu and McAvoy, 1989; McAvoy and Chamberlain, 1989). FGF2 is more highly expressed in the posterior chamber of the eye than the anterior chamber in mammals. Approximately 1 ng/ml FGF2 is present in the aqueous humor of humans, cats, dogs, and pigs (Tripathi et al., 1992), the human intraocular FGF2 level may be around 10-20 ng/ml (Baird et al., 1985a), and more than 200 ng/g FGF2 can be purified from bovine retinal tissue (Baird et al., 1985b). In *Xenopus*, FGF2 was observed in both the cornea and retina (Chapter 2; Fukui and Henry, 2011). As for FGF1, 290 ng/ml FGF1 is required for half maximal stimulation in the rat (Chamberlain and
McAvoy, 1989). Similar to the case for FGF2, FGF1 is expressed more in the retina than the cornea: 35 ng/ml FGF1 has been measured in the bovine aqueous humor, 295 ng per gram of tissue in the vitreous body, and 479 ng/g in the retina (Caruelle et al., 1989). We have also detected less FGF1 mRNA in the Xenopus cornea than the retina (Chapter 2; Fukui and Henry, 2011), but the FGF1 concentration levels measured in mammalian eye tissues would be sufficient to induce lens formation in this study’s Xenopus cornea culture system. A limitation of this study lies in the use of human recombinant FGFs to treat Xenopus tissues: there are differences in composition between human and Xenopus FGFs and FGFRs, including the FGF-FGFR binding sites (Plotnikov et al., 2000; Stauber et al., 2000), so our current study may have resulted in sufficiency of different concentrations of FGFs than endogenous FGF levels. Finally, the Xenopus cornea’s response to FGF1 described here may explain the results of previous xenoplastic grafting experiments in which eyecups from other frog species (incapable of lens regeneration) were used to implant Xenopus corneas that then exhibited lens regeneration (Filoni et al., 1979; Bosco et al., 1991, 1993a,b): the formation of lenses in these situations was probably due to the fact that these eyecups contain FGFs.

The roles of insulin and FBS in lens regeneration

Lens development studies have observed that insulin can enhance lens fiber differentiation (Piatigorsky, 1973; Reddan et al., 1975), but there have been no past studies correlating insulin with Xenopus lens regeneration. Our experiments show that 5 µg/ml insulin treatment is sufficient for lens regeneration in in vitro cultured corneas. This concentration of insulin (5 µg/ml) is routinely used as part of supplemented hormonal epithelial medium (SHEM; Jumblatt and Neufeld, 1983) or as a supplement to MCDB 151 medium (Peehl and Ham, 1980; Tsao et al., 1982; Kruse and Tseng, 1991) for primary mammalian cornea epithelial cultures to facilitate cell proliferation. However, mammals do not regenerate lenses from corneal tissue. In our case, the role of insulin may be independent of cell proliferation, as lens regeneration has been shown to occur even without cell proliferation (Filoni et al., 1995). Interestingly, Wolffian lens regeneration in newts does not appear to
be induced by insulin treatment \textit{in vitro}. A study investigating the effect of various factors on \textit{in vitro} newt lens regeneration in irido-corneal complexes observed that although 0.14 IU/ml insulin (roughly 5 µg/ml) was able to induce increased iris depigmentation, the lens regeneration rate was comparable to that of controls (Connelly, 1980). In contrast, our experiments using a similar concentration of insulin elicited lens regeneration in \textit{Xenopus} cornea cultures. Therefore, our findings may indicate a distinct, insulin-sensitive aspect of lens regeneration specific to \textit{Xenopus} cornea tissue.

In our insulin treatments, the insulin applied is likely to have also activated IGF receptors (Varewijck and Janssen, 2012). IGFs appear to enhance lens fiber differentiation in vertebrate lens epithelial cell cultures (Reddan and Wilson-Dziedzic, 1983; Beebe et al., 1987; Richardson et al., 1993). For newt lens regeneration, there is conflicting evidence for the sufficiency of IGF signaling. An early study found that IGF1 treatment is able to induce newt lens regeneration (Connelly and Green, 1987), but a later study using a higher concentration of IGF1 did not observe lens regeneration (Hayashi et al., 2002). Introduction of IGF1 \textit{in vivo} also did not trigger Wolffian lens regeneration (Hayashi et al., 2004). As for \textit{Xenopus} lens regeneration, an EST screen identified one encoding a protein that interacts with IGFs, insulin-like growth factor-binding protein 2 (\textit{IGFBP2}), which was upregulated in the cornea during lens regeneration (Malloch et al., 2009). This finding suggests that IGFs could play a role during lens regeneration. Since IGF has not been directly examined yet in the context of \textit{Xenopus} lens regeneration, an interesting further experiment would be to examine the effect of applying IGFs on \textit{Xenopus} cornea cultures and to characterize the expression of these proteins and their receptors in the eye.

Fetal bovine serum is routinely used for tissue culture studies. Indeed, the modified L-15 medium commonly used for \textit{Xenopus} tissue culture typically contains 10% v/v FBS (Kay and Peng, 1991). A striking result from our experiments is that 10% v/v FBS was sufficient for lens formation under our cornea culture conditions, contrasting with the findings of Bosco et al. (1997b) in which FBS supplementation (10% v/v) did not result in lens formation. One explanation may be the presence of variable amounts of growth factors between different lots of FBS that may trigger lens regeneration. In particular, FBS
typically contains both IGF1 and IGF2 (Honegger and Humbel, 1986), as well as FGFs, and these growth factors may have enhanced lens formation in our cultures, as discussed above. A key difference in cornea morphology between our experiments with flattened epithelia and those of Bosco et al. (1997b) with round vesicles may also have affected sensitivity for lens regeneration factors.

**Cornea culture morphology**

Our experiments are unique for *Xenopus* in that corneas attach and spread in the bottom of the culture well, resulting in cornea cultures spanning a larger area. Although Bosco et al. (1997b) utilized cultured *Xenopus* corneas, those corneas did not attach to any surface and become vesicularized instead. This was similar to an early investigation of cultured *Xenopus* corneas that observed hollow vesicle formation in cases in which the cornea tissue was not attached to any substrate (Campbell and Jones, 1968). On the other hand, our morphology is similar to other mammalian cornea epithelial culture models, which are typically attached onto a coated surface to form an epithelial sheet (reviewed by Reichl et al., 2011; Sabater et al., 2013), including the earliest uses of human cornea cultures (Thygeson, 1938). Our culture method tends to maintain a more natural state, as these corneas exhibit epithelial morphology *in vivo*. Normally the inner surface of the cornea epithelium has a specialized basement membrane (Bowman’s layer), which is mimicked in our cultures by attachment to a gelatin coated surface. Bosco et al. (1997b) found that following FGF treatment the entire cultured cornea appears to stain positively for lens proteins after 7-10 days. In contrast, our culture method results in distinct lens-like structures forming within the cultured corneas (Figure 3.5, Figure 3.8). The lens structures formed have a three dimensional rounded appearance distinct from the flattened epithelial-like appearance of the rest of the cornea in culture. The differences in culture method may have caused the difference in cornea sensitivity to lens regeneration factors *in vitro* in that a flattened epithelial morphology may favor the regeneration of a new lens. In both cases, however, the resulting lens structures appear to be similar in size (on the order of 100 µm in diameter).
Chapter 4

Conclusions and Future Directions

This dissertation explored the relationship between FGF and FGFR signaling and *Xenopus laevis* lens regeneration, which has not been extensively studied in the past. Although much is known about the topic of FGF signaling and about lens regeneration, the only evidence for *Xenopus* lens regeneration being related to FGF signaling was a correlation between FGFR2 expression and lens regeneration competence (Arresta et al., 2005), and a study in which a high concentration of FGF1 induced lens cell differentiation in culture (Bosco et al., 1997b). In this dissertation, the expression of FGFs and FGFRs in the cornea and retina were characterized, as well as the effect of FGFR inhibition on lens regeneration, and the sufficiency of several FGFs was tested in inducing lens formation in culture. This dissertation also includes a preliminary investigation of the role of insulin in *Xenopus* lens regeneration, which has not been examined in the past.

This work expanded upon the current understanding of how FGFs and FGFRs may play roles in lens regeneration. We have found through expression studies that *FGF1*, *FGF8*, and *FGF9* mRNA are differentially expressed between the larval retina and cornea. These FGFs exhibited a much higher level of mRNA expression in retinal tissue than cornea tissue, as surveyed using a semi-quantitative RT-PCR method. As the signal to trigger lens regeneration appears to originate from the neural retina and act upon the cornea (see Bosco et al., 1979, 1980; Cioni et al., 1982), these three FGFs (1, 8, and 9 identified in Chapter 2; see Fukui and Henry, 2011) were deemed to more likely play the role of the key retinal signals that trigger lens regeneration. In addition, this research found that *FGFR1*, *FGFR2*, and *FGFR3* mRNA (but not *FGFR4*) were expressed in the larval cornea, potentially acting as receptors for these ligands. Together, the findings support the idea that FGF1, 8, and/or 9 may interact with FGFR1, 2, and/or 3 to trigger lens regeneration.
In addition, this dissertation further explored the functional roles of FGF ligands and FGF receptors on lens regeneration. FGFR function appears to be necessary for lens regeneration, as our investigations using an inhibitor (SU5402) and by expressing a dominant negative FGFR1 resulted in inhibition of lens regeneration in \textit{in vitro} eye cultures. This suggests that one or more of these FGFRs and their isoforms are necessary for \textit{Xenopus} lens regeneration. One limitation of these experiments is that it is unclear exactly which combination of the three receptors are necessary, since the dominant negative FGFR1 inhibits the function of other FGFRs (Ueno et al., 1992). A future experiment is needed to determine the exact FGFR necessary via introduction of morpholinos to the corneal tissue using lipofection or electroporation, though some functional redundancy may also be present.

As for the FGF ligands, we have found that treating isolated cultured corneas with FGF1 alone was sufficient for the differentiation of lens cells expressing lens specific antigens at a relatively low concentration of 20 ng/ml FGF1, using an \textit{in vitro} cornea culture approach. In contrast, FGF8 also induces lens formation only in a few cases when tested at a high concentration, and FGF2 and FGF9 do not appear to induce lens formation. Together, these results (Chapter 3) suggest that FGF1 is likely to be a key ligand interacting with one or more FGFR in the corneal tissue to induce lens regeneration.

Research described in chapter 3 examined the potential effects of insulin and FBS on the \textit{in vitro} cornea culture system. In particular, 5µg/ml insulin treatment induced lens formation in \textit{in vitro} cornea cultures. As insulin is a ligand for IGF receptors in addition to insulin receptors, it may be the case that either insulin or an IGF ligand plays some role in lens formation. To explore this option in the future, one could test the direct effect of various concentrations of insulin, IGF1 or IGF2 on cultured corneal tissue using the \textit{in vitro} cornea culture system developed here. To examine the potential necessity of these ligands in lens regeneration one could express the dominant negative insulin receptor or IGF receptors in transgenic animals, and then assess the potential for lens regeneration in the resulting transgenic corneas. Alternatively, the expression of these receptors could
be knocked down by electroporation or lipofection of morpholinos targeting IGF receptors specifically into corneal tissue.

These experiments have highlighted differences in molecular mechanism between *Xenopus* lens regeneration and Wolffian lens regeneration. Although FGF1 does not induce Wolffian lens regeneration (Hayashi et al., 2004), the experiments described here indicate that FGF1 is a key factor in *Xenopus* lens regeneration. In addition, Chapter 3 also determined that FGF2 treatment is not sufficient for *Xenopus* lens formation, unlike the case in Wolffian lens regeneration in which FGF2 triggers lens regeneration (Hayashi et al., 2002, 2004). Furthermore, the results concerning insulin inducing lens regeneration contrasts with the lack of effect of insulin or IGF on inducing Wolffian lens regeneration (Connelly, 1980; Hayashi et al., 2002, 2004). Similarly, a previous study in our lab determined that retinoic acid signaling must be decreased in the regenerating *Xenopus* lens (Thomas and Henry, 2014), as opposed to the upregulation of retinoic acid signaling during Wolffian lens regeneration (Tsonis et al., 2000, 2002). These differences, perhaps due to differences regarding the specific tissues that form the lens (iris in Wolffian regeneration vs. cornea in *Xenopus*), emphasize the importance of comparing this phenomenon in different animals in order to understand why it is that some can regenerate lenses while others cannot.

A future avenue of research is to investigate the downstream effectors of the FGF-FGFR interaction in regards to *Xenopus* lens regeneration. Despite the existence of literature implicating a role for the downstream MAPK pathway in lens development (reviewed in Robinson, 2006; Lovicu et al., 2011), it is unknown which FGF signaling downstream pathways act in *Xenopus* lens regeneration. One method may be to use small molecule inhibitors to test the necessity of each downstream signaling pathway such as U0126, a MEK inhibitor (Favata et al., 1998). It should be noted that a preliminary examination performed in our lab by Cheng (2006) investigating the MAPK pathway by application of U0126 did not result in inhibition of lens regeneration, possibly suggesting that a different downstream signaling pathway (such as the PI3K/Akt pathway) may be responsible for lens regeneration. As Sef and Sprouty are known inhibitors of the MAPK pathway, one experiment to further assess the necessity of the MAPK pathway would be to
overexpress these proteins in corneal tissue using transgenesis and then examine the effect on lens regeneration. More recently, a plethora of inhibitors have been developed for the MAPK and PI3K/Akt downstream pathways (reviewed in Chappell et al., 2011; Rodon et al., 2013), which may also be utilized to investigate the role of specific downstream factors on lens regeneration. The PI3K/Akt downstream pathway may be interesting to investigate due to evidence that it is activated during lens fiber differentiation in the mammalian system (Iyengar et al., 2009; Wang et al., 2010) and is necessary for lens cell proliferation and differentiation (Iyengar et al., 2007; Wang et al., 2009). Another method to investigate these downstream pathways would be to knock down expression of members of the pathways using morpholinos electroporated directly into cornea epithelial cells, and then challenging these corneal cells to form lenses when combined with retinal tissue in the \textit{in vitro} eye culture assay.

This dissertation has expanded the knowledge of the factors involved in \textit{Xenopus} lens regeneration by demonstrating stronger evidence for the key role of FGF-FGFR function in lens regeneration. FGF1 has been presented as a strong candidate for the key factor in inducing lens regeneration, and FGFR signaling has been shown to be necessary for lens regeneration. This knowledge will aid future work in other organisms to elucidate common themes regarding development and regeneration of lenses, and may be applied towards developing novel treatments for eye diseases to restore vision in humans. Ultimately, this work may contribute to the formation of optimal refractive lenses, as opposed to artificial lenses, to restore vision in humans.
Chapter 5

Materials and Methods

Protocols

Animal care and handling

Adult pigmented *X. laevis* were obtained from Nasco (Fort Atkinson, WI). Fertilized eggs were prepared and larvae were reared to various stages, as described by Henry and Grainger (1987) and Schaefer et al. (1999). Briefly, animals are injected with human chorionic gonadotropin (hCG; 1500 IU for the female and 1000 IU for the male; Chorulon, Intervet, Summit, NJ) the evening before egg collection. Animals are put in a dark room at this time and kept in the dark throughout the mating. Frogs are introduced together in the dark the next morning and eggs start to be collected typically within two hours. Collected eggs are dejellied by washing with 6 mM DTT and 50 mM HEPES in 1/20X NAM (see page 94). After dejellying, eggs are washed two times in 1/20X NAM by serial transfer between solutions and then spread onto 100 mm petri dishes with 1/20X NAM. Embryos are reared for 7 days at 18°C in 1/20X NAM up to stage 43 (all stages according to Nieuwkoop and Faber, 1956), changed daily, into fresh 100mm petri dishes. Larvae are then transferred to room temperature into larger tanks of carbon-filtered water after this period and fed yeast (Red Star Yeast, Milwaukee, WI) until they are two weeks old. Larvae are then fed powdered Frog Brittle (eNasco, Fort Atkinson, WI) until the stage desired. Larvae were staged according to Nieuwkoop and Faber (1956).

Transgenesis

F0 transgenic animals were generated for use in subsequent experiments. Several protocols have been merged together to produce an optimum method (Amaya and Kroll, 1999; Sive et al., 2000; Smith et al., 2006; Ishibashi et al., 2008).
Glass micropipettes (Drummond Scientific, Broomall, PA; catalog #1-000-0500) were pulled to generate a needle tip using a PUL-1 micropipette puller (World Precision Instruments, Sarasota, FL) using the maximum delay setting (4) and maximum heat setting (10) on Auto mode. This produced needles that were tapered for approximately 2 cm in length before breaking off the tip. Needle tips were broken off immediately before use to produce a 30 -100 µm diameter opening, as described below. Depending on the number of eggs laid by each female, up to 15 needles were typically used to generate embryos in a single transgenesis experiment, one needle for each dish of injected eggs (each dish holding a few hundred oocytes). A syringe injection apparatus (GENIE; Kent Scientific, Torrington, CT) was used to control the flow of sperm nuclei during the transgenesis protocol. For transgenesis, this flow rate is set to 0.6 µl/min. Also, for transfer of sperm nuclei we prepared cut p200 pipette tips in advance: the p200 filter tip (Denville Scientific, Metuchen, NJ) is cut at approximately the 10µl mark on the tip with a sharp razor blade by hand to make an opening diameter of approximately 1.5 mm and then autoclaved in a pipette tip box. The larger opening is to ensure that sperm nuclei will not be sheared during transfer.

Agarose dishes were prepared by pouring 1% (w/v) agarose (Bio-Rad, Hercules, CA; catalog #161-3101) in 1X MMR (on page 93) into small petri dishes (60 mm). Small plastic weigh boats (1 5/8” square, Fisher Scientific, Pittsburgh, PA) were placed on top of the molten agarose to create circular depressions (approximately 2-5 mm deep) to accommodate the eggs, and these boats were weighted down to keep them from floating, similar to the method in Sive et al. (2000). Once the agarose solidified, weigh boats were removed and the agarose dishes were wrapped in parafilm and stored at 4°C for up to two months until use. Any agarose dish that developed visible bacterial or fungal growth while in storage was discarded.
Preparation of sperm nuclei for transgenesis

Sperm nuclei were prepared in advance for use in the transgenesis procedure, following the protocol of Sive et al. (2000). After preparation, sperm nuclei stock may be stored at -80°C for several years. Details are as follows:

1. Cool 1X MMR (see page 93) on ice for washes, and prepare 30 ml fresh 2X Nuclear Preparation Buffer (NPB; see page 94) solution. From this, make fresh solutions to be used that day and store on ice (scale up volumes as needed):

   (a) 40 ml 1X NPB
   (b) 10 ml 1X NPB with 3% (w/v) BSA (Sigma-Aldrich, St. Louis, MO; catalog #A7906), 10 µl/ml leupeptin (from 10 mg/ml stock in DMSO; Roche, Indianapolis, IN), 0.3 mM PMSF (from 0.3 M stock in ethanol; Roche, Indianapolis, IN)
   (c) 5 ml 1X NPB with 0.3% (w/v) BSA
   (d) 1 mL storage buffer (500 µL 2X NPB, 300 µl glycerol, 170 µl sterile H2O, 30 µl 10% BSA)
   (e) lysolcithin solution: 100 µL 10 mg/ml L-α-lysophosphatidylcholine (make fresh or use stock from -20°C; Sigma-Aldrich, St. Louis, MO; catalog #L4129)

2. Euthanize male frogs and surgically isolate testes as previously described (Sive et al., 2000, 2007a). Briefly, male animals are sacrificed by pithing and then placed belly up on paper towels. To remove a testis, cut a vertical slit lateral to the ventral midline, pull out the fat body (yellow tissue) from the animal, and the testis (lighter white colored tissue) should be attached to the fat body. The testis is then freed from the surrounding tissue using forceps.

3. Place each pair of testes collected in a 60 mm petri dish in cold 1X MMR. Remove any large pieces of fat or tissue attached to the testes. Try to avoid puncturing a testis and releasing sperm.

4. Rinse in cold 1X MMR three times, and then two times in cold 1X NPB.

5. Transfer a pair of testes to a dry 35 mm petri dish and macerate with sharp, clean forceps until no clumps are visible. This takes a lot of effort, but is essential for getting a good yield of sperm nuclei.

6. Resuspend the macerate in 2 ml of cold 1X NPB (use pipettes with pre-cut tips), and filter the macerate through 2-4 layers of sterile cotton cheesecloth into a 15 ml tube. Use one tube for each pair of testes.

7. Use an additional 8 ml of cold 1X NPB to suspend the residual macerate and repeat the filtering process through the same cheesecloth into the same 15 ml plastic screw top tube for each pair of testes. Using gloves, fold and squeeze the cheesecloth above the 15 ml tube to maximize the amount of fluid collected. After this step, approximately 10 ml of fluid should have been collected per pair of testes.
8. Centrifuge at 3000 rpm at 4°C for 10 minutes to pellet the sperm. Remove supernatant, wash pellet in 8 ml cold 1X NPB by inverting, and repeat the centrifugation.

9. Resuspend in 1 ml of 1X NPB, bring to room temperature, and add 50 µl lysolecithin. Gently mix by inverting and incubate for 5 minutes at room temperature.

10. Add 10 ml cold 1X NPB with 3% BSA, 10 µl/ml leupeptin, 0.3 mM PMSF to stop the lysolecithin reaction. Mix gently and centrifuge at 3000 rpm for 10 minutes at 4°C.

11. Wash pellet in 5 ml cold 1X NPB with 0.3% BSA, mix gently, and centrifuge again at 3000 rpm for 10 minutes at 4°C.

12. Resuspend pellet using a cut tip in 500 µl 1X NPB with 0.3% BSA and 30% glycerol. Transfer to a 1.5 ml tube and count sperm density in a hemacytometer. To enhance sperm visibility, dilute 1:100 in 1X sperm dilution buffer (see page 97) and add 1µl of 1:10000 Hoescht dye stock.

13. Repellet and resuspend sperm nuclei in storage solution (1X NPB, 0.3% BSA, 47% glycerol) to make a final solution with approximately 100 sperm nuclei/ml. Aliquot in single-use tubes (4 µl in each PCR tube), mixing the stock occasionally to maintain a uniform nuclei concentration. Store at -80°C.

**Transgenesis**

Agarose dishes and MMR solutions should be kept in the 16°C incubator overnight before injection, to minimize differences in temperature. Making fresh 0.1X MMR solution (see page 93) the day before transgenesis tends to improve early embryo survival (most critical before hatching).

1. Inject female animals with 1500 IU hCG (Chorulon, Intervet, Summit, NJ) the night before egg collection. Keep the females isolated in individual buckets overnight in approximately one gallon of activated carbon-filtered water and throughout the egg-laying period. Replace the bucket water in the morning if it becomes unclean overnight.

   - Note: If the females are kept at room temperature, egg collection can start approximately 8 hours after injection. If kept in the 18°C incubator, the female will start to produce eggs around 10 hours after injection. It is also possible to keep females at 16°C so eggs are produced 12 hours after injection, but this cold temperature may cause lower quality egg production.

2. In the morning, prepare 2-2.5% solution of cysteine (Sigma-Aldrich, St. Louis, MO) in 1X MMR (i.e. 8-10 g cysteine for 400 ml MMR). Adjust the pH of the solution to
between 7.8 and 8.0 using 10 N NaOH. Keep this solution at room temperature, as this solution will be used to dejelly the collected eggs.

3. Collect the first batch of eggs from the female into a 250 ml beaker. The female frog may be squeezed to collect (“strip”) the freshest eggs (see Figure 5.1), or alternatively, eggs can be collected by pipetting from the bottom of each bucket. Stripping eggs appears to be more effective at collecting the freshest (best quality) eggs, but Sive et al. (2007b) have suggested that the natural egg collection without stripping is less stressful to the female. Also, it should be noted that some females did not yield many eggs by stripping.

4. Add enough cysteine solution prepared in step 2 to the 250 ml beaker to submerge the collected eggs. The dejellying process should take about 5-10 minutes, and is completed when the eggs appear to stack after swirling in solution without any transparent space (indicating remaining jelly) between the eggs.

   • If eggs are attached to the beaker wall or inside the plastic pipette, they can be coaxed down by gently rinsing with cysteine solution.

5. Once dejellied, wash the eggs in 1X MMR in a beaker three times by pouring in MMR, swirling, and decanting the solution at room temperature. While washing, remove eggs that have a large white patch, are larger or smaller than the other eggs from the same female, or have not maintained their spherical form. More washes may be necessary for batches of lower quality eggs (with many white-patched or odd shaped eggs), as these eggs are easily crushed and can cloud the MMR - the MMR should remain clear after swirling during the last wash.

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**Figure 5.1 – Stripping eggs from a female animal**

The female frog can be squeezed to collect the freshest eggs. Stars indicate the locations to put the experimenter’s fingers to hold the hindlimbs up away from the cloaca. To strip, the ventral side of the frog is massaged by the experimenter’s thumb in the shaded areas. The arrows indicate the direction to massage the abdomen for maximal egg collection.
6. Using a disposable plastic squeeze bulb pipette (Fisherbrand, Pittsburgh, PA; catalog #13-711-7M) with the tip cut off (making a tip diameter of approximately 3-4 mm), transfer the eggs to 1% agarose dishes filled with 0.4X MMR, 4% ficoll, and 10 µg/ml gentamicin. This injection solution should be filtered sterile using a 0.2 µm syringe filter (Whatman, Piscataway, NJ; catalog #6780-2502) and can be made beforehand and stored at 4°C for up to a few weeks. After transferring, incubate the eggs at 16°C for a minimum of 5 minutes. At this point, the eggs in dishes can safely stay in the incubator at 16°C for a few hours, although the resulting embryos are most viable when injected immediately.

- Note: fresh eggs can be collected hourly from each female as needed for approximately 5 hours after eggs are first produced.

7. Carefully add approximately 600 ng of linearized DNA to 4 µl of sperm nuclei stock (aliquots of sperm nuclei are stored at -80°C). Mix by moving the pipette tip slowly in a circular motion; do not pipet up and down as this will shear the sperm nuclei.

8. Incubate the sperm-DNA mixture at room temperature for 15 minutes. After the incubation, dilute the sperm nuclei with 300 µl of sperm nuclei dilution buffer (SDB; see page 97). Store the diluted sperm in the 16°C incubator. The sperm nuclei ideally should be used within the first couple of hours after preparation: the quality of these diluted nuclei will decrease over time.

9. Cut the tip of a pulled glass needle to produce a 30 - 100 µm diameter opening at the tip. This cut can be produced quickly by stabbing the needle into a single layer of a sheet of Kimwipe (Fisher Scientific, Pittsburgh, PA) which also tends to put a bevel on the broken tip.

10. Using a pre-cut p200 pipet tip, mix the diluted sperm nuclei solution by pipetting multiple times and then draw 70 µl into the tip. Insert the larger, unpulled end of the glass needle into the pipet tip, and use capillary action to fill the needle with liquid. Return the unused portion of the sperm nuclei solution to 16°C for future use.

11. If there are air bubbles in the needle holder, which is filled with mineral oil, depress the syringe on the injection apparatus to expel the bubbles. Attach the needle to the needle holder, avoiding making air bubbles in the needle. The oil should be in direct contact with the sperm suspension. Turn on the injection apparatus, making sure the liquid flows at a rate of 0.6 µL/min.

- The liquid from the needle tends to flow faster for the first minute underwater due to pressure applied during needle attachment to the injection apparatus, leading to a much increased rate of sperm nuclei ejected. The speed of flow can be stabilized by keeping the needle underwater towards the side of the agarose dish for a minute before starting injections on each dish of eggs.

12. Inject the sperm nuclei into the eggs, aiming to keep the needle in each egg for roughly 0.3 seconds per egg. Aim to insert and withdraw the needle in one smooth, rapid movement. This time generally allows for exactly one sperm nucleus to enter
each egg on average. Aim to inject the pigmented animal pole to make it easier to keep track of which eggs have been injected, visible by a white dot at each injection site.

13. After injection, return the dish with eggs to a 16°C incubator and leave them undisturbed.

14. Repeat steps 9 to 13 for each collected dish of eggs, using a new needle for each dish.

15. When the embryos reach the four-cell stage (approximately 3 to 4 hours after injection), select the healthy embryos and transfer them to 0.1X MMR in 10 cm petri dishes. The healthiest embryos have very thin visible divisions between cells, with each of the four cells occupying an equal volume of the embryo. Unhealthy embryos have large white caps at the injection site, and their cells appear more separated within the embryo. Embryos with no cell divisions, a multiple of three cells, unequal sized cells, or many cells can be discarded. The selected embryos will show the best development if there are no more than 20 embryos placed per dish and they are spatially separated from each other. After sorting, rear the embryos at 18°C.

16. When the embryos reach stage 12 (late afternoon on the next day), re-sort the embryos and put them in fresh 0.1X MMR, distributed 25 embryos per dish, using approximately 30 ml 0.1X MMR per dish. After this step, healthy embryos should be sorted daily into fresh 0.1X MMR.

17. Sort the transgenic embryos 4 to 6 days after injection - those embryos that have incorporated the transgenic construct can be identified by their fluorescent green lenses. However, some of the identified embryos may not be entirely transgenic due to transient expression of injected linear DNA (Sparrow et al., 2000) or from variability in the location for genomic integration of the transgene (Marsh-Armstrong et al., 1999). Even so, the mosaicism exhibited by some embryos is to a much lesser degree than with plain DNA injections, as first noted by Amaya and Kroll (1999).

Larval tissue collection and tissue culture

Eye tissues were cultured in vitro to analyze conditions required for lens regeneration. For the SU5402 experiments, larvae were treated with 100 U/ml Penicillin and 100 µg/ml Streptomycin in 1/20X NAM (see page 94) for three days before surgery to help reduce the level of bacterial contamination. For other experiments, larvae were pulled directly from the tadpole stock on the day of surgery. Pre-cut p200 filter tips were prepared beforehand for gently transferring tissue between dishes. These tips were cut at roughly the 10 µl mark (to make an approximately 1.5 mm diameter opening) with a sharp razor blade and then autoclaved beforehand.
Lentectomy and eye tissue collection

For surgical lentectomy, lenses were excised from the larval eye using number 5 Dumont forceps and ultrafine iridectomy scissors made from number 5 Dumont forceps, following a protocol developed previously in our lab (Schaefer et al., 1999; Carinato et al., 2000; Figure 5.2). First, an incision was made in the posterior margin of the outer cornea of anesthetized larvae, spanning approximately a third of the circumference of the eye. A second incision was made in the inner cornea of the eye, spanning the pupillary space to expose the lens (Figure 5.2). The lens was then pulled out using forceps and visually inspected for any damage. Intact lenses appear spherical and transparent. Larvae with damaged lenses or incomplete lens extraction were discarded.

Corneal tissue was collected from tadpoles. First, the entire circumference of the outer margin of the cornea was cut in an anesthetized larva. Then, the outer cornea was gently
detached from the inner cornea by cutting the connecting stalk. Upon this detachment, the outer cornea is now completely excised from the larva. If corneas were to be frozen, they were transferred immediately using forceps to a 1.5 ml eppendorf tube in a dry ice and EtOH bath. Corneas were stored at -80°C. Otherwise, for cornea-containing cultures, corneas were pipetted and transferred to a wash dish with culture medium using a cut pipet tip, as described below.

Retinal tissue was also collected from tadpoles. In the case of retina collection, larvae were first lentectomized, using the protocol above. Once the lens was removed, the outer cornea was detached from the inner cornea and folded anteriorly to expose the eyecup. The eyecup, consisting mostly of retinal tissue, was then detached from the larva by cutting the optic stalk and muscles. For future RNA analysis, this tissue was then transferred using forceps to a 1.5 ml tube in a dry ice and EtOH bath, then stored at -80°C. Alternatively, the retina was pipetted to a wash dish for subsequent culturing with transgenic corneas, as described below.

**Eye culture**

Larval eyes were cultured in modified L-15 media, based on a protocol previously developed in this lab (Henry and Mittleman, 1995; Elkins, 2006). Since the tissues collected were not necessarily sterile due to corneas having direct contact with the external environment, antibiotics were used to prevent bacterial contamination. The antibiotics used were marbofloxacin (10 µg/ml, Sigma-Aldrich, St. Louis, MO) to prevent the growth of *Pseudomonas* and Pen-Strep (100 U/ml Penicillin and 100 µg/ml Streptomycin, Mediatech, Herndon, VA). Amphotericin B (2.5 µg/ml, Sigma-Aldrich, St. Louis, MO) was also used to prevent fungal contamination. Any contaminated culture was discarded.

Eyes for culture were assembled in a similar manner as previously described (Bosco et al., 1993c; Elkins, 2006). Steps for preparing the eye culture are illustrated in Figure 5.3. First, the lens was removed from a given eye, similar to lentectomy (Fig. 5.3A-B). Using iridectomy scissors an incision was made around the eye into the outer cornea epithelium, while maintaining the central attachment between the inner cornea endothelium
Figure 5.3 – in vitro eye culture

_in vitro_ eye culture system used to assay lens regeneration in stage 47-49 larvae of _X. laevis._

(A) The larval eye is shown with both the inner cornea and outer cornea intact. (B) The lens is removed following incision of the outer and inner corneas. (C-D) The outer cornea is tucked into the vitreous chamber of the enucleated eye. (E) The eye is excised from the tadpole and cultured in modified L-15 media with or without FGFR inhibitor (SU5402). Structures are as labeled.

and outer cornea epithelium to help keep the tissue together. An incision was then made in the inner cornea endothelium and the lens was removed with forceps. The outer cornea was tucked into the opening of the vitreous chamber of the enucleated eye (Fig. 5.3C-D). Next, the entire eye was excised from the tadpole by cutting the optic nerve and muscle attachments, and gently washed three times in modified L-15 culture media (see page 93) by serial transfer to separate dishes. Eyes were transferred to a 96-well plate for SU5402 experiments (100 µl culture medium per well) using a pre-cut p200 filter tip, as described above (Fig. 5.3E). Each eye was cultured separately, and fresh culture medium was changed daily. If the culture developed any visible bacterial or fungal contamination, it was discarded.

To inhibit FGFR function using SU5402, various concentrations of SU5402 diluted from a 10 mg/ml stock in DMSO (Calbiochem, Billerica, MA) was added to modified L-15 media. Control cultures in these experiments included an equivalent final concentration of DMSO (0.25%) in modified L-15 media, corresponding to the concentration of DMSO used in the maximal dose of SU5402. Eyes were fixed in 3.7% formaldehyde (Sigma-Aldrich, St Louis, MO) in modified L-15 media six days after surgery, which is ample time for the formation of lens cells detectable by anti-lens antibody staining (Henry and Mittleman, 1995).
Figure 5.4 – transgenic in vitro eye culture
Eye culture system used to assay lens regeneration using transgenic corneas. (A) A wildtype larval eye is shown. (B) The lens is removed after incision of the outer and inner corneas. (C) The cornea is removed from the wildtype eye cup. (D) The intact transgenic eye is shown, identified by green fluorescence in the lens. (E-F) The outer cornea is excised from the eye. (G) The eyecup from the wildtype eye and the transgenic cornea are combined and placed in a culture well. Structures are as labeled.

Transgenic corneas were assayed for lens regeneration competence using a similar method as above, shown in Figure 5.4. In contrast to the method outlined above for small molecule inhibition of FGFRs, retinas were derived from wildtype animals and corneas were from transgenic animals. Two animals were used in order to avoid detecting any remaining lens fragments in the retina after lentectomy and to exclude any detectable transgene expression from being in the retina. From the wildtype eye, the lens was removed, similar to lentectomy (Fig. 5.4A-B). The retina was excised from the tadpole and transferred to a wash dish with culture medium using a pre-cut p200 filter tip (1.5 mm diameter opening, as described above). Transgenic eyes were identified by green fluorescence in the lens. From the transgenic eye, the cornea epithelium was excised and placed in culture medium with the retina (Fig. 5.4D-F). In the wash dish, the eye structure was assembled by gently inserting the transgenic cornea into the wildtype retina using a pair of forceps. The assembled “eye” was then washed gently two more times by serial transfer in modified L-15 culture medium. Single eyes were transferred to individual wells of a 24-well plate (1 ml culture medium per well; Fig. 5.4G). Culture medium was changed fresh
daily. Plates were heat shocked daily (40 minutes at 34°C) to express the transgene. To heat shock, plates were placed in a constant temperature oven (DN-63, Baxter, Deerfield, IL) set to 34°C. The oven also contained shallow tupperware dishes of water to maintain a moist environment and to prevent desiccation. Gamma-crystallin expression is initiated at the later stages of lens regeneration (Schaefer et al., 1999), so the associated expression of GFP in our transgene is expected to be present later than the case of anti-lens antibody staining which detects multiple crystallins. For these transgenesis experiments, eyes were cultured for a longer period and assessed for lens regeneration by observing GFP expression 14 days after surgery.

**Cornea culture**

In order to promote cell attachment to the bottom of the culture wells, 24-well plates were coated with gelatin before use for cornea cultures. To coat the wells, 500 µl 0.1% gelatin (see page 90) is added to each well of a 24-well plate and left in the biological hood for a minimum of 30 minutes. After coating, the gelatin solution is removed and wells are washed twice with 500 µL culture medium (67% L-15, page 91) just before use. Alternatively, gelatin-coated plates can be sealed with parafilm and stored at 4°C (with 0.1% gelatin solution) for a few months.

Corneas were cultured using various culture media in an attempt to find the optimal culture conditions. CnT-50 medium (Zen-Bio, Research Triangle Park, NC) yielded cornea cultures that appeared to survive for approximately two weeks. CnT-20 medium (Zen-Bio, Research Triangle Park, NC) diluted 2:3 with deionized tissue culture water appeared to yield increased cornea attachment relative to the CnT-50 trial and showed migrating cells after 3 days in culture. With 67% L-15 medium (without FBS, see page 91), the cultures survived for approximately two weeks before massive cell death occurred. The cultures in modified L-15 medium (with FBS, see page 93) had the longest survival time (up to the 3 months observed), as assayed by cell morphology. All media was supplemented with marbofloxacin (10 µg/ml, Sigma-Aldrich, St. Louis, MO), Pen-Strep (100 U/ml Penicillin
Figure 5.5 – *in vitro* cornea culture

Cornea culture method to assay factors sufficient for lens formation. (A) The intact larval eye is shown. (B) The cornea epithelium is excised, keeping the rest of the eye structures intact. (C) The cornea is attached to the bottom of the culture well by pushing gently with forceps to indent the plastic and adhere the tissues. (D, E) A cornea cultured for two days using this method in 67% L-15 medium imaged using brightfield illumination (D) and fluorescent hoescht nuclear staining (E). The scale bar in E indicates 500 µm. Structures are as labeled.

and 100 µg/ml Streptomycin, Mediatech, Herndon, VA), and amphotericin B (2.5 µg/ml, Sigma-Aldrich, St. Louis, MO) to prevent contamination, as described above.

The cornea epithelium is the only eye tissue used to prepare *in vitro* cornea cultures (Fig. 5.5). Other eye tissues are not strictly necessary for assaying lens regeneration, as regenerated lenses form from cells of the inner layer of cornea epithelium (Freeman, 1963). To make cornea cultures, the cornea epithelium is first excised from the larval eye (Fig. 5.5A-B) and briefly washed once in culture medium (either 67% L-15 or modified L-15) for a few seconds, by transferring between dishes using a cut pipet tip. A longer wash can allow the cornea to curl up on itself, which is not desirable for the next step. After briefly washing, the cornea is transferred to a culture well and oriented so that the inner layer of the epithelium is facing the gelatin-coated plastic well bottom. The inner layer of the cornea epithelium can be identified by the presence of loose tissue attached to the corneal stalk or by the pattern of pigmentation on the pericorneal tissue when removing the cornea. Alternatively, one can mark the cornea with two small incisions of differing length along the side of the cornea to help indicate which side is the outer or inner layer (using the different lengths of the incisions to orient the cornea). Once oriented, the corneal tissue is pinned down to the bottom of the well by gently pushing down on the cornea with the tips of the forceps (Fig. 5.5C), which resulted in indentations in the bottom of the plastic well (Fig. 5.5D). Each cornea was anchored at 3-4 points (i.e. pressing twice, each time with both tips of the forceps) for best attachment to generate cells that attach and migrate...
along the bottom of the well. Up to four corneas were cultured together in a single well of a 24-well plate, and culture medium was changed every three days by exchange of 0.5 ml medium per well. Corneal cultures were maintained for up to a few months in modified L-15 medium using this method.

Cultured corneas were exposed to FGF1 (R&D Systems, Minneapolis, MN), FGF2 (R&D Systems, Minneapolis, MN), FGF8 (R&D Systems, Minneapolis, MN), FGF9 (R&D Systems, Minneapolis, MN), or insulin (Sigma-Aldrich, St. Louis, MO) to investigate their effects on cornea-lens regeneration. FGF1 and FGF2 stock were initially made at a concentration of 100 μg/ml in PBS with 0.1% BSA and stored at -80°C. FGF8 and FGF9 stock were made at a concentration of 100 μg/ml in PBS with 0.1% BSA and also stored at -80°C. FGF stock solutions were thawed and added to culture media immediately before use. FGF treatment concentrations ranged from 10 ng/ml to 1 μg/ml in culture media.

Control cultures without FGF were treated with an equivalent amount of PBS and BSA as the highest concentration used for each FGF. Insulin (5 μg/ml final concentration) was added to media during formulation of either the 67% L-15 or modified L-15 media, and the pH of media was adjusted to 7.6 after insulin addition using NaOH. Insulin-containing media was then filtered sterile and stored at 4°C until use.

**Tissue fixation**

For future lens detection by anti-lens antibody staining, cultured eye tissues were fixed in 3.7% formaldehyde in 1/20X NAM or culture medium (modified L-15 for eye cultures or 67% L-15 for cornea cultures) after the culture period. In the case of whole eye cultures, eyes were detached from the bottom of culture wells by pipetting or gently pushing them with a tungsten needle. Eyes were transferred into a vial from the culture wells using a cut pipet tip prior to fixation. Whole eyes were fixed for 1-2 hours at room temperature or overnight at 4°C. Cornea cultures were fixed for 15 minutes at room temperature. Fixed tissues were then washed twice each (5-10 minutes per wash) in 30% EtOH, 50% EtOH, and 70% EtOH (all EtOH diluted with H2O). Tissue was then stored at 4°C until further analysis (see antibody staining on page 78 below).
DNA/RNA techniques

Restriction enzyme cutting of DNA

Restriction enzymes were used to linearize plasmids or isolate DNA fragments. Restriction enzymes and corresponding buffers were obtained from Invitrogen (Grand Island, NY) or New England Biolabs (Ipswich, MA). For a small 10 µl digestion reaction to verify ligation results, 0.5 µl of enzyme was used. For digestion of large amounts of DNA, enzyme amounts were adjusted to result in a minimum of 5-fold overdigestion, as calculated from number of enzyme units per µl and the DNA concentration. In cases with concentrated DNA, reactions were diluted so the restriction enzyme(s) added did not exceed 10% of the total reaction volume. 10X reaction buffers (supplied with restriction enzymes) and nuclease-free water were added to make a final 1X buffer concentration. Reactions were incubated for two hours at 37°C.

Cut DNA segments were purified using the GENECLEAN Turbo kit (MP Biomedicals, Solon, OH) according to kit instructions for gel purification (to isolate a specific DNA segment) or enzymatic solution purification. In the case of gel purification, DNA yield appeared to improve by letting the melted gel in salt solution cool to room temperature before initiating the centrifugation steps. All DNA was eluted using nuclease-free water instead of the kit’s Elution Solution.

DNA ligation

Fragments of DNA were inserted into vectors to form custom plasmids. For ligations, the Rapid DNA ligation kit (Roche Diagnostics, Indianapolis, IN) was used according to instructions (including the recommendation for a 1:3 molar ratio of vector to insert), with the exception that the ligation reactions were carried out for 30 minutes instead of 5 minutes to increase yield. 1 µl of the reaction was used for each transformation experiment described below. Completed ligation reactions were stored at -20°C.
Growing competent E. coli stocks

Stbl3 chemically competent cells (Invitrogen, Grand Island, NY) were grown to make additional stock for transformations. This method was kindly shared by the Fei Wang lab, loosely based on the protocol optimized by Hanahan (1983).

1. On the first day, streak competent cell stock onto a LB plate (without antibiotics).
2. The next day, inoculate a single isolated colony in 3 ml LB media (without antibiotics) and grow overnight shaking at 37°C.
3. The next morning, transfer 1 ml of the overnight culture to 100 ml LB.
4. Incubate the cells while shaking until OD600 is 0.5 - 0.7 across 1 cm; do not let the OD get higher than 0.8.
   - Note that in the case of using the NanoDrop spectrophotometer (model ND-1000, NanoDrop, Wilmington, DE) these OD values should be tenfold lower (0.05 - 0.07) due to measuring across a 1 mm distance instead of 1 cm.
5. In the cold room, split the culture into equal amounts in 50 ml tubes. Incubate on ice for 15 minutes.
6. Centrifuge at maximum speed (3350 rpm, 1750 g on model CL, International Equipment Co., Needham Heights, MA) for 10 minutes in the cold room to pellet the bacteria.
7. Discard the supernatant and add 16.6 ml of ice-cold RF1 solution (see page 96) to each tube and resuspend. Incubate on ice for 15-30 minutes.
8. Centrifuge again at maximum speed for 10 minutes in the cold room.
9. Discard the supernatant, add 4 ml ice-cold RF2 solution (see page 96) to each tube and resuspend. Incubate on ice for 15 minutes.
10. In the cold room, aliquot the competent cells, performing all actions under a flame to keep the environment sterile. Make a combination of single-use 50 µl aliquots and 1 ml aliquots. All aliquots should be stored at -80°C.
11. To test the competent cells, plate cells on a plain LB plate (should develop many colonies) and an LB plate with antibiotic (e.g. ampicillin, should develop no colonies). As another positive control, one can transform cells with a control plasmid and plate them on an LB plate with the plasmid’s associated antibiotic (should develop colonies).

DNA transformation into electrocompetent cells

Transformation of DNA into ElectroMAX DH10B cells (Invitrogen, Grand Island, NY) was performed similarly to the method provided by the company.
1. Start warming LB plates at room temperature with the agar side up to avoid water condensation. Alternatively, to make slightly less wet plates, warm the plates in a 37°C incubator.

2. Thaw a tube of competent cells on ice for 5-10 minutes. Mark the lid with a dot to add to the freeze-thaw cycle count as the tube may be refrozen if there are remaining cells. Cells from a tube with a high number of freeze-thaw cycles will not remain as competent as cells from a fresh tube.

3. Start cooling a micro-electroporation chamber (LABRepCo, Horsham, PA) on ice.

4. In a 600 µl tube, combine 15 µl of competent cells with 1 µl of the ligation reaction, trying not to introduce any air bubbles into the solution. Mix gently by stirring with the pipet tip.

5. Gently pipet the competent cell mixture into the 1.5 mm electrode gap of the electroporation chamber (LABRepCo, Horsham, PA). Make sure there are no bubbles introduced in this process - it is preferable to leave a small volume of cells in the pipet tip than to add a bubble.

6. Turn on the Cell-Porator *E. coli* Electroporation System Pulser (Gibco, Grand Island, NY). The “voltage range” should be set to the medium setting (for an approximately 2.5kV pulse). Put the electroporation chamber in the cooled base container (the base is kept at 4°C for storage), and screw on the lid.

7. Push the “charge” button, and wait for the “ready” light to turn on, indicating that the machine is fully charged. Once the machine is ready, the reading in the voltage indicator should stay steady. Push the “trigger” button to send the electrical pulse to the cuvette.

8. After the pulse, make sure that the mass of cells did not explode in the chamber (i.e. see if the liquid is still in the electrode gap, not scattered all over the chamber, the latter usually due to inclusion of air bubbles). Add 500 µl of SOC medium (included with the ElectroMAX cells, Invitrogen, Grand Island, NY) to the chamber, rinsing the cells off of the electrodes.

9. Use a thin sterile plastic pipet to transfer the cells to a 15 ml Falcon tube.

10. Shake the angled tube horizontally at 37°C for 1 hour to allow the cells to recover. The horizontal angle provides an increased surface area for better aeration of the solution. Tape the Falcon tubes onto the shaker surface to secure them.

11. If the DNA vector contains blue/white selection sequences (pGEM-T Easy), spread 100 µl of 100 mM IPTG and 20 µl of 50 mg/mL X-gal onto each LB plate while waiting for the hour-long cell incubation. This allow time for the solutions to be absorbed by the plates before cell plating.

   - Later, this addition of IPTG and X-gal will allow blue/white selection to occur, as blue colonies will indicate ligation products with no insert.
12. Spread between 10 µl and 300 µl of cells under sterile conditions (near an open flame) onto pre-warmed LB plates with antibiotic. If using the glass spreader bar, keep spreading the cells until no visible liquid is left on the plate. Typically, two plates with a 10-fold difference in volume were plated (such as 30 µl and 300 µl).

13. Incubate the plates at 37°C overnight, agar side up.

14. The next morning, confirm the presence of individual colonies on the plates. Wrap the plates with parafilm and store at 4°C. Plates can be stored for up to a week.

**DNA transformation into chemically competent cells**

Stbl3 cells (Invitrogen) were transformed with heat shock, based on the method provided by the company. Before starting this protocol, make sure that at least one of the heating blocks in lab is set to 42°C and LB antibiotic plates are being pre-warmed at room temperature.

1. Thaw one aliquot of Stbl3 cells on ice for each transformation reaction.

2. Add between 1 pg to 100 ng DNA (or 1 µl of miniprepped DNA) to the cells and mix by stirring the pipet tip, not by pipetting up and down. Incubate the mixture on ice for 30 minutes.

3. Heat shock the cells for 45 seconds at 42°C.

4. Return the tube to ice and incubate for 2 minutes.

5. Add 250 µl SOC medium or LB medium to each tube and transfer the solution to a 5 ml Falcon tube. Using SOC medium will yield more colonies than LB medium.

6. Shake the Falcon tube horizontally at 37°C for 1 hour. This incubation can be cut short to as little as 30 minutes, but will decrease transformation efficiency.

7. Spread between 10 µl and 300 µl onto the pre-warmed LB antibiotic plates.

8. Incubate the inverted plates (agar side up) at 37°C overnight.

9. Plates can be stored at 4°C wrapped in parafilm after the overnight incubation.

**Plasmid preparation via miniprep**

Plasmid DNA was prepared and purified using the Wizard Plus SV Minipreps kit (Promega, Madison, WI) with a few modifications to the protocol as described below.

1. Fill glass test tubes with 5-10 ml LB medium with antibiotic. If using ampicillin, the frozen ampicillin stock must be fresh (within one year of aliquoting) or the antibiotic selection may not work.
2. Inoculate the LB medium from a fresh plate of transformed bacteria, picking a single colony of *E. coli* per test tube. Alternatively, the LB can be inoculated by bacteria from a frozen stock at -80°C.

3. Grow the bacteria by shaking overnight at 37°C.

4. The next morning, check to see if the bacteria culture grew - the LB should look cloudy instead of clear. Make glycerin stocks by combining 738 µl of the overnight bacterial culture with 262 µl of 50% autoclaved glycerin solution in a microcentrifuge tube, and store at -80°C.

5. Harvest the bacteria by centrifuging the bacterial culture in a 1.5 ml microcentrifuge tube for 5 minutes at 10,000g (11,000 rpm on Eppendorf model 5415C). Repeat the centrifugation until all of the bacteria grown is harvested.

6. Add 250 µl of Resuspension Solution to each tube. Resuspend the cells by pipetting up and down.

7. Add 250 µl of Lysis Solution to each tube. Mix by inverting the tube a few times.

8. Add 10 µl of Alkaline Protease Solution. Mix by inverting the tube a few times. Incubate for 5 minutes.

9. Add 350 µl of Neutralization Solution. Mix by inverting the tube a few times.

10. Centrifuge at maximum speed for 10 minutes at room temperature.

11. Set up the kit’s spin columns in collection tubes. Label the spin columns.

12. Decant the supernatant from the centrifuged lysate into a spin column. Centrifuge for one minute at maximum speed and then discard the flowthrough in the collection tube.

13. Add 750 µl of Column Wash Solution and centrifuge for one minute at maximum speed. Discard the flowthrough.

14. Add 250 µl of Column Wash Solution and centrifuge for two minutes at maximum speed.

15. Transfer the spin column to a 1.5 ml microcentrifuge tube. Add 50 µl of nuclease free water to the center of the spin column. Elute by centrifuging for one minute at maximum speed. Discard the spin column after elution.

16. Measure the DNA concentration using a Nanodrop spectrophotometer. Store the DNA at -20°C.

**RNA extraction**

RNA was extracted from *Xenopus* tissues with TRIzol (Invitrogen, Grand Island, NY), loosely following the protocol supplied with the product, as described below. Before extraction, tissues were flash frozen in a dry ice and EtOH bath and stored at -80°C. Corneas
and retinas were collected from *X. laevis* larvae (stage 48-51) using fine iridectomy scissors. Regenerating tissues were generated by removing the lens from the right eye of stage 48-51 larvae, as described above. Corneas and retinas during lens regeneration were collected 1-, 3-, 5-, and 7- days after lentectomy. In the case of corneas, due to their small size, it is optimal to use a minimum of 50 corneas for each RNA extraction to get the best yield per cornea. Positive control RNA was extracted from stage 37-38 embryos.

1. Homogenize the tissue in TRIzol. Record the volume \((V)\) of TRIzol used at this step - later steps will use this volume to calculate other values.

   • The volume used will depend on the amount of tissue to homogenize: the supplied protocol recommends 1 ml for every 50-100 \(\mu\) l tissue. This equates to roughly to 500 \(\mu\) l TRIzol for every 25 cornea or retina samples, or in the case of whole embryos, 50 \(\mu\) l per embryo.

   • To start homogenizing, grind the tissue with a pestle in a small volume of TRIzol. Then, quickly add the remaining volume of TRIzol and vortex to complete the homogenization.

2. Incubate for 5 minutes at room temperature.

3. Add 0.2 \(\times V\) chloroform and vortex. Incubate another 3 minutes at room temperature.

4. In the cold room (4°C), centrifuge for 15 minutes. While waiting, prepare a ice-cold 75% EtOH/25% DEPC treated H\(_2\)O mixture to be used in a later step.

5. Extract the aqueous phase (top layer) into a new tube.

6. Add 0.5 \(\times V\) isopropanol, then gently invert multiple times to mix the solution. Incubate 10 minutes at room temperature.

7. Centrifuge for 20 minutes in the cold room. Immediately after centrifuging, remove the supernate. This step may be repeated to combine RNA from a larger number of tissue samples: add additional isopropanol solution from the previous step to the same tube and centrifuge again.

8. Wash with \(V\) ice-cold 75% EtOH/25% DEPC H\(_2\)O. Centrifuge for 5 minutes in the cold room. Be careful not to lose the RNA pellet.

9. Remove the EtOH wash and air dry the RNA pellet for 5 to 10 minutes at room temperature.

10. Add RNAs-free water (Sigma) to dissolve the RNA pellet. Use 5 \(\mu\) l for every 25 corneas and 25 \(\mu\) l for every 25 retinas.

11. Incubate for 10 minutes at 55 to 60°C to finish dissolving the RNA pellet. Measure the RNA concentration using the NanoDrop spectrophotometer. Store the RNA at -80°C.
**DNase treatment of RNA**

In order to remove any residual genomic DNA from the RNA collected, RNA was treated with DNase. The treated RNA was then purified using NucAway spin columns (Ambion, Grand Island, NY).

1. Dilute the RNA to 200 ng/µl, adding 5X FS buffer to make 1X final concentration (Invitrogen, Grand Island, NY) and a 1:25 dilution of 0.1 M DTT. For each [50 µl] reaction, add 1 µl TURBO DNase (Ambion, Grand Island, NY).

2. Incubate the reaction at 37°C for 30 minutes.

3. For each reaction, prepare one NucAway spin column (Ambion, Grand Island, NY).
   
   (a) Settle the dry gel into the bottom of the column by tapping it on the bench.
   
   (b) Hydrate the column with 650 µl nuclease-free H$_2$O, replace cap, and vortex. Let sit at room temperature for at least 5 minutes.
   
   (c) Confirm that all of the dry gel is hydrated by visual inspection. There should be a uniform-appearing gray color to the contents of the column. Put the gel column in a collection tube and spin at 750g for 2 minutes at room temperature.
   
   (d) Remove the H$_2$O from the collection tube, then spin for 1 minute at room temperature.

4. Apply up to 100 µl of the treated RNA to the top of the column, centering the liquid as much as possible.

5. Put the column in an elution tube and spin at 750g for 2 minutes.

6. Measure the concentration of the eluate using the NanoDrop spectrophotometer. Store the RNA at -80°C.

**First-strand cDNA synthesis**

The cDNA synthesis protocol was based on the protocols provided for SuperScript III Reverse Transcriptase (Invitrogen, Grand Island, NY) and SuperScript II Reverse Transcriptase (Invitrogen, Grand Island, NY). The following steps assume the desired result is 20 µl cDNA, enough for 10 PCR reactions.

1. Calculate the volumes of RNA needed to add to each cDNA reaction. At this step, make sure each sample will represent equal amounts of RNA (typically 10 to 100 ng per PCR reaction, which translates to 100 to 1000 ng per cDNA reaction). Up to 8 µl of RNA stock solution may be used.
In the case of RT-PCR of FGFs and FGFRs, the RNA used was 100 ng per 20 µl cDNA, so each PCR reaction will use 10 ng total RNA.

2. In a PCR tube, combine: 1 µl of oligo dT primer, 1 µl of 10 mM dNTPs (Roche Diagnostics, Indianapolis, IN), RNA as calculated above, and use Sigma ultrapure water (Sigma-Aldrich, St. Louis, MO) to a final volume of 10 µl.

3. Using a thermocycler, incubate the tube at 65°C for 5 minutes, then cool to 4°C for at least one minute.

4. Add 4 µl of 5X First-Strand buffer (Invitrogen, Grand Island, NY), 1 µl of 0.1 M DTT, 1 µl of RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen, Grand Island, NY), 1 µl SuperScript II, and 3 µl Sigma water.

5. Incubate the tube at 42°C for 50 minutes, 70°C for 15 minutes, then cool to 4°C.

6. Add 1 µl RNAse H (Invitrogen, Grand Island, NY) and incubate at 37°C for 20 minutes.

7. The resulting cDNA can be stored at -20°C.

**PCR portion of RT-PCR**

PCR reactions were performed using Taq polymerase (New England BioLabs, Ipswich, MA), amplified for 35 cycles. Reactions were performed as programmed below on an DNA Engine Thermal Cycler (Biorad, Hercules, CA). PCR products were confirmed by sequencing (Biotechnology Center, Urbana, IL).

**PCR program for amplification of FGF/FGFR fragments**

1: 94°C for 1 minute
2: 94°C for 1 minute
3: 60°C for 1 minute
4: 72°C for 2 minutes
5: Return to step 2 for 34 times
6: 72°C for 10 minutes
7: 4°C forever

**Antibody staining**

**Embedding and sectioning tissues in paraplast**

Eye tissues were stored in 70% EtOH at 4°C after fixation, as described above. Tissue sections were produced and placed on subbed slides for later immunohistochemistry, such as anti-lens staining.
1. Wash in 95% EtOH for 15 minutes.
2. Wash 3 x 15 minutes in 100% EtOH.
3. In the chemical hood, wash 3 x 10 minutes in xylene.
4. Transfer the vials to the 60\degree C oven. Treat 6 hours or overnight in 50% xylene / 50% paraplast (Paraplast Plus, McCormick Scientific, St. Louis, MO).
5. Wash 3 x in 100% paraplast for 6 hours or overnight each. At the third wash, also fill plastic boats (Peel-A-Way Mold, Truncated T12, Polysciences, Inc., Warrington, PA) with paraplast and warm them in the embedding oven at 60\degree C.
6. Transfer the specimens to the wax filled boats, and incubate 6 hours or overnight.
7. Orient the specimens, making sure everything takes up at most a quarter of the area at the bottom of the boat. Do not let specimens contact one another. Cool at room temperature to solidify the paraplast. These boats can be stored at 4\degree C until sectioning.
8. Remove the paraplast blocks from the boats and cut off the solidified meniscus so there is a flat surface on the side opposite from the specimens. Using a heated spatula to melt the flat paraplast surface, attach the paraplast block to a wooden block. Carve the top of paraplast block down so that only a small rectangular area around the specimens is present on the top surface of the block.
9. Section the specimens using the 8 \mu m setting on the AO rotary microtome (model 820, American Optical, Buffalo, NY). Arrange the sections over water on an albumin-subbed slide (Humason, 1962). Heat the slides at 37\degree C overnight to dry. The slides with attached sections can be stored in sealed containers at 4\degree C.

Cross-absorption of primary \(\alpha\)-lens antibody serum

Polyclonal rabbit serum containing \(\alpha\)-lens antibody was kindly donated by the Grainger lab (Henry and Grainger, 1990). The rabbit serum containing antibody requires cross-absorption against non-lens \textit{Xenopus} proteins. Stage 19-24 embryos are used for this purpose, as they have not yet developed lenses. For cross-absorption, sets of 25 embryos can be collected and stored at -80\degree C until use.

1. Homogenize the embryos in an equal volume of homogenate buffer in a 1.5 ml tube using a plastic pestle.

\begin{align*}
\text{Homogenate buffer (1 ml)} \\
20 \mu l & \text{2M KCl} \\
10 \mu l & \text{1M Pipes} \\
10 \mu l & 100mM PMSF (stock PMSF is stable in isopropanol) \\
5 \mu l & \text{NP40} \\
955 \mu l & \text{H}_2\text{O}
\end{align*}
2. Add 50 µl of rabbit serum and mix.

3. Incubate at 12°C for 1-2 hours, mixing occasionally.

4. Spin down the mixture for 10 minutes, then carefully remove the supernatant (between the lipid layer and the insoluble pellet) into a new 1.5 ml tube. Avoid collecting any lipids.

5. If needed, repeat step 4 two more times to ensure that the resulting solution is completely free of lipids.

6. Store the cross-absorbed serum at -20°C.

**Antibody staining of sectioned eye tissues on slides**

Embedded eyes in paraplast blocks were sectioned on a microtome to 8 µm thickness. Slides were stored at 4°C until staining.

1. Warm the slides to room temperature before unwrapping slides, so as to avoid water condensation.

2. To dewax the slides, soak for 2 x 10 minutes in 100% xylene or Histo-Clear (National Diagnostics). Note that xylene seems to work faster than Histo-Clear but is potentially more toxic.

3. Air dry the slides at room temperature, and make sure the slides are completely clear of wax. This should take less than one minute for xylene, and longer for Histo-Clear.

4. Transfer slides to PBS (see page 95) for 5 minutes.

5. Transfer slides to 0.1 M glycine in PBS for 30 minutes to denature proteins.

6. Wash slides in PBS for 5 minutes.

7. Put slides in 5% (w/v) Carnation nonfat dry milk in PBS for 45 minutes to block.

8. Place the slides in moisture chambers and add 200-300 µl of primary antibody solution to completely cover the top surface of the slide. The moisture chambers are essential for preventing the slides from drying out. Slides are elevated using the tops of 35 mm petri dishes to prevent wicking of the solution. Incubate the slides in the moisture chamber for at least two hours at room temperature or overnight at 4°C. Slides must be completely level to prevent pooling of the solution.

   - In the case of lens antibodies (Henry and Grainger, 1990), dilute the cross-absorbed lens antibody solution 1:50 into the blocking solution (5% dry milk).

9. Collect the primary antibody solution from the slides, and transfer the slides to PBS. The primary antibody solution can be stored at -20°C and used approximately three times.
10. Wash slides 2 x 10 minutes in PBS.

11. Dilute the secondary antibody 1:100 in 5% dry milk and 1X PBS. For lens staining, the secondary antibody is goat anti-rabbit rhodamine IgG (Jackson ImmunoResearch Laboratories, Inc.), and lens cells should be fluorescent red.

12. Place slides in moisture chambers and add 200 µl of secondary antibody solution to each slide, covering the entire top surface of the slide. Let the slides incubate for at least one hour at room temperature or overnight at 4°C.

13. Wash the slides for 2 x 10 minutes in PBS and mount using a 80% glycerol / 20% PBS solution, with 1:10000 dilution of hoescht 33342 (Molecular Probes) for nuclear staining. Store slides at 4°C.

**Antibody staining of whole corneas**

Corneas cultured in 24-well plates can be stained with a similar protocol as for sectioned eye tissues, but the tissue remains attached to the culture wells.

1. Fix corneas in their wells in 3.7% formaldehyde in 67% L-15 (without serum) for 15 minutes at room temperature.

   - At this point, corneas can be washed in 70% ethanol twice for 10 minutes each and then stored at 4°C until staining.

2. Wash the corneas in 2 ml/well PBTw (PBS with 0.1% Tween 20) for 5 minutes.

3. Wash in 2 ml/well PBS for 2 x 10 minutes.

4. Block using 1 ml/well 5% dry milk in PBS; incubate for 45 minutes.

5. Incubate with 200 µl/well primary antibody for 2 hours at room temperature or overnight at 4°C.

   - To make the α-lens antibody solution, dilute cross-absorbed antibody 1:50 in PBS with 5% milk, or use a previously used, diluted stock taken from -20°C. Diluted primary antibodies can be used up to three times.

6. Wash with 2 ml/well PBS for 2 x 10 minutes.

7. Incubate with 200 µl/well secondary antibody for 1 hour at room temperature or overnight at 4°C. For lens staining, use a 1:100 dilution of the rhodamine goat anti-rabbit secondary antibody in PBS with 5% dry milk.

8. Wash 2 x 10 minutes in PBS.

9. Add mounting media (80% glycerol / 20% PBS / 1:10000 Hoescht) to wells, and store plates in dark.
Statistical analysis for lens counting

Since the inhibition assays were expected to yield significantly low regeneration rates, Fisher’s exact test under the one-tailed condition was used (Yates, 1934; Irwin, 1935). Comparisons with p values less than 0.05 were considered to be significant.

Protein analysis

Tissue collection and quantification for protein analysis

Tissue samples were flash frozen using a dry ice and ethanol bath at the time of collection and stored at -80°C until analysis. For tail samples, individual tail halves were transferred using clean forceps into separate 1.5 ml tubes and tissue homogenization was performed in 100 µl Lysis buffer (see page 92) for each tail sample. For cornea cultures, culture medium was removed and 100 µl Lysis buffer was added to each well containing 4 corneas. The culture plates were then rocked for 10 minutes, and the lysed solution was collected. To remove insoluble material, homogenized tissue samples were spun down at maximum speed for 5 minutes at room temperature, and the supernatant was collected.

Protein quantification was performed using a BCA kit (Thermo Scientific, Waltham, MA), with some modifications to the provided instructions due to the low volumes required for the Nanodrop spectrophotometer. Although attempts were made to use the Bradford method (Bradford, 1976), there was too much detergent in the lysis buffer for effective protein quantification. In the modified BCA quantification method, only 5 µl of each homogenized tissue sample was combined with 5 µl lysis buffer and 10 µl BCA working solution. In the case of larval tail samples, serial dilutions were made (by addition of equal volumes of lysis buffer and BCA working solution) to accommodate the wide variety in tail sizes. Samples were incubated at 37°C for 2 hours before measurement using the BCA mode on the Nanodrop software. After quantification, protein concentrations were adjusted to be equal by addition of protein sample buffer (see page 96).
Preparation of polyacrylamide gels for Western blotting

1. Assemble the gel casting apparatus (Bio-Rad, Hercules, CA), making sure that the glass plates are clean and oriented correctly. We use the 0.75 mm spacers for mini gels with either a 10-well or 15-well comb.

2. Prepare the resolving gel solution (see table below) according to the desired percentage gel. Do not add TEMED until ready to pour. Required reagents are listed below for a single gel:

<table>
<thead>
<tr>
<th>reagent</th>
<th>7.5% gel</th>
<th>10% gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M Tris-HCl, pH 8.8</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>20% (w/v) SDS</td>
<td>20 µl</td>
<td>20 µl</td>
</tr>
<tr>
<td>acrylamide/bis-acrylamide (30%)</td>
<td>1 ml</td>
<td>1.33 ml</td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>40 µl</td>
<td>40 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>2.4 µl</td>
<td>2.4 µl</td>
</tr>
<tr>
<td>H₂O</td>
<td>2 ml</td>
<td>1.67 ml</td>
</tr>
</tbody>
</table>

3. Using a p1000 pipet, gently pipet 4 ml of the resolving gel solution per gel, or until the solution reaches 1-2 cm below the lip of the glass plate. Then, carefully add 1 ml of H₂O or 100% ethanol to the top of the resolving gel. Ethanol is preferred for adding to the top of the resolving gel, since it is much easier to make the mistake of pipetting H₂O too quickly than to pipet ethanol too quickly. Make sure there are no bubbles in the gel and the solution isn’t leaking out at the bottom of the gel. Allow 30 minutes for the gel to set.

4. Prepare the stacking gel solution. Do not add TEMED until ready to pour.

<table>
<thead>
<tr>
<th>reagent</th>
<th>4% stacking (2 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M Tris-HCl, pH 6.8</td>
<td>500 µl</td>
</tr>
<tr>
<td>20% (w/v) SDS</td>
<td>10 µl</td>
</tr>
<tr>
<td>acrylamide/bis-acrylamide (30%)</td>
<td>333 µl</td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>20 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>2 µl</td>
</tr>
<tr>
<td>H₂O</td>
<td>1135 µl</td>
</tr>
</tbody>
</table>

5. Pour out the H₂O/EtOH overlay on the resolving gel and use a kimwipe to remove the excess fluid.

6. Gently pipet the stacking gel solution until the solution reaches the lip of the glass plate. Insert the comb carefully, making sure there are no bubbles under or between the teeth. Let the gel set for 30 minutes.

7. Once the gel sets, carefully remove the comb so that the wells maintain their rectangular shape. For the cleanest Western blots, use the gels immediately. Alternatively, gels can be wrapped in saran wrap with wet paper towels and stored at 4°C for up to a few weeks.
Western blot

To prepare the protein samples, add 2X protein sample buffer (see page 96) and boil for 5 minutes. These protein samples can then be stored at -20°C until use.

1. Assemble the gel apparatus (Biorad, Hercules, CA) and fill the middle chamber completely with Tris-SDS-Glycine buffer (page 97). Fill the outer chamber with Tris-SDS-Glycine buffer, but this buffer is only required up to the bottom of the gel in the outer chamber. If the buffer leaks from the middle chamber, re-assemble the gel apparatus so it doesn’t leak.

2. Wash all the wells with buffer using a p200 pipet.

3. Load the unused wells with protein sample buffer to limit the lower expansion of the other lanes.

4. Load the protein standard. Use 5 µl of the Precision Plus Protein Standard (Biorad, Hercules, CA) or 2 µl of PageRuler Prestained Protein Ladder (Thermo Scientific, Waltham, MA) in a single lane.

5. Load the protein samples slowly into wells, making sure the samples flow into the correct well.

6. Run the gel at 150 V until the dye front moves to the bottom of the gel. This should take approximately one hour. While running, check the gel apparatus periodically to make sure that the buffer hasn’t leaked out of the middle chamber.

7. Soak the fiber pads, filter paper (Whatman Chromatography Paper, Whatman, Piscataway, NJ, catalog number 3030917), and nitrocellulose membrane (Bio-Rad, Hercules, CA) in Western blot transfer buffer (see page 98) in a shallow tupperware container.

8. Disassemble the gel apparatus and assemble the gel transfer apparatus in the cold room. Make sure there is ice in the ice dam and fill partially with transfer buffer. Add a small stir bar to the chamber.

9. Carefully remove the gel from the glass plates and put it with the nitrocellulose membrane in transfer buffer.

10. To assemble the protein blot sandwich, put the fiber pads on the outside, then two pieces of filter paper, then the nitrocellulose membrane and gel in the middle. To keep track of the sandwich orientation, make sure the gel is on the black side of the gel holder cassette and the transfer membrane is towards the clear side of the cassette.

11. Place the cassette into the transfer apparatus so that the black side of the cassette is oriented towards the black (negative) side of the electrode assembly. Fill the rest of the chamber with transfer buffer.

12. Connect the electrodes and transfer at 80V for an hour in the cold room. Set the stir bar to the slowest possible setting without stalling.
13. Remove the nitrocellulose membrane and stain with Ponceau S stain (page 95) for 5 minutes.

14. Destain with dH\textsubscript{2}O for 5 minutes.

15. With a clean razor blade, cut the nitrocellulose membrane down to the area that contains the ladder or where it is stained. The cut-down membrane should fit in the small clear plastic boxes (approximately 7 cm by 5 cm) for washing.

16. Wash in 1X TBST (page 97) for 2 x 10 minutes.

17. To make Blocking Buffer, add 5% dry milk to 1X TBST (2.5 g for 50 ml). Vacuum filter the blocking buffer using Whatman paper to remove large particles.

18. Block for 1-2 hours at room temperature.

19. Dilute primary antibody in 5 ml 1X TBST with 1% dry milk (4 ml TBST and 1 ml Blocking Buffer) or thaw a used primary antibody solution. Immerse the membrane in antibody solution, shaking overnight at 4°C. The primary antibody solution can be collected and stored at -20°C to be used up to three times.

20. Wash in 1X TBST for 2 x 10 minutes.

21. Dilute the secondary antibody 1:5,000 in 1X TBST with 1% dry milk. Incubate the membrane for 1-2 hours at room temperature in secondary antibody solution.

22. Wash in 1X TBST for 2 x 10 minutes.

23. Develop the membrane using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA) according to the manufacturer’s instructions. Specifically, mix the kit solutions 1:1, making 4ml total volume per membrane. Drain the membrane using a paper towel, lay it on top of a sheet of saran wrap (protein side up), and pipet the mixed solution onto the membrane. Incubate for 5 minutes, and then drain off the substrate.

24. Wrap the membrane using saran wrap and expose to film (Denville Scientific, Metuchen, NJ) in a dark room for 30 seconds. The exposure time can be adjusted depending on the intensity of the signal. Develop the film using a Kodak X-OMAT 2000A processor (Eastman Kodak, Rochester, NY).

**In situ Hybridization**

**Preparation of torula RNA to be used in hybridization buffer**

A phenol-chloroform extraction was performed on crude yeast RNA to remove any possible contaminants. This RNA is then used in the Hybridization buffer (page 91) for the *in situ* experiment described below.
1. Combine 1 g crude yeast Torula RNA (Sigma, St. Louis, MO, catalog number R-6625) and 10 ml DEPC treated H$_2$O (page 90) into a 50 ml conical screw cap tube. Heat at 56°C for 20 minutes while rocking - there will be a lot of solid, insoluble material left in the water.

2. Centrifuge at the highest speed setting (3350 rpm, 1750 g on model CL, International Equipment Co., Needham Heights, MA) at 4°C for 5 minutes to pellet the insoluble material. Transfer the supernatant to a new conical tube.

3. Add 8 ml pure phenol (stored at 4°C), 1960 µl chloroform, and 40 µl isoamyl alcohol. Shake well and then centrifuge at maximum speed for 10 minutes.

4. Recover the aqueous layer into another conical tube. Extract again with an equal volume of chloroform/isoamyl alcohol (24:1 ratio). Mix well and centrifuge for 10 minutes at maximum speed.

5. Recover the aqueous layer into another conical tube. For every 1 ml of liquid recovered, add 100 µl NaOAc (3M stock at pH 5.2 in DEPC H$_2$O) and 2.75 ml ice-cold 100% ethanol. The RNA should immediately start precipitating.

6. Complete the RNA precipitation by cooling at -20°C overnight or -80°C for 1 hour.

7. Centrifuge sample at maximum speed for 15 minutes at 4°C.

8. Remove the supernatant. Wash the pellet with ice-cold 70% ethanol in DEPC H$_2$O. Spin again for 5 minutes at 4°C.

9. Remove the supernatant. Dry the pellet in the tube.

10. Resuspend the pellet in 5 ml DEPC H$_2$O.

11. Quantitate the RNA sample and dilute to a final concentration of 40 mg/ml using DEPC H$_2$O. Aliquot 1.25 ml per tube (enough for 50 ml Hybridization buffer) and store at -80°C.

**Preparation and quantification of DIG-labeled RNA probes**

RNA probes are labeled with digoxigenin (DIG) for later detection. It is important that gloves are worn and aerosol-free tips are used, in order to prevent RNase contamination.

1. PCR amplify the DNA fragment to be used as the template for the later RNA transcription reaction. Use SP6, T3, or T7 primers as required.

**PCR program for making DIG-labeled probes**

1: 94°C for 1 minute  
2: 94°C for 1 minute
2. Set up the DIG-labeling reactions for each primer used as shown below, to make a total of two 20µl reactions.

<table>
<thead>
<tr>
<th></th>
<th>SP6</th>
<th>T3</th>
<th>T7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambion RNA polymerase (20U/µl, primer specific, Ambion, Grand Island, NY)</td>
<td>2µl</td>
<td>2µl</td>
<td>1µl</td>
</tr>
<tr>
<td>10X transcription buffer (primer specific, Ambion, Grand Island, NY)</td>
<td>2µl</td>
<td>2µl</td>
<td>2µl</td>
</tr>
<tr>
<td>DIG-labeling mix (Roche Diagnostics, Indianapolis, IN)</td>
<td>2µl</td>
<td>2µl</td>
<td>2µl</td>
</tr>
<tr>
<td>PCR reaction from step 1</td>
<td>7µl</td>
<td>7µl</td>
<td>7µl</td>
</tr>
<tr>
<td>RNase-free H2O</td>
<td>6.5µl</td>
<td>6.5µl</td>
<td>7.5µl</td>
</tr>
<tr>
<td>RNase inhibitor (Ambion, Grand Island, NY)</td>
<td>0.5µl</td>
<td>0.5µl</td>
<td>0.5µl</td>
</tr>
</tbody>
</table>

3. Incubate the reactions at 37°C for 90 minutes.

4. Use Nuc-Away columns (Ambion, Grand Island, NY) to purify the RNA reactions immediately after the reactions are complete, using the steps described in the Nuc-Away column instruction manual.

(a) Settle the dry gel into the bottom of the column by tapping it on the bench. Hydrate the column with 650 µl nuclease-free H2O, replace cap, and vortex. Let sit at room temperature for at least 5 minutes.

(b) Confirm that all of the dry gel is hydrated by visual inspection. There should be a uniform-appearing gray color to the contents of the column. Put the gel column in a collection tube and spin at 750g for 2 minutes at room temperature.

(c) Remove the H2O from the collection tube, then spin for 1 minute at room temperature.

(d) Apply the results of the DIG-labeling reaction to the top of the column, centering the liquid as much as possible. Put the column in an elution tube and spin at 750g for 2 minutes.

(e) An optional step to verify the result of the transcription reaction is to run 1 µl of the purified product on a RNA gel.

5. For quantification of the transcription results, a dot blot is performed to quantify RNA. First, use DEPC treated H2O to make 10-fold serial dilutions of labeled control RNA (Roche Diagnostics, Indianapolis, IN) and the transcribed DIG-labeled probes. Essentially, add 1 µl RNA to 9 µl DEPC H2O to make the 1:10 dilution, and then make a 1:100 dilution using the 1:10 dilution and DEPC H2O for each transcribed RNA sample.

6. On a positively charged nylon membrane, spot 1 µl of each sample dilution. Label the samples on the membrane using a Skilcraft government pen.
7. Fix RNA to the membrane using a Spectrolinker UV Crosslinker (model XL-1500, Spectronics, Westbury, NY) using the “Optimal Crosslink” button.

8. Wet the membrane with MAB (page 92).

9. Incubate the membrane in 5 ml Northern blocking solution (add 3 ml 10% BMB stock in -20°C freezer, to 12 ml MAB) for 5 minutes at room temperature.

10. Dilute 2 µl anti-DIG-AP antibody (Roche Diagnostics, Indianapolis, IN) in 10 ml Northern blocking solution. Incubate the membrane in anti-DIG-AP for 5 minutes at room temperature.

11. Wash the membrane twice for 5 minutes each in MAB at room temperature.

12. Make AP buffer (5 ml 1 M Tris pH 9.5, 2.5 ml 1 M MgCl₂, 1 ml 5 M NaCl, bring up to 50 ml with DEPC H₂O). Incubate the membrane in 10 ml AP buffer for 2 minutes at room temperature.

13. Make color substrate solution by adding 33.75 µl NBT (100 mg/ml stock) and 35 µl BCIP (50 mg/ml stock) to 10 ml AP buffer. Incubate the membrane in color substrate solution in the dark for 1 hour, checking every 15 minutes for color development.

14. To stop the reaction, wash with AP buffer for 5 minutes at room temperature.

15. Wash the membrane twice for 5 minutes each in MAB and then let the membrane dry on a paper towel.

16. Observe the developed color on the membrane. Ideally, the spots corresponding to the probe RNA should develop exactly as dark as the control RNA.

**In situ hybridization**

1. For corneal tissues to be stained using *in situ* hybridization, fix in MEMFA (see page 93) within the 24-well plate for 30 minutes. Samples can then be washed with 100% methanol twice for 10 minutes each and stored at -20°C until use.

2. Hydrate each well by washing at room temperature in each solution below for 5 minutes each, making sure to keep the fixed tissue wet at all times, using 2 ml per well:
   
   (a) 100% methanol
   (b) 75% methanol and 25% PTw (see page 96)
   (c) 50% methanol and 50% PTw
   (d) 25% methanol and 75% PTw
   (e) 3 washes of 100% PTw

3. Make Proteinase K solution by adding 25 µl Proteinase K (10 mg/ml stock) to 25 ml PTw. Wash in Proteinase K solution for 5 minutes at room temperature.
4. Make 2 mg/ml glycine solution by adding 0.05g glycine to 25 ml PTw. Wash in glycine solution for 5 minutes at room temperature.

5. Wash twice in PTw for 5 minutes each.

6. Thaw 20% paraformaldehyde stock (see page 95) from -20°C freezer. Add 5 ml 20% paraformaldehyde to 15 ml PTw to make 4% paraformaldehyde solution. Refix in paraformaldehyde for 20 minutes.

7. Wash twice in PTw for 5 minutes each at room temperature, using 2 ml per well.

8. Remove half of PTw and add 250 µl hybridization buffer (page 91) to each well.

9. Replace all of the solution in each well with 500 µl hybridization buffer. Place on rocker in oven set to the slowest speed at 60°C for 10 minutes.

10. Incubate with fresh hybridization buffer (500 µl per well) at 60°C for a minimum of 2 hours while rocking.

11. Make probe solution by adding 5 µl DIG-labeled probe to 500 µl hybridization solution for each well. Incubate probe solution for 30 minutes at 60°C.

12. Replace the hybridization buffer in the wells with probe solution (500 µl per well), and rock overnight at 60°C.

13. In the morning, wash with hybridization buffer for 10 minutes at 60°C.

14. Make 2X SSC + 0.3% CHAPS solution: add 0.15 g CHAPS (Sigma-Aldrich, St. Louis, MO) to 5 ml 20X SSC (page 97) in a 50 ml conical. Fill to 50 ml with DEPC H₂O.

15. Wash with 50% hybridization buffer and 50% 2X SSC + 0.3% CHAPS at 60°C for 10 minutes while rocking, using approximately 500 µl solution per well.

16. Wash with 25% hybridization buffer and 75% 2X SSC + 0.3% CHAPS at 60°C for 10 minutes while rocking, using approximately 500 µl solution per well.

17. Decrease the oven temperature to 37°C. Wash twice in 2X SSC + 0.3% CHAPS for 20 minutes each at 37°C, using approximately 1 ml per well.

18. Make RNase solution by adding 40 µl RNase A (Roche Diagnostics, Indianapolis, IN) and 20 µl RNase T (Sigma-Aldrich, St. Louis, MO) to 20 ml 2X SSC + 0.3% CHAPS. Treat wells with RNase solution for 30 minutes at 37°C.

19. Make 2X SSC by diluting 20X SSC with DEPC H₂O. Wash twice in 2X SSC at room temperature for 10 minutes each.

20. Wash twice in 0.2X SSC for 30 minutes each at room temperature.

21. Wash twice in MAB (page 92) for 10-15 minutes each.

22. Add 3 ml BMB (10% stock in -20°C freezer) to 12 ml MAB to make 2% BMB buffer. Wash in this buffer for 60 minutes at room temperature.
23. Combine 9 ml MAB, 3 ml BMB, and 3 ml lamb serum (Gibco, Grand Island, NY). Wash in this solution for 60 minutes at room temperature, using 0.5 ml per well.

24. Add anti-DIG antibody (Roche Diagnostics, Indianapolis, IN) to the solution above (with MAB, BMB, and lamb serum) at a 1:2000 dilution. Fill each well with 0.5 ml of antibody solution and shake overnight at 4°C.

25. Wash five times for 60 minutes each in MAB.

26. Wash twice in Alkaline Phosphatase Buffer (5 ml 1 M Tris pH 9.5, 2.5 ml 1 M MgCl₂, 1 ml 5 M NaCl, 50 µl Tween 20, 0.06 g lavamisol (Sigma-Aldrich, St. Louis, MO), fill to 50 ml with DEPC H₂O) for 5 minutes each at room temperature.

27. Add 500µl BM Purple (Roche, Indianapolis, IN) per well and develop in the dark at room temperature for a few hours. It is important to avoid any light exposure to the solution. If there is no signal after a few hours, develop in the dark overnight at 4°C. The signal may take a few days to develop, in which case BM Purple should be replaced daily.

28. Wash in Alkaline Phosphatase Buffer for 10 minutes at room temperature to stop the reaction.

29. Wash twice in 1X PBS for 5 minutes each at room temperature.

30. Refix in 4% paraformaldehyde for a few hours.

31. Wash twice in 1X PBS for 5 minutes each.

32. Add 80% glycerol / 20% PBS solution and store refixed samples at 4°C. Make sure the plates are sealed well with parafilm to avoid desiccation.

Solutions and recipes

DEPC (diethylpyrocarbonate) treated H₂O

Add 1ml DEPC (Sigma-Aldrich, St. Louis, MO) to 1 L dH₂O in chemical hood. Vigorously shake the bottle and vent twice. Keep the bottle slightly open in the chemical hood overnight, and then autoclave. Note that DEPC is dangerous!

0.1% gelatin

Use a clean, detergent-free bottle to make this solution.

0.1 g Knox unflavored gelatine (Kraft Foods, Northfield, IL)
100 ml H₂O
Autoclave to sterilize. Note that the gelatin should dissolve in the H$_2$O in the autoclave.

**Hybridization buffer (100 ml)**

- 50 ml 50% deionized formamide (stock in -20°C freezer)
- 25 ml 20X SSC (see page 97)
- 2.5 ml Torula RNA (see page 85)
- 2 ml 5 mg/ml Heparin (stock stored in -20°C freezer)
- 2 ml 50X Denhardt’s solution (stock stored in -20°C freezer)
- 100 µl Tween 20
- 0.1 g CHAPS
- 1 ml 0.5M EDTA

Fill to 100 ml with DEPC H$_2$O.

**67% L-15 medium (250 ml)**

- 2.28 g L-15 powder (Invitrogen, Grand Island, NY, catalog #41300-021)
- 2.5 ml Pen-Strep (10,000 U/ml Penicillin and 10 mg/ml Streptomycin stock; Mediatech, Herndon, VA, catalog #30002CI)

Fill to 250 mL with 18 MΩ deionized, tissue culture grade H$_2$O

Adjust pH to 7.6, filter sterilize using a 500 ml Nalgene vacuum filter (Thermo Scientific, Waltham, MA, catalog #291-4520) in biological hood, and store at 4°C.

**LB medium**

- 25 g Miller LB powder (Fisher Scientific, Pittsburgh, PA)

Dissolve in 1 liter H$_2$O

Aliquot into 250 ml bottles. Autoclave on same day.
LB ampicillin plates (makes one sleeve of plates)

12.5 g Miller LB powder (Fisher Scientific, Pittsburgh, PA)
7.5 g Bacto agar (Fisher Scientific, Pittsburgh, PA)

Dissolve LB in 500 ml H$_2$O

Autoclave the LB/agar broth immediately after mixing, keeping the stir bar in the bottle. After autoclaving, let bottle cool down to approximately 55°C while stirring slowly. Add 500 µl 100 mg/ml ampicillin to make a final concentration of 100 µg/ml ampicillin. Quickly pour into medium sized (100 mm) petri dishes, completely covering the bottom of each dish (approximately 1/4 full) while trying to avoid producing air bubbles.

Lysis buffer

Make this solution fresh for each use. Final concentrations of each ingredient are listed below.

1X protease inhibitor (cOmplete, Mini, EDTA-free, Roche, Indianapolis, IN; catalog #11836170001; make the 10X stock according to instructions and store at -80°C)
1X phosphatase inhibitor (PhosSTOP, Roche, Indianapolis, IN; make 10X stock according to instructions and store at -20°C)
1 mM EDTA (dilute from 0.5 M EDTA stock)
1X PBS (dilute from 20X stock; see page 95)
1% NP-40
Fill with H$_2$O

MAB (Maleate buffer, 1 L)

11.61 g maleic acid
8.76 g NaCl

First, fill a 1L bottle to approximately 800 ml with H$_2$O and stir to dissolve ingredients. Adjust the pH to 7.5. This pH adjustment will require a lot of NaOH: drop NaOH
pellets into the solution until the pH gets close to 7, and then fine tune using 10N NaOH. Fill to 1L with H₂O, treat with DEPC, and autoclave.

MEMFA (50 ml)

This solution is only effective for one week after being made.

- 5 ml 1 M MOPS pH 7.4
- 1 ml 100 mM EGTA pH 8.0
- 500 µl 100 mM MgSO₄
- 5 ml 37 % formaldehyde
- 38.5 ml DEPC H₂O

10X MMR (Marc’s Modified Ringers, 1 liter)

This recipe is from Sive et al. (2000). Use a detergent-free bottle when making this solution.

- 58.44 g NaCl
- 1.4912 g KCl
- 2.033 g MgCl₂•6H₂O (to make 10 mM)
- 2.940 g CaCl₂•2H₂O (to make 20 mM)
- 11.925 g HEPES (to make 50 mM, Sigma-Aldrich, St. Louis, MO)

Fill to 1 liter with H₂O, adjust pH to 7.5 and autoclave. Dilute when needed with autoclaved H₂O in a clean, detergent-free bottle to make 1X MMR and 0.1X MMR.

modified L-15 medium (250 ml)

This recipe was adapted from Kay and Peng (1991).

- 2.09 g L-15 powder (Invitrogen, Grand Island, NY, catalog #41300-021)
- 25 ml fetal bovine serum (Fisher Scientific, Pittsburg, PA)
- 2.5 ml penicillin-streptomycin stock (10 000 U/ml Penicillin and 10 000 µg/ml Streptomycin, Mediatech, Herndon, VA; catalog #30-002-CI)

Fill to 250 ml with 18 MΩ deionized culture grade water
Adjust pH to 7.6, filter purified using a 500 ml Nalgene vacuum filter (Thermo Scientific, Waltham, MA, catalog #291-4520) in the biological hood, and store at 4°C.

10X NAM (Normal Amphibian Media) salts

This recipe is presented in Sive et al. (2000), based on the NAM formulation described in Slack and Forman (1980).

65 g NaCl
1.5 g KCl
2.4 g Ca(NO$_3$)$_2$$\cdot$4H$_2$O
0.37 g Na$_2$EDTA$\cdot$2H$_2$O

Fill to 1 liter and autoclave sterile.

1/20X NAM (1 liter)

Make sure the bottle used is detergent-free. Shake vigorously before use to introduce O$_2$ into solution.

5 ml 10X NAM from above
20 ml 0.1 M NaPO$_4$ pH 7.5
500 µl 0.1 M NaHCO$_3$ (aliquots are stored at -20°C)

Fill to 1 liter with autoclaved H$_2$O and shake to aerate the solution.

2X NPB (Nuclear Preparation Buffer, 30 ml)

This recipe is from Sive et al. (2000). All reagents should be sterilized before use.

10 ml 1.5 M sucrose
0.9 ml 1 M HEPES pH 7.7
0.120 ml 0.5 M EDTA pH 8.0
3.0 mL 10 mM spermidine (Sigma-Aldrich, St. Louis, MO, catalog #S2501)
1.2 mL 10 mM spermine (Sigma-Aldrich, St. Louis, MO, catalog #S1141)
0.608 mL 100 mM DTT
Fill to 30 mL with sterile H₂O.

20% paraformaldehyde (20 ml)

In a 50 ml conical tube, add 4 g paraformaldehyde (Fisher Scientific, Pittsburg, PA) and 20 ml DEPC H₂O (see page 90). Add 2.5 µl 10N NaOH. Make sure the cap on the conical tube is tight. Rock in a 60°C oven until solution is clear. Store at -20°C.

10X PBS (DEPC treated, 1 liter)

80 g NaCl
2 g KCl
14.4 g Na₂HPO₄
4.8 g KH₂PO₄

Fill to 1 liter with H₂O, and adjust pH to 7.4. Add 1 ml DEPC in chemical hood and vigorously shake to mix. Let vent in chemical hood overnight and autoclave.

20X PBS (1 liter)

160 g NaCl
4 g KCl
28.8 g Na₂HPO₄ (to make 0.2 M)
4.8 g KH₂PO₄ (to make 40 mM)

Fill to 1 liter with H₂O, and adjust the pH so it is 7.4 when diluted 1:20. The pH of the 20X solution may not measure at 7.4 due to extreme concentration. Autoclave. To make 1X PBS, add 50 ml of 20X PBS to 950 ml H₂O.

Ponceau S stain

0.25 g Ponceau S (Matheson, Coleman & Bell, East Rutherford, NJ)
12.5 ml glacial acetic acid
Fill to 250 ml with dH₂O
2X protein sample buffer (50 ml)

23.5 ml deionized water
6.25 ml 0.5 M Tris-HCl pH 6.8
12.5 ml glycerol
5 ml 20% SDS
250 µl 2% bromophenol blue (catalog # B-5525, Sigma, St. Louis, MO)

Add β-mercaptoethanol 1:20 to an aliquot immediately prior to use. Store at -20°C once the β-mercaptoethanol is added.

PTw (500 ml, for in situ hybridization)

450 ml DEPC H2O (see page 90)
50 ml DEPC treated 10X PBS (see page 95)
500 µl Tween 20

RF1 solution (500 ml)

6 g RbCl (Sigma-Aldrich, St. Louis, MO)
5 g MnCl2•4H2O
15 ml 1 M potassium acetate
0.75 g CaCl2•2H2O
75 ml glycerol
Fill to 500 ml with H2O

Adjust pH to 5.8 with 0.2 M acetic acid.

RF2 solution (100 ml)

2 ml 0.5 M MOPS
0.12 g RbCl2
1.1 g CaCl2•2H2O
15 ml glycerol
Fill to 100 ml with H₂O

Adjust pH to 6.8 with NaOH.

1X SDB (sperm nuclei dilution buffer, 20 ml)

2.5 ml 2 M sucrose
750 µl 2 M KCl
20 µl 0.5 M spermidine (catalog # S2501, Sigma-Aldrich, St. Louis, MO)
8 µl 0.5 M spermine (catalog #S1141, Sigma-Aldrich, St. Louis, MO)
16.7 ml H₂O

Add 20 µl 0.1 N NaOH, and confirm that the pH is between 7.3 and 7.5 using pH paper. Filter sterile, split into 500 µl aliquots, and store at -20°C.

20X SSC (1 liter)

175.3 g NaCl
88.2 g sodium citrate
Fill to 1 liter with H₂O

Adjust pH to 7.0 with HCl. Treat with DEPC in the chemical hood and autoclave.

1X TBST (1 liter)

10 ml 1 M Tris pH 7.5
20 ml 5 M NaCl
500 µL 100% Tween 20
Fill to 1 liter with H₂O

1X Tris-SDS-Glycine buffer (1 liter)

25 ml 1 M Tris pH 9.5
14.42 g glycine
5 ml 20% SDS
Fill to 1 liter with H₂O
10X Western blot incomplete transfer buffer (1 liter)

30.3 g Tris base (Fisher Scientific, Pittsburg, PA)
144.2 g glycine
Dissolve in 1 liter of H$_2$O

1X Western blot transfer buffer (1 liter)

100 ml 10X Western blot incomplete transfer buffer from above
700 ml H$_2$O
200 ml methanol
Store at 4°C

It is important to add the H$_2$O before adding methanol to prevent precipitate formation.

Primers

RT-PCR verification of $FGF$ and $FGFR$ expression

Oligonucleotides were used for assaying $FGF$ and $FGFR$ expression. Primer sequences were designed from $X.~laevis$ $FGF$ and $FGFR$ sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/nucleotide/) where available. For $X.~laevis$ $FGFs$ not in this database, primers were designed from putative genes in the JGI $X.~tropicalis$ genome project database (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html; FGFs marked by *). All FGF primer pairs were designed to sandwich an intron sequence, aligning the $X.~laevis$ sequences with putative JGI genomic sequences to locate introns, in order to minimize the effects of DNA contamination. In all FGFRs, the amplified region was limited to the transmembrane domain to include both isoforms of each FGFR and to exclude the secreted forms of FGFRs (Hanneken et al., 1994; Groth and Lardelli, 2002). Salt adjusted melting temperature ($T_m$) values are noted for each primer.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5’ - 3’)</th>
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<td>R  CAAACCAGTTCATGTCTGG</td>
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Table 5.1 – continued on next page
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<th>Tm(°C)</th>
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<tr>
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<td>TTAAAATGAACCGCCGCTG</td>
<td>61.0</td>
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<tr>
<td>R</td>
<td>CGAGACTCCAGACAACATGG</td>
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<tr>
<td><em>FGFR2</em> F</td>
<td>TCTGCATGGTAGTGGTCTGC</td>
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</tr>
<tr>
<td>R</td>
<td>GATCCTCACGAGTGGAGTGGG</td>
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<td>R</td>
<td>GGTTGACCACAATAAGGACGG</td>
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<td><em>FGFR4</em> F</td>
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<tr>
<td>R</td>
<td>CAGTTTATGGACAGTTGGCC</td>
<td>59.2</td>
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</tbody>
</table>

**Table 5.1** – Oligonucleotides used for RT-PCR verification of *FGF* and *FGFR* expression.

**Manipulation of transgenesis construct**

**FGFR insertion**  Primers were constructed to insert dominant negative FGFR sequences into the CGxHG transgenesis vector adjacent to the hsp70 promoter at the 3’ end (Figure 3.1). The forward primers contain a SacI or StuI site (double underlined below), and the reverse primers contain an EcoRI, XbaI, or StuI site (underlined below). Reverse primers were designed so that expression of the amplified product would be limited to the extracellular domains and the transmembrane domain of each FGFR, effectively excluding the intracellular tyrosine kinase domains. Several bases (italicized below) were added to the 5’ end of each primer to facilitate restriction enzyme digestion.

- **dnFGFR1 Forward primer:** `tttt gagctc atggtctccgaaggtccctc`
- **dnFGFR1 Reverse primer:** `aaaaa gaattc cggtgtctcttatattaaagataatg`
- **dnFGFR2 Forward primer:** `tttt aggcct atgctgctactcgccgtacttg`
Figure 5.6 – primers for insertion into 5’ end of transgenes
Short sequences were added to the 5’ end of transgenes using primers. (A) A pair of primers constructed the SP6 promoter sequence and 5’ untranslated region of β-globin, flanked by AgeI and SacI restriction enzyme sites. (B) A pair of primers were used to form inserts containing the 5’ untranslated region of β-globin, the ATG start site, and an HA tag. These inserts were flanked by the AgeI and StuI restriction enzyme sites, or alternatively, AgeI and SacI restriction enzyme sites.

dnFGFR2 Reverse primer: aaaa tactaga ctatggagcggtattcgtttggtgag

dnFGFR3 Forward primer: tttt gagctc atggtctctgtgaatggtgtccc

dnFGFR3 Reverse primer: aaaa aggcct ctaggggaaccttgagacttttatgcacc

**SP6 and 5’ β-globin UTR** Primers were constructed to insert a SP6 promoter sequence (double underlined below) and the 5’ untranslated region of β-globin (Krieg and Melton, 1984; Figure 5.6A; underlined below). These sequences were flanked by the AgeI restriction enzyme site (italicized) and SacI site. Furthermore, the restriction sites were essentially pre-cut by not including the ends of the restriction sites, so that the annealed pair of primers can ligate directly with a double-digested plasmid. These primers were annealed using the thermal cycler, gradually decreasing the temperature so that the primers would align properly while annealing. On the thermal cycler, the SLOWCOOL program (95°C for 4 minutes, decrease 0.1°C per second until reaching 4°C, then wait at 4°C indefinitely) was entered for this purpose. Primer sequences were verified by sequencing after insertion into the transgenesis plasmid.

Forward primer: cccggt gatttaggtgacactatagaa

tgtttcggcagaagctcagaataaaacgctcaacctttgcc gagct

Reverse primer: c gcacaaagttgacgctttatctgaagcctactgcaacaaacaagaac

ttcagtaggtgcagctttaatc a
HA tags were inserted onto the C-terminal end of XFD (dominant negative FGFR1; Chapter 3). A pair of primers were used to form a short insert containing three copies of DNA encoding the HA tag, flanked by EcoRI and StuI restriction enzyme sites.

**5’ β-globin UTR and N-terminal HA tag** Two different pairs of primers were constructed to generate plasmids for expressing proteins with a N-terminal HA tag (Figure 5.6B). In each case, the AgeI site is upstream (acccgt; italicized below), followed by the 5’ β-globin untranslated region (underlined below), a start codon (atg), an HA tag (encoding YPYDVPDYA; double underlined below), and then a second restriction enzyme site (italicized and underlined below). One pair has a StuI site (agccct) for the downstream cut site and the other pair of primers has a SacI site (gagctc). Each pair was then annealed using the SLOWCOOL program as described above. The annealed primer pairs were then double digested with their corresponding restriction enzymes, and inserted into the transgenesis plasmid. Sequencing was used to verify proper annealing and primer sequence.

Forward (StuI version): \texttt{tagg accggt ttgttctttttgcagaagctcagaataaacgctcaactttgg}
\texttt{atg tacccttagcttcthtaaccgattatgcccatatggcc agccct tcg}
Reverse (StuI version): \texttt{cga agccct ggcccataatgggtacatctgtaagggta cat ccaaaagttgacgctttctatgtacaaagctcaacttttg}
\texttt{tagt tagg atgtccttcttttgcagaagctcagaataaacgctcaacttttg}
\texttt{atg tacccttagctttagcataaccgattatgcccatatggcc gagctc tcg}
\texttt{cga gagctc ggcccataatgggtacatctgtaagggta cat ccaaaagttgacgctttctatgtacaaagctcaacttttg}
\texttt{tagt tagg atgtccttcttttgcagaagctcagaataaacgctcaacttttg}
\texttt{atg tacccttagctttagcataaccgattatgcccatatggcc gagctc tcg}
\texttt{cga gagctc ggcccataatgggtacatctgtaagggta cat ccaaaagttgacgctttctatgtacaaagctcaacttttg}

**C-terminal HA tag (3 copies)** Insertion of three copies of the HA tag was accomplished with a pair of complementary primers (Figure 5.7). Three slightly different codon sequences (separated by spaces below) encoding the protein sequence YPYDVPDYA were sandwiched between the EcoRI (underlined below) and StuI (italicized below) pre-cut

\texttt{C-terminal HA tag (3 copies)} Insertion of three copies of the HA tag was accomplished with a pair of complementary primers (Figure 5.7). Three slightly different codon sequences (separated by spaces below) encoding the protein sequence YPYDVPDYA were sandwiched between the EcoRI (underlined below) and StuI (italicized below) pre-cut
restriction sites. The primer pair was annealed using the SLOWCOOL program prior to ligation into the transgenesis plasmid. After ligation, the inserted primer sequence was verified by sequencing.

Forward primer: `aattc tacccttacgatgtaccgattatgcc
taccatacgtgctcagactacgt taccatatgtatgttcgactatatgca agg`

Reverse primer: `cct tgcatagttccgaacatcatatggata agcgtagtctggacgtatggtggtggacgtaatcgggtacatcggtaaggta g`
References


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Appendix: Published Papers


