AN APPROACH TO LENS REGENERATION IN MICE FOLLOWING LENTECTIONMY AND THE IMPLANTATION OF A BIODEGRADABLE HYDROGEL ENCAPSULATING IRIS PIGMENTED TISSUE IN COMBINATION WITH BASIC FIBROBLAST GROWTH FACTOR

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AN APPROACH TO LENS REGENERATION IN MICE FOLLOWING LENTECTOMY AND THE IMPLANTATION OF A BIODEGRADABLE HYDROGEL ENCAPSULATING IRIS PIGMENTED TISSUE IN COMBINATION WITH BASIC FIBROBLAST GROWTH FACTOR

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ABSTRACT

AN APPROACH TO LENS REGENERATION IN MICE FOLLOWING LENTECTOMY AND THE IMPLANTATION OF A BIODEGRADABLE HYDROGEL ENCAPSULATING IRIS PIGMENTED TISSUE IN COMBINATION WITH BASIC FIBROBLAST GROWTH FACTOR

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Organ or tissue regeneration is the process by which damaged or lost tissue parts or whole body organs are repaired or replaced. When compared to amphibians, mammals possess very limited regenerative capabilities. Mammals are capable of lens regeneration following lentectomy only if the lens capsule is left behind. Regeneration is achieved by the residual lens epithelial cells (LECs) adherent to the remaining lens capsule. Urodele amphibians, however, have been reported to regenerate their lenses, following whole organ removal, by the transdifferentiation of the pigmented epithelial cells (PECs) of the dorsal iris. These cells, namely PECs, have been shown to possess a potential for transdifferentiation in vitro as well as in vivo. In this study, the feasibility of coaxing iris PECs to regenerate a lens in vivo was tested by encapsulating an iris pigmented epithelial
tissue by a hydrogel bead combined with FGF and implanting the resulting matrix in lentectomized mice. This study also investigates the ability of aligned Poly-ε-caprolactone (PCL) nanofibers in inducing the differentiation of LECs and the subsequent alignment of lens fiber cells.

Keywords: Lens Regeneration, Iris pigmented epithelial cells, Lens epithelial cells, Transdifferentiation, Differentiation, Fibroblast growth factor (FGF), Hydrogel, Poly-ε-caprolactone (PCL), Nanofibers, Mouse.
Dedicated to my parents, without whom I would not be where I am today
I would like to thank my research advisor Dr. Tsonis without whose expertise and help I would not have been able to conduct and complete my research.

I would also like to thank the members of my committee: Dr. Robert Wilkens and Dr. Amit Singh to whom I owe appreciation for their continuance guidance throughout this journey.

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<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FGFr</td>
<td>Fibroblast Growth Factor Receptor</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>LECs</td>
<td>Lens Epithelial Cells</td>
</tr>
<tr>
<td>IPEc</td>
<td>Iris-Pigmented Epithelial cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Cell Growth Factor</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-Like Growth Factor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
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<tr>
<td>CTGF</td>
<td>Connective Tissue Growth Factor</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted Protein Acidic and Rich in Cysteine</td>
</tr>
<tr>
<td>IOL</td>
<td>Intraocular Lens</td>
</tr>
<tr>
<td>PCO</td>
<td>Posterior Capsular Opacification</td>
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<tr>
<td>α-SMA</td>
<td>Alpha-Smooth Muscle Actin</td>
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INTRODUCTION

Organ regeneration or repair is the ability to replace damaged or lost organ or tissue parts. This exceptional ability is intrinsic to certain organs, and may be totally absent in others [as reviewed in (Baddour, Sousounis et al. 2012)]. Lower vertebrates exhibit superior regenerative capabilities when compared to mammals, and organ regeneration as seen in amphibians has not been yet reported in humans. Today, transplantation and implantation have been the most commonly adopted means to alleviate humans from the life-threatening pains due to severely damaged organs.

One organ of particular importance is the ocular lens, a transparent tissue in the eye cup responsible for light transmission to the retina to enable vision (see Chapter I). The mechanisms of lens induction and development are conserved in the vertebrate eye, and key molecules and signaling pathways that play role in early lens morphogenesis have been identified (see Chapter I). As in other developmental systems, the lens is susceptible to pathological conditions. Aberrant growth and differentiation of epithelial cells can lead to cataract (see Chapter II), an eye disease that is a major cause of blindness worldwide (West 2007). The standard care for cataract over the past 30 years have consisted in surgically removing the cataractous lens fibers and leaving the lens capsule behind to host an intraocular lens (IOL) for vision restoration. Despite the advances in current surgical techniques and the design of novel IOLs, post-operative cataract surgery complications still occur (see Chapter II). Posterior capsular
opacification (PCO) remains a major complication of modern cataract surgery; a condition that often causes secondary loss of vision and calls for yet another surgical intervention to restore vision, a drawback that can be prevented if regeneration of the human lens was feasible. In that regard, studies that focus on the regeneration of the lens in higher vertebrates are of paramount importance for the treatment of diseases such as cataract. An overview of lens regeneration in various animal models such as the newt, frog, rabbit, mouse and rat is given in Chapter III. Today, lens regeneration has been extensively reported in amphibians, and more recently in mammals. In general, regeneration of the lens has been shown to follow one of two pathways: transdifferentiation of the iris pigmented epithelial cells (Call, Grogg et al. 2005; Tsonis 2006) or the inner corneal epithelium (Freeman 1963) as seen in newts and tadpole frogs, respectively, and proliferation and differentiation of residual lens epithelial cells as reported in New Zealand Albino rabbits (Stewart and Espinasse 1959; Stewart 1962; Pettit 1963; Agarwal, Angra et al. 1964; Gwon, Enomoto et al. 1989; Gwon, Gruber et al. 1993) and rodents, such as mice (Call, Grogg et al. 2004) and rats (Lois, Dawson et al. 2003; Huang and Xie 2010). In mammals, the regeneration of the lens is dependent on a number of conditions such as inflammation and lens capsule integrity (see Chapter IV). Also, the regenerated mammalian lenses have been studied for their DNA and RNA content, as well as their crystallin composition (see Chapter V).

In this study, a new approach for inducing lens regeneration in mice in the absence of a lens capsule was attempted. A biodegradable hydrogel of oligo(poly(ethylene glycol)fumarate) cross-linked with poly(ethylene glycol)-diacrylate (see Chapter VI) was used to deliver a growth factor in situ and to provide the structural
framework needed for the lens to regenerate in mice following lentectomy (the removal of the intact lens). The hydrogel was first combined with basic fibroblast growth factor (bFGF) and used to encapsulate an iris pigmented tissue prior to its implantation into lentectomized mice. After 50 days, the eyes were collected and prepared for histological studies to evaluate the ability of the animals for lens regeneration.

This study also assesses the proliferation and differentiation of mouse lens epithelial (MLE) cells in vitro. Lens epithelial cells were harvested from mouse lens capsule explants and grown in regular as well as serum-starved medium on aligned PCL nanofibers. The ability of aligned (PCL) nanofibers to induce the differentiation of MLE cells and the subsequent alignment of lens fibers was also evaluated.
LITERATURE REVIEW

A. An Overview of Organ Regeneration:

Organ or tissue regeneration is the process by which damaged or lost tissue parts or whole body organs are repaired or replaced. This distinctive regenerative ability varies from one class to another (i.e., amphibians versus mammals), and is intrinsic to certain organisms and not to others from the same class. Today, lower vertebrates have been reported to exhibit superior regenerative capabilities when compared to higher vertebrates. Among vertebrates, the zebrafish, for example, has the ability to regenerate its heart two months following the resection of 20% of its ventricular mass (Poss, Wilson et al. 2002). The axolotl, a neotenic salamander, is a pioneer in the field of limb regeneration due to its exceptional ability to regenerate, as an adult, an exact replica of the limb upon amputation at any level (Gardiner, Carlson et al. 1999; Stocum and Cameron 2011). Perhaps the most fascinating animal for regenerative studies is the newt, an aquatic amphibian of the Salamandridae family, that has been shown capable of regenerating whole body organs or body parts such as the limb, tail (including the spinal cord), heart, jaw, brain and ocular tissues (such as the lens and retina), even as adults (Brockes and Kumar 2005; Call and Tsonis 2005). Regeneration of the lens has also been reported in mammals, but only after the lens capsule is left behind following the extraction of the lens fibers. The lens is an avascular tissue that is separated from other ocular tissues by its own extracellular matrix, the lens capsule. Aqueous and vitreous
humors bathe the lens capsule from the anterior and posterior sides, respectively (Tholozan, Gribbon et al. 2007). Over the years, the eye, and in particular the lens has become a popular system for regeneration studies. First, the work of Hans Spemann in 1901 suggested that the optic vesicle is required for the development of the lens. Since then, many investigators have repeated Speemen’s original experiment with varying results. In some cases lenses would form after optic primordium ablation, but in other cases, they would not. These results suggested that different species vary with respect to the timing of lens induction (Servetnick, Cook et al. 1996) [as reviewed in(Lang 2004)]. Much of the work in the early nineteenth century focused on embryonic eye and lens induction. However, the identification of key regulatory molecules that mediate eye and lens development and growth (Del Rio-Tsonis and Tsonis 2003) was enabled after the recent development of molecular technologies that provided great insight at the molecular level. These findings also provided the founding basis for lens regeneration studies.

The exceptional ability for lens regeneration as observed in lower vertebrates has not been yet reported in humans. However, when the underlying mechanisms of regeneration are unraveled, safe organ repair and regeneration can then be induced in humans. This holds tremendous therapeutic potential for the induction of lens regeneration in humans as a mean for treating cataract.
CHAPTER I
THE VERTEBRATE OCULAR LENS

The ocular lens of different species differ in the size and shape, as well as the arrangement of suture patterns (Land and Nilsson 2002) and the major crystallin proteins (Duncan, Cvekl et al. 2004) that composes it. For the purposes of this study, the vertebrate lens will be the main point of focus.

The vertebrate lens is a transparent, biconcave in shape, ocular tissue. Its transparency depends mainly on its hydration, the organization of its fibers, and the solubility of its proteins. The main function of the lens is to focus light passing from the cornea onto the retina which in turn, converts light into electrical signals that get transmitted by the optic nerve to the brain to enable vision. In the eyecup, the incoming light is converged onto the retina because of the higher refractive index of the lens compared with that of its surrounding aqueous and vitreous fluids [as reviewed in (La Croix 2008)].

A thick basement membrane, called the lens capsule, completely surrounds the lens cells which have been shown to be organized in an antero-posterior manner. In the embryo, the cells in the posterior half of the lens elongate and differentiate into lens fibers that make up the bulk of the lens, and the cells in the anterior half of the lens vesicle proliferate to form a monolayer of cuboidal epithelial cells covering the anterior
surface of the lens fibers. This distinct distribution pattern accounts for lens polarity and transparency (Stocum and Cameron 2011).

A. Embryological Origin of the Vertebrate Lens and Its Development:

Embryological manipulation (i.e., Spemann optic rudiment ablation experiments, optic vesicle transplantations) as well advances in genetic techniques and the use of lineage tracers have enabled the design of experiments that can further decipher the mechanisms underlying lens induction and development (Lang 2004).

The embryonic development of the lens and its postnatal growth are multi-step processes that have been studied by many researchers over the years. The group of Robert Grainger has defined four stages by which the lens develops: the first, they named, a period of lens-forming competence ((Servetnick and Grainger 1991), the second they attributed to the gaining of the head ectoderm of a “lens-forming bias” during neurulation (Grainger, Mannion et al. 1997), and the third and fourth stages were characterized by the acquisition of the cells of fate specificity at the end of neurulation and differentiation, respectively (Grainger 1992).

Briefly, in vertebrates, the lens originates from a tissue layer called the head surface ectoderm. The first step in the induction of the lens vesicle is the evagination of the optic vesicle that comes in close contact with the presumptive head ectoderm which, in addition to signals from the retina primordium, thickens into a structure called the lens placode. The optic vesicle and presumptive lens ectoderm do not come in complete contact with each other, but rather maintain a narrow gap across which the basal surfaces of the cells face each other, and in which a fibrillar extracellular matrix (ECM) builds up and forms the basis for the strong adhesion between the two tissues (Wakely 1977;
This strong interaction is what is thought to mediate the invagination of the lens placode and the formation of the lens pit and the optic cup. The lens pit then detaches and forms a hollow vesicle called the lens vesicle which is completely surrounded by the lens capsule, a basement membrane with various roles will be discusses in Chapter IV.

Embryological lens development is then carried out by the proliferation and differentiation of the lens epithelial cells that got trapped on the interior surface of the lens vesicle with the closure of the lens pit. The lens cells, previously at the center of the lens placode, now reside in the posterior half of the lens vesicle. These cells, in response to signals from the neural retina, start to elongate along the equatorial zone of the lens, synthesize crystallin and extrude their nuclei as they differentiate into primary lens fibers that eventually fill the lumen of the vesicle. As for the peripheral cells of the lens placode, they form the lens epithelium, a layer of epithelial cells that cover the anterior half of the lens vesicle. These cells continue to proliferate along the anterior capsule and, as they pass through the equatorial region, withdraw from the cell cycle and begin to differentiate into secondary lens fibers that get compressed around the primary lens fibers. After birth, lens fibers continue to be laid down throughout life, adding layers of fibers around the bulk of the lens (Gwon 2006). Both primary and secondary fiber cells undergo a series of characteristic changes, among the first of which is permanent withdrawal from the cell cycle followed by morphological elongation and eventual degeneration of all intracellular organelles, and all changes of which are orchestrated by changes in gene expression pattern. Lens fiber cell differentiation is also characterized by the appearance, or the massive increase in abundance, of the specific crystallin proteins
\(\beta, \gamma\)-and \(\alpha\)-crystallins [as reviewed in (Robinson 2006)]. In mammals, the expression of \(\beta\)- and \(\gamma\)-crystallins are generally associated with the differentiation of the lens fibers (Wang, Garcia et al. 2004).

The lens is surrounded by the aqueous and vitreous humors; the aqueous humor bathes the anterior lens capsule and separates the lens from the cornea, whereas the posterior side of the lens is exposed to the vitreous humor that fills the chamber between the lens and the retina. As previously noted, the distinct division in the lens of lens epithelial cells at the anterior surface and lens fibers at the posterior side is what accounts for lens polarity, a feature that has been shown to be highly dependent on the position of the lens in the eye. In an experiment by Coulombre and Coulombre, the lens of a chick embryo (5 days of age) was surgically removed, reversed, and inserted back in the eye. As a result, Coulombre and Coulombre found that the lens polarity was also reversed as evidenced by the new layer of epithelial cells that covered the anterior capsule (formally the posterior capsule) and the differentiation of the epithelial cells on the posterior (previously the anterior) side of the lens capsule. This elegant experiment clearly demonstrate the important role of the ocular environment in determining the lens polarity, as well as modulating the growth of the lens (Coulombre and Coulombre 1963). This experiment was later reproduced in mice by Yamamoto in 1976. Similar results were observed where mouse lenses were removed then implanted with the anterior side now facing the retina and the posterior capsule facing the cornea. Yamamoto went further by implanting lenses from 6- to 8-day old mice in adult (2 months of age) lentectomized mice. The implanted lenses continued to grow, always showing transparency and a well-established connection with the ciliary body of the recipient mouse (Yamamoto 1976).
Nevertheless, these results were not observed in recipient mice that lacked a retina, an observation that is somewhat suspected in the view of the important role of the retina in the induction of lens and the differentiation of the lens fiber cells (Bosco 1988; Bosco, Venturini et al. 1997; Arresta, Bernardini et al. 2005). Interestingly, not only does the ocular environment influence the development of the lens, but also the lens the growth of neighboring ocular tissues. For one, the lens has been shown to indirectly affect the shape of the eye by affecting the accumulation of the vitreous humor as demonstrated by an experiment by Coulombre and Coulombre in which the absence of the lens resulted in the failure to accumulate the vitreous humor, thus, resulting in smaller eyes (Coulombre and Coulombre 1964).

B. Signaling Pathways/Molecules Required/Involved in the Induction and Development of the Vertebrate Lens:

The major genetic signaling pathways and molecules that play role in early lens morphogenesis have been shown to be highly conserved in the vertebrate eye (Grogg, Call et al. 2006; Makarev, Call et al. 2007). These include the gene Pax6 (Del Rio-Tsonis, Washabaugh et al. 1995; Tsonis and Fuentes 2006), the three signaling pathways FGF (Robinson 2006), Wnts (Stump, Ang et al. 2003; Smith, Miller et al. 2005) and Hedgehog (Macdonald, Barth et al. 1995; Tsonis, Vergara et al. 2004), BMPs (Furuta and Hogan 1998; Wawersik, Purcell et al. 1999), and the transcription factors FoxE3 (Blixt, Mahlapuu et al. 2000), Sox1 (Kamachi, Uchikawa et al. 1998; Nishiguchi, Wood et al. 1998), c-Maf (Kawauchi, Takahashi et al. 1999; Kim, Li et al. 1999; Ring, Cordes et al. 2000), Sox2(Kelberman, de Castro et al. 2008), Six3(Lengler, Krausz et al. 2001; Carl, Loosli et al. 2002), and Prox1 (Wigle, Chowdhury et al. 1999). Briefly, Six3 (Lengler,
Krausz et al. 2001; Carl, Loosli et al. 2002) is expressed in the lens and neural retina and play important roles in regulating ocular development (Goudreau, Petrou et al. 2002). FoxE3 regulates the proliferation and survival of the lens epithelial cells (Blixt, Mahlapuu et al. 2000), whereas Prox1 have been found to regulate the expression of \( p57^{kip2} \) (Wigle, Chowdhury et al. 1999), a cell cycle regulator essential for mediating the cell cycle exit – by inhibiting the activity of cyclin-dependent kinases (Zhang, Wong et al. 1998) – and the differentiation of proliferating lens epithelial cells into fibers at the equatorial region of the lens. On the other hand, both the transcription factors c-Maf (Kawauchi, Takahashi et al. 1999; Kim, Li et al. 1999; Ring, Cordes et al. 2000) and Sox1 (Kamachi, Uchikawa et al. 1998; Nishiguchi, Wood et al. 1998) have been shown to up-regulate the expression of crystallin genes in the lens. As for the gene Pax6, the FGF signaling pathway, and the BMPs, they will be discussed in further detail in the sections to follow.

The Pax6 Gene

The Pax6 gene is both necessary and sufficient for the induction and development of the eye. Pax6 controls the transcription factor cascade involved in lens induction starting from the placodal stage (Lang 2004). Studies have shown that Pax6 is expressed in a number of eye tissues, two of which are the head surface ectoderm and the lens placode, both of which have been extensively studied for their role in early in early lens morphogenesis (Grindley, Davidson et al. 1995; Lang 2004). Loss- and gain-of-function, as well as misexpression experiments, helped identify the critical role of the Pax6 gene in eye induction and development. Eyes failed to develop in humans (Glaser, Walton et al.
1992; Jordan, Hanson et al. 1992), mice (Hill, Favor et al. 1991), and flies (Quiring, Walldorf et al. 1994) that carried mutations in the Pax6 homologue gene. Furthermore, misexpression of Pax6 in Xenopus (Chow, Altmann et al. 1999; Zuber, Gestri et al. 2003) and flies (Halder, Callaerts et al. 1995) led to the formation of an ectopic eye. The ability of the Pax6 gene to induce ectopic eye formation in both invertebrates and vertebrates is a testimony of its evolutionary conserved function (Callaerts, Halder et al. 1997; Lang 2004).

Pax6 is also a key regulator of lens induction and development. Studies have shown that failure to express Pax6 in the surface ectoderm (Fujiwara, Uchida et al. 1994), or the lens placode (Ashery-Padan, Marquardt et al. 2000) does not lead to the proper formation of the lens, whereas Pax6-induced expression in Xenopus laevis (Altmann, Chow et al. 1997; Chow, Altmann et al. 1999) results in ectopic lens formation. In mammals, the expression pattern of Pax6 in the eye and its role in the induction of the lens, have been studied in the mouse. In addition to regulating the early stages of lens induction in the mouse, Pax6 is also involved in the development of the lens through the activation of αB-crystallin (Piatigorsky 1998). Furthermore, two distinct phases of Pax6 expression have been detected in the presemptive lens ectoderm of the mouse embryo, with the second phase being dependent on the first (Grindley, Davidson et al. 1995).

The complex expression pattern of Pax6 suggests that this “gene master,” as referred to by Tsonis and Fuentes (Tsonis and Fuentes 2006), is at the top of a genetic hierarchy that regulates eye and lens induction and development. A number of studies have revealed that downstream of Pax6 are the transcription factors FoxE3, Sox2, Mab21/1, Six3, and Prox1 [as reviewed in (Lang 2004)].
**FGF Signaling Pathway**

FGF and Pax6:

The expression levels of *Pax6* in the lens placode are believed to be regulated by FGF receptor activity, whereby the inhibition of the FGF receptor kinase activity results in lower levels of *Pax6* in this eye tissue (Faber, Dimanlig et al. 2001), thus, suggesting that FGF receptor activity lies upstream to *Pax6*. Given the critical role of *Pax6*, FGF signaling is thought to play a role in eye and lens induction and development.

**In vitro** studies:

The early experiments by Coulombre and Coulombre (Coulombre and Coulombre 1963) and Yamamoto (Yamamoto 1976) established the important role of the neural retina in promoting lens growth *in vivo*. Subsequent experiments *in vitro* aimed at further elucidating the mechanism by which the retina promotes lens growth (Muthukkaruppan 1965; McAvoy 1980; McAvoy and Fernon 1984). In one study, rat lens epithelial cells were cultured with neural retina or retina-conditioned medium, and were found to elongate and accumulate lens fiber-specific crystallins (\(\beta\)-, \(\gamma\)- and \(\alpha\)-crystallins). It was then that Richardson and McAvoy (1990) attributed this fiber-inducing role to retina-derived factors, later isolated and termed FGFs (Richardson and McAvoy 1990). First in the FGF family to be isolated were FGF1, also known by acidic FGF, endothelial cell growth factor, retina-derived growth factor, eye-derived growth factor-II, and brain-derived growth factor factor-II, and FGF2, also known by basic FGF, eye-derived growth factor-I, and brain-derived growth factor-I [as reviewed in (Robinson 2006)]. Furthermore, *Fgf1* and *Fgf2* (de Iongh and McAvoy 1992; de Iongh and McAvoy 1993;
Lovicu and McAvoy 1993; Lovicu, de Iongh et al. 1997) were found to be expressed throughout the rat eye, and in particular in the lens and the lens capsule (Lovicu and McAvoy 1993). It was then with a series of *in vitro* experiments with rat lens epithelial cell explants that the role of FGF1 and FGF2 in inducing lens epithelial cell elongation and accumulation of β- and γ-crystallins was demonstrated (McAvoy 1980; Chamberlain and McAvoy 1987; Chamberlain and McAvoy 1989; McAvoy and Chamberlain 1989). When cultured in the presence of FGF1 and FGF2, lens cells from rat lens explants were found to differentiate and accumulate crystallin, more so with FGF2 than with FGF1 (Chamberlain and McAvoy 1987; Chamberlain and McAvoy 1989).

Interestingly, FGF was found to induce differentiation of lens cells from explants of neonatal as well as postnatal rats explants (Cenedella 1989), supporting the hypothesis that FGF is implicated in lens growth throughout life. Moreover, lens cells from older rats were less responsive to FGF (Richardson and McAvoy 1990; Richardson, McAvoy et al. 1992) with the central epithelium being less susceptible to FGF activity than the peripheral regions of the lens (Chamberlain and McAvoy 1997). Furthermore, the modulation of cellular behavior by FGF is concentration-dependent (McAvoy and Chamberlain 1989; Liu, Chamberlain et al. 1996). In one experiment by McAvoy and Chamberlin, lens epithelial cells from neonatal rat lenses were found to either proliferate, migrate or differentiate into fiber cells with the increasing concentration of FGF2 in culture (McAvoy and Chamberlain 1989). When subjected to low concentrations of FGF, lens cells proliferated and migrated (McAvoy and Chamberlain 1989) resulting in a multilayered epithelium with the strongly adherent cells closest to the lens capsule and the actively migrating cells on top (Lovicu and McAvoy 1989). As the concentration of
FGF increases, proliferation and migration seized as the lens cells started to elongate, extruded their cytoplasmic organelles, and prepared to differentiate (McAvoy and Chamberlain 1989). Furthermore, FGF was shown to enhance transdifferentiation of the pigmented epithelial cells to lens cells *in vitro* (Hyuga, Kodama et al. 1993).

FGF gradient:

The results showing that different FGF concentrations elicit different responses in lens cell cultures, together with the findings from early experiments (Coulombre and Coulombre 1963; Yamamoto 1976) [see section on “Embryological Origin of the Vertebrate Lens and its Development] are strongly indicative of an “antero-posterior” gradient of FGF that is involved in determining the distinct lens polarity (Chamberlain and McAvoy 1997). First step towards validating this hypothesis was the conduction of a series of experiments on humans (Baird, Esch et al. 1986; Caruelle, Groux-Muscatelli et al. 1989; Baudouin, Fredj-Reygrobellet et al. 1990; Sivalingam, Kenney et al. 1990), rats (Noji, Matsuo et al. 1990; Fu, Spirito et al. 1991; Connolly, Hjelmeland et al. 1992; de Iongh and McAvoy 1992; de Iongh and McAvoy 1993; de Iongh and McAvoy 1993; Lovicu and McAvoy 1993), bovine (Folkman, Klagsbrun et al. 1988; Caruelle, Groux-Muscatelli et al. 1989; Hanneken, Lutty et al. 1989; Jacquemin, Halley et al. 1990; Schulz, Chamberlain et al. 1993), and mouse (Jacquemin, Halley et al. 1990) to determine the bioavailability and distribution patterns of FGFs in the ocular tissues. Both FGF1 and FGF2 were detected in cells of various ocular tissues such as the lens, lens capsule, cornea, iris, retina, aqueous and vitreous humors. *Fgf1* and *Fgf2* have been found to be expressed in the mouse neural retina and in lens cells during development (McAvoy
In rat lens, mRNA expression studies revealed a weak for Fgf mRNA in the anterior central epithelium, versus a more intense signal towards the lens equator with the strongest signal detected in the differentiating fibers of the transitional zone. However, no signal could be detected at the later stages of fiber maturation (Lovicu, de Iongh et al. 1997). Similarly, different FGF distribution patterns were detected between the vitreous and aqueous humors (Schulz, Chamberlain et al. 1993; Lovicu, Chamberlain et al. 1995). The gradient distribution of FGF in the eyeball was further demonstrated by a study by Caruelle and coworkers in which a higher concentration of FGF was detected in the posterior than in the anterior chamber of the eye, further supporting the hypothesis that FGF is an inducer of lens cell differentiation at the posterior side of the lens capsule (Caruelle, Groux-Muscetelli et al. 1989).

*In vivo* studies:

The lens fiber-inducing role of FGF was first detected in vitro and later verified by a series of in vivo experiments employing transgenic mice with altered FGF expression levels to detect the resulting effect on in situ lens fiber differentiation. In one study, mice expressing the transgene FGF1 containing the signal peptide sequence derived from FGF4 revealed abnormal elongation of the epithelial cells at the anterior side of the capsule. These cells also expressed the differentiation marker β-crystallin (Robinson, Overbeek et al. 1995). To further elucidate the role of FGF1 in lens regeneration, exogenous FGF1 was applied to lentectomized newt eyes, and after 25 days, histological examination revealed several eye abnormalities (including double lens
formation, and lenses with abnormal shapes and polarities). These observations were attributed to the mitogenic role of FGF in inducing the uncontrolled proliferation of the depigmented iris cells (Del Rio-Tsonis, Jung et al. 1997). In another study, transgenic mice expressing a dominant-negative form of FGFR1 in the lens showed fiber cells with reduced ability to elongate and differentiate, combined with increased apoptosis of fiber cells in the lens central region (Chow, Roux et al. 1995). Similar results (abnormal differentiation of lens epithelial cells or developmental eye abnormalities) were observed with transgenic mice expressing Fgf3 (Robinson, Ohtaka-Maruyama et al. 1998) or Fgf4, Fgf7 or Fgf9 (Lovicu and Overbeek 1998) in lens cells. Collectively, these studies demonstrated the important role of FGF signaling in lens fiber differentiation as evidenced by the findings that altered levels of FGF in the eye can inappropriately induce lens epithelial cells to exit from the cell cycle prematurely and differentiate into fiber cells (Robinson, Overbeek et al. 1995; Lovicu and Overbeek 1998; Robinson, Ohtaka-Maruyama et al. 1998).

FGF and heparan sulfate proteoglycans:

The activity and bioavailability of FGF in the lens is modulated by binding of the growth factor to the heparan sulphate side chains of the heparan sulphate proteoglycans (Rifkin and Moscatelli 1989; Jackson, Busch et al. 1991; Nugent and Edelman 1992; Ross, Kubinak et al. 1993). Several heparan sulphate proteoglycans have been isolated from the rat lens capsule (Schulz, Chamberlain et al. 1995). When bound to heparan sulphate proteoglycans, FGFs are protected against proteolytic degradation and denaturation (Rifkin and Moscatelli 1989). In addition, heparan sulphate proteoglycans
promote the proper functioning of the FGFrs by forming a co-receptor with the cell-surface FGFrs and mediating FGF binding and the subsequent cellular response (Rapraeger, Krufta et al. 1991; Yayon, Klagsbrun et al. 1991; Ornitz 2000; Pellegrini, Burke et al. 2000; Schlessinger, Plotnikov et al. 2000). By promoting FGF signaling, heparan sulphate proteoglycans, thus, play an important role in normal lens development. To further elucidate the critical role of heparan sulphate, Pan and coworkers inactivated Ndst1 in mouse embryos which resulted in an altered formation of the lens and retina through the disruption of the lens vesicle invagination and the proper differentiation of the lens cells (Pan, Woodbury et al. 2006). That is the case because failure to express Nsdt1, the gene encoding for a heparan sulphate-synthesizing enzyme, leads to reduced FGF ligand binding to FGFrs and a disrupted FGF signaling, but interestingly not BMP or Wnt signaling pathways as observed in the case of Drosophila Ndst mutant (Lin, Buff et al. 1999; Lin and Perrimon 1999; Belenkaya, Han et al. 2004; Pan, Woodbury et al. 2006). This further confirms that FGF signaling is required for proper lens development.

Redundancy:

The ability of FGFs in promoting lens growth depends on the ability of the FGF ligand to bind a specific FGF receptor isoform. Lens cells express at least three of the FGF receptors. Seeing that multiple members of the Fgf gene family are expressed in the eye, it is possible that the same FGF ligand can bind more than one FGF receptor isoform. This is supported by the finding that mice null for a number of different FGFs present no abnormal lens phenotype.
FGF receptors:

FGFs act through their membrane bound receptors. A FGF ligand mediates cellular responses by binding to specific cell-surface receptor tyrosine kinases (Johnson and Williams 1993) and activating the ERK-MAP-kinases pathway in the lens, resulting in increased levels of phosphorylated Erk1 and Erk2 (Lovicu and McAvoy 2001; Iyengar, Wang et al. 2007). FGF signaling has been implicated in lens induction, lens cell proliferation and survival, lens fiber differentiation and lens regeneration. Fgfr1-4 are four of the FGFr family that have been shown to be expressed in the developing vertebrate lens (Orr-Urtreger, Givol et al. 1991; Peters, Werner et al. 1992; Orr-Urtreger, Bedford et al. 1993; Peters, Ornitz et al. 1993). Expression of Fgfr1, Fgfr2, and Fgfr3 has been detected in lens cells, whereas Fgfr1 and Fgfr2 have been implicated in the development of retina (Orr-Urtreger, Givol et al. 1991; Launay, Fromentoux et al. 1994; Tcheng, Fuhrmann et al. 1994). All four of these Fgfr genes have been found to undergo alternative splicing which lead to the generation of different isoforms. One example of which is FGFr2, with its two isoforms IIIb (also known as keratinocyte growth factor receptor (KGFR)) and IIIc (also termed bek), both of which have been found to demonstrate different affinities for different FGFs (Miki, Bottaro et al. 1992). Studies on rat embryonic and postnatal developing lenses showed that Fgfr1, the two variants bek and KGFR of Fgfr2, and Fgfr3 genes were expressed in the lens. This expression varies with age, as evidenced by a decrease in the expression of Fgfr2 variants, and to a higher extent Fgfr3, in postanal lenses compared to lenses at the embryonic stages of development. This distinct, yet sometimes overlapping, expression pattern of the different FGFr family members suggests that FGFr1-3 may play different roles during lens
development (de Iongh, Lovicu et al. 1997). Experiments with transgenic mice, expressing truncated Fgfrs or over-expressing secreted Fgfrs, helped elucidate the role of FGFRs in lens development. In one study, mice with null mutations in Fgfr3 or Fgfr4, or both Fgfr3 and Fgfr4 revealed no abnormalities in developed lenses (Deng, Wynshaw-Boris et al. 1996; Weinstein, Xu et al. 1998). In another study by the group of Robinson and coworkers, germ line and conditional gene targeting were used to delete Fgfrs in transgenic mice, resulting in abnormal lens fiber differentiation in mice lacking any two of the four FGFRs1-3. Conversely, mice with combined deletion of three of the FGFRs revealed defects in the developing lenses. Another interesting finding by this group revealed that mice with at least one wild type allele of either Fgfr2 or Fgfr3, but not Fgfr1, developed normal clear lenses. Conversely, mice that lacked all six alleles of Fgfr1-3 revealed defective lenses. This study also reveals redundancy among Fgfr1-3 as reflected by the expression of Fgfr3 (a single allele only needed) by the lens vesicle which can compensate for the loss of both Fgfr2 alleles (Zhao, Yang et al. 2008). Fgfr1 on the other hand, has been rendered not so essential for the differentiation of the lens fibers in mice (Govindarajan and Overbeek 2001; Zhao, Yang et al. 2004; Zhao, Yang et al. 2006). The conditional deletion of Fgfr1 was not found to compromise the development of the lens in mice, at either the embryonic or following birth stages. Furthermore, the expression of the transcription factors necessary for normal lens development (e.g., c-Maf and Sox1) as well as the expression of α-, β-, and γ-crystallins are comparable in the FGFR1 deficient and wild-type lenses. These findings suggest that the absence of FGFR1 does not affect the normal development of the lens or the ability of lens cells to undergo differentiation (Zhao, Yang et al. 2006). This does not, however,
contradict with previous studies in which transgenic mice expressing a dominant negative FGFr1 in the lens resulted in the inhibition of fiber cell elongation and fiber cell death (Ueno, Gunn et al. 1992; Chow, Roux et al. 1995; Robinson, MacMillan-Crow et al. 1995; Stolen and Griep 2000). The reason was partly attributed to redundancy amongst the FGFr1s, where FGFr1 is believed to heterodimerize with other FGFr1s and abolish the functions of multiple FGFr1s simultaneously (Ueno, Gunn et al. 1992; Zhao, Yang et al. 2006).

Conservation of FGFr signaling during lens regeneration:

FGF signaling through FGFr1s is not only conserved during lens development in vertebrates, but also during lens regeneration in mammalian as well as non-mammalian systems. In this section the role of FGFs during lens regeneration in amphibians and mammals will be briefly outlined, and a more detailed description of the mechanisms underlying lens regeneration will be given in Chapter III. Amphibians have the exceptional ability to regenerate the lens following lentectomy. Lens regeneration in *Xenopus laevis* is achieved by the transdifferentiation of the outer cornea under the influence of FGFs supplied by the neural retina (Bosco 1988; Bosco, Venturini et al. 1997; Arresta, Bernardini et al. 2005). In the newt, however, lens regeneration is achieved by the transdifferentiation of the pigmented epithelial cells from the dorsal iris of the eye under the influence of FGFs (Del Rio-Tsonis, Jung et al. 1997; Del Rio-Tsonis, Trombley et al. 1998; Hayashi, Mizuno et al. 2004; Tsonis, Madhavan et al. 2004). These findings were the result of a series of experiments that examined the expression pattern of FGFs during lens regeneration, as well as the effect of exogenous FGFs on the
regeneration of the lens. In one study, the gene expression of Fgf1, and Fgfr2 (in both its variant forms KGFR and bek) was detected in the dedifferentiating pigmented epithelial cells, and to a lower extent in the retina. In the intact adult newt eye, Fgf1 is expressed in all the layers of the retina, and in the lens epithelium. During lens regeneration, Fgf1 levels throughout the retina layers remained relatively unchanged, with increased observed levels of Fgf1 in the differentiating iris pigmented cells and in lens fibers at later stages. Similar expression patterns were associated with bek, whereas KGFR was found to be down-regulated in the retina and up-regulated in the lens during lens regeneration in the newt. Lastly, Fgfr3 expression is detected in the retina, ciliary body, and the dorsal and ventral iris of the intact newt eye, and this level increases in the dedifferentiating pigmented epithelial cells and lens fibers during lens regeneration (Del Rio-Tsonis, Jung et al. 1997). On a separate note, exogenous treatment of lentectomized newt eyes with FGF1 and FGF4 results in abnormalities (i.e., double lens regeneration, and lenses with abnormal polarities), verifying the conserved role of FGFs during normal lens morphogenesis and regeneration (Del Rio-Tsonis, Jung et al. 1997). Another interesting finding revealed the sequential disappearing of proteoglycans (proteins known for binding FGF) from the dorsal iris of the newt eye following lentectomy (Zalik and Scott 1973; Tsonis and Del Rio-Tsonis 1995), suggesting that FGFs are now made accessible to bind their corresponding receptors and promote dedifferentiation of the iris pigmented epithelial cells (Del Rio-Tsonis, Jung et al. 1997).

In conclusion, FGF signaling play an important role in lens induction, development and regeneration, and is essential in regulating lens cell proliferation, differentiation and survival [as reviewed in (Robinson 2006)].
Bone Morphogenetic Proteins (BMPs)

BMPs are essential for lens induction and are believed to converge upstream with FGFs to regulate the expression of Pax6 in the eye tissues (Furuta and Hogan 1998; Wawersik, Purcell et al. 1999; Faber, Dimanlig et al. 2001). In particular, BMP4 and BMP7 have both been studied for their roles in early lens development. The distinct and overlapping expression patterns of Bmp4 and Bmp7 have been detected in the early eye tissues (Dudley and Robertson 1997; Furuta and Hogan 1998; Wawersik, Purcell et al. 1999). Bmp4 expression is the strongest in the optic vesicle, suggesting that BMP4 is required for the optic vesicle to acquire lens-induction competence (Furuta and Hogan 1998). However, studies suggest that BMP4 act in conjunction with at least one additional signal for lens development to occur (Furuta and Hogan 1998). Interestingly, no change in Pax6 expression is seen in Bmp4(tm1) mutant eyes, and Bmp4 expression appears unaffected in the eyes of homozygous Pax6(Sey-1Neu), suggesting that Pax6 and BMP4 function independently. Nonetheless, BMP4 is thought to act in parallel with Sox2 in the pathway of lens induction [as reviewed in (Lang 2004). As for BMP7, studies in which the Bmp7 gene was deleted revealed penetrant eye development defects, suggesting that BMP7 is required for normal eye development (Dudley, Lyons et al. 1995; Luo, Hofmann et al. 1995). The critical role of BMP7 was further demonstrated in a study where Bmp7null and Tfr7/Tfr7 mice (transgene homozygotes expressing a dominant-negative Fgfr1 in the lens placode) were crossed and produced compound genotype mice with a very small lens pit that failed to form the lens vesicle (Faber, Dimanlig et al. 2001). Subsequent studies showed that BMP7 is in particular essential for lens placode development, and affects the expression of Pax6 in the lens placode
(Wawersik, Purcell et al. 1999). This strongly suggests that BMP7 generally lies upstream of the \textit{Pax6} gene enhancers that regulate the expression of \textit{Pax6} in the lens placode [as reviewed in (Lang 2004)].

\textit{Other Growth Factors that Play Role in Lens Morphogenesis}

Finally, it is worth noting that, in addition to FGFs and BMPs, the lens expresses a number of growth factors and cytokines during development and those include but are not limited to TGF\(\alpha\) (Decsi, Peiffer et al. 1994; Reneker, Silversides et al. 1995; Reneker, Silversides et al. 2000), TGF\(\beta\) (Srinivasan, Lovicu et al. 1998; Zhao and Overbeek 2001; Flugel-Koch, Ohlmann et al. 2002), EGF (Reneker, Silversides et al. 2000), VEGF (Ash and Overbeek 2000), PDGF (Reneker and Overbeek 1996; Reneker and Overbeek 1996), IGF-I (Shirke, Faber et al. 2001), IL-1\(\beta\) (Vinores, Xiao et al. 2003), insulin (Reneker, Chen et al. 2004), and optineurin (Kroeber, Ohlmann et al. 2006). Although these growth factors have been shown to influence lens fiber cell differentiation, they have been reported to do so indirectly, possibly by lying downstream of the initiating events (Lovicu and McAvoy 2005).
CHAPTER II
CATARACT

Cataract is an eye disease characterized by the clouding of the normally clear lens of the eye. It affects generally elderly people and can often lead to blindness.

A. Formation and Current Surgical Treatments

As previously noted, the lens is a transparent ocular tissue, and loss of transparency in the lens (Coulombre 1979) is referred to as cataract. One of the major causes of cataract is, thus, the loss of the precise arrangement of the crystallins within the lens fibers or of the lens fibers themselves, a condition which disrupts light transmission through the lens and can eventually lead to vision impairment. Cataract has, therefore, been closely associated with blindness worldwide (West 2007) and surgical intervention is often required to restore vision in the affected population.

For the past thirty years, cataract removal techniques have progressed from extracapsular cataract extraction (Rooks, Brightman et al. 1985) to phacoemulsification (Sigle and Nasisse 2006). The former has been the most widely used since 1982; it consists in removing a segment of the anterior capsule and making a relatively big opening in the lens through which the lens fibers are extracted. The capsular bag (which now consists of a portion of the anterior and the entire posterior capsules) remains in situ to maintain a separation of the aqueous and vitreous humors, and in most cases, to also
house an IOL. Following this surgical procedure, light can freely pass through the transparent IOL and the posterior lens capsule (usually free of epithelial cells towards the center) to reach the retina (Minassian, Rosen et al. 2001). As for phacoemulsification, this relatively recent surgical technique has gained attention due to its use of state of the art equipments. During the procedure, cataract is removed by utilizing an ultrasonically driven oscillating needle that emulsifies the lens which is then removed from the eye with an automated irrigation/aspiration system (Minassian, Rosen et al. 2001). This method results in the making of a small incision through which a foldable IOL is inserted into the remaining capsular bag to restore vision (Riaz, Mehta et al. 2006). 

Phacoemulsification has been reported to possess a 90% success rate (Sigle and Nasisse 2006).

Cataract surgery success rate is highly affected by a number of factors such the stage of cataract development, postoperative ocular hypertension, sex and age of patient at time of surgery, and the presence or absence of an IOL (Biros, Gelatt et al. 2000). More so, cataract treatment is highly dependent on the progression stage of the cataract to be operated on. The four most common stages of cataract progression have been defined, with the least mature to the most mature being incipient, immature, mature, and hypermature. These stages typically differ from one another by (1) the volume of the lens that is affected by the cataract, (2) the extent to which the host vision is impaired, (3) the absence or presence of inflammatory responses and the degree to which they are susceptible to anti-inflammatory medication, and (4) the severity of the cataract which dictates the feasibility or not of surgical intervention. Generally, the less mature the
cataract the better the results obtained with cataract surgery – with the best results observed with incipient cataract (La Croix 2008).

The processes by which the different forms of cataracts develop is complex as evidenced by a study by Mansergh and coworkers that confirmed the differential expression of 54 genes during cataract progression in Sparc null mice (Mansergh, Wride et al. 2004).

**Different Types of Cataract**

Several factors can lead to cataract formation: genetic (Gelatt and Mackay 2005), metabolic [e.g., diabetes mellitus (Basher and Roberts 1995; Beam, Correa et al. 1999)], environmental [e.g., oxidative damage (Truscott 2005; Williams 2006; Williams and Munday 2006)], and senility [e.g., nuclear sclerosis (Dziezyc and Brooks 1983; Truscott 2000; Truscott 2005)]. Diabetes for example, alters the normal metabolism of the lens resulting in cataract formation (Basher and Roberts 1995), whereas age leads to the degeneration of the fibers’ soluble proteins causing the embryonic nucleus of the lens to become denser, less flexible and harder which renders the lens opaque, thus, also leading to cataract formation (Truscott 2000). In addition, cataract can be of a number of types: anterior subcapsular cataract, posterior capsule opacification, posterior subcapsular cataract, and Sparc-related cataract (Martinez and de Iongh ; Hejtmancik 2008), depending on the affected position of the lens.
Cataract-Associated Inflammation

In some cases of severe cataract (i.e., hypermature cataracts), an inflammatory response has been detected that causes abnormalities in the eye (i.e., retinal detachment) and renders the cataract inoperable (van der Woerdt, Wilkie et al. 1993; La Croix 2008). Cataract-associated inflammatory responses are of two forms: phacolytic uveitis (van der Woerdt, Nasisse et al. 1992) and phacolastic uveitis (Van Der Woerdt 2000). The former occurs when a larger than normal amount of crystallins is extruded out of the lens and leads to the recruitment of T cells (van der Woerdt, Nasisse et al. 1992; Denis, Brooks et al. 2003), whereas the latter occurs as a result of ruptured lens capsule, and is more painful and often results in glaucoma and affected vision (Grahn and Cullen 2000; Van Der Woerdt 2000).

B. Cataract Surgery Complications

Despite the benefits of improved current cataract surgical techniques, cataract surgery is frequently accompanied with post-operative complications that can lead to secondary vision impairment in the patients. These complications are often associated with the abnormal behavior of residual lens epithelial cells. Traditional cataract surgery requires that the posterior and part of the anterior capsules remain behind to host the IOL. Epithelial cells remain stubbornly attached to the periphery of the residual lens capsule and undergo phenotypic changes giving rise to lens fibers and migratory myofibroblasts that render the lens capsule opaque (Marcantonio and Vrensen 1999; de Iongh, Wederell et al. 2005; Lois, Taylor et al. 2005). The myofibroblasts are generated by EMT, a process that often leads to fibrosis and the increased wrinkling and contraction of the lens capsule. Moreover, lenticular fiber regeneration often occurs in the peripheral regions of
the capsular bag leading to the formation of Sommerring’s ring, and an aggregation of swollen globular cells known as Elschnig’s pearl, both of which can greatly affect the visual axis (Kappelhof, Vrensen et al. 1987). Furthermore, anterior capsular phimosis (generated by the complete occlusion of the capsulorhexis opening following phacoemulsification and the implantation with an intraocular lens) and capsular contraction syndrome (characterized by a severe fibrotic response that reduces the diameter of the capsular bag following extracapsular cataract surgery) are two conditions that could also greatly affect vision in patients following cataract surgery (Sciscio and Liu 1999; Waheed, Eleftheriadis et al. 2001). The aforementioned complications, when severe, often require corrective surgery to restore vision.

*Posterior Capsular Opacification (PCO)*

Opacification of the posterior capsule remains the most common complication of cataract surgery, and is frequently associated with secondary loss of vision in a significant number of cataract surgery patients (Lois, Taylor et al. 2005). The common methods for evaluating post-operative capsular opacification is by slit lamp examination or by histological studies (followed by analytical methods such as by immunohistochemistry), both techniques of which give great insight on the behavior of residual lens epithelial cells following cataract surgery and, in many cases, of the foreign-body response to the implantation of an IOL [as reviewed by (Saika 2004)]. Over the years, these studies have revealed that despite the surgical trauma, a group of lens epithelial cells remain attached to the residual lens capsule. These resilient cells proliferate along the anterior capsule, migrate onto the surface of the IOL, and colonize
the cell-free posterior capsule. The cells then continue to divide, and ultimately encroach on the visual axis and progressively restricts the ability of the lens to transmit light (Wormstone 2002) so that corrective surgery might be necessary. In vivo models have also been used to study the stages leading to PCO formation. In one study, mice underwent extracapsular lens extraction, and lens epithelial cell behavior was noted 0 and 24 hrs, 3 and 14 days following surgery. Immediately after surgery, lens epithelial cells were present lining the inner of the anterior capsule and the lens bow. By 24 hours, the cells migrated toward the center of the posterior capsule, and by 3 days, the cells have multilayered and resulted in capsular wrinkling. PCO formation was marked by day 14 with the formation of lens fibers and the development of Soemmerring’s ring. Furthermore, PCO in mice was associated with macrophage migration (Lois, Taylor et al. 2005).

The opacification of the posterior capsule is a complex process that requires cross talk between several signaling pathways including those induced by FGFs (Mansfield, Cerra et al. 2004; Symonds, Lovicu et al. 2006), osteopontin (Saika, Shirai et al. 2007), hepatocyte growth factor (Choi, Park et al. 2004), epidermal growth factor (Jiang, Zhou et al. 2006), integrins (Walker and Menko 2009), and Wnts (Chong, Stump et al. 2009). The complexity of the signaling regulating PCO formation remains to be determined.

The overall rate of PCO requiring surgical intervention has decreased with the development of improved surgical cataract treatments and the fabrication of enhanced IOLs capable of trapping migrating lens epithelial cells at the lens capsule periphery. Nonetheless, PCO still occurs in 3 to 40% of the operated eyes (Bertelmann and Kojetinsky 2001; Li, Chen et al. 2008). As a result, more and more studies are focusing
on PCO prevention and management. One study, for example, emphasized on the importance of well performed cataract surgery on the rate of PCO occurrence, whereby the complete overlapping of the anterior capsule and the IOL was reported to reduce the incidence of PCO formation (Smith, Daynes et al. 2004). Another technique that proved efficient, especially in young patients that tend to develop PCO relatively fast, is referred to as the “bag-in-the-lens” procedure during which identical capsulorhexis are made in both the anterior and posterior capsules and the resulting capsule edges are inserted into the edges of the IOL [from where the designation bag (the capsule) in the lens (the IOLs) comes from] (Tassignon, De Groot et al. 2002; De Groot, Tassignon et al. 2005; De Groot, Leysen et al. 2006). This technique has been shown to reduce proliferation of the lens epithelial cells in vitro (Tassignon, De Groot et al. 2002; De Groot, Tassignon et al. 2005) as well as in vivo using rabbit eyes and human donor eyes (De Groot, Tassignon et al. 2005). In situ drug delivery has also been used in an attempt to prevent and/or manage PCO. In one instance, the drugs are delivered by means of implanted IOLs (Duncan, Wormstone et al. 1997) or capsular rings (Cochener, Bougaran et al. 2003; Cochener, Pandey et al. 2003). However, limitations associated with the optimal concentration and minimal dosage of drug delivered, as well as successful targeted cell delivery remain the major drawbacks of drug deliver therapies (Wormstone, Wang et al. 2009). Alternatively, the Perfect Capsule method has been adopted for PCO prevention (Milvella Ltd, Epping, Australia). In this procedure, a sealed capsule irrigation (SCI) system is used in which a silicone disc closes the capsulorhexis and allows, through its extension arms, the delivery of a solution and the subsequent removal of lens epithelial cells (Duncan, Wormstone et al. 1997; Maloof, Pandey et al. 2005; Duncan, Wang et al. 2007; Rabsilber, Limberger et
al. 2007; Zhang, Duncan et al. 2007). Needless to say, these techniques are not always successful in preventing PCO, which once formed, could be treated by the use of neodymium yttrium aluminum garnet (Nd-YAG) laser capsulotomy, a procedure that photodisrupts the thickened posterior capsule, and thereby clears the visual axis of the lens (Aslam, Devlin et al. 2003; Auffarth, Brezin et al. 2004; Findl, Buehl et al. 2007).

**Epithelial to Mesenchymal Transition (EMT)**

Opacification of the lens can be the result of EMT, a process regulated by TGFβ. Following cataract surgery and the implantation of an IOL, lens epithelial cells (cuboidal in shape) transdifferentiate to elongated myofibroblast cells, a process commonly referred to as EMT (Hay 1995). These cells then encroach on the surface of the IOL and migrate on to the inner surface of the residual lens capsule resulting in the restricted ability of the lens to transmit light (Hay 1995; Hay and Zuk 1995). The myofibroblast cells express α-SMA, a marker that is not typically expressed in normal lens cells (Saika, Kawashima et al. 1998; Wormstone 2002; de Iongh, Wederell et al. 2005). Extracellular matrix macromolecules (i.e., collagen types I, III, V, VI, VII, or XIV, fibronectin, hyaluronan, fibrillin, osteopontin, and lumican) have also been detected in the epithelial cells during EMT (Saika, Kawashima et al. 1998; Saika, Kawashima et al. 1998; Saika, Miyamoto et al. 2001; Saika, Miyamoto et al. 2001). In addition, up-regulation of prolyl-4-hydroxylase, a key enzyme involved in collagen biosynthesis, has been also detected in the fibroblastic lens cells (Saika, Kawashima et al. 1998). During healing, the cells have also been found to alter their cell surface receptor expression pattern, such as by up-regulating CD44 (Desai, Wang et al. ; Saika, Kawashima et al. 1998) or expressing integrin α-subunits that differ from those expressed in uninjured adult lens epithelial cells.
(Barbour, Saika et al. 2004). As a result, capsule thickening by fibrosis and capsular contraction by α-SMA-expressing cells have become characteristic features of PCO (Tomasek, Gabbiani et al. 2002).

Recently, two studies in mice revealed (1) the role of a complement receptor C5a antagonist in decreasing the incidence of EMT following lens cataract surgery (Suetsugu-Maki, Maki et al. 2011) and (2) the implication of miR-184 and miR-204 in the control of secondary cataract formation in mouse lens capsular bag explants (Hoffmann, Huang et al. 2011).

*Soemmerring’s Ring and Elschnig’s Pearls*

The trauma of cataract surgery induces a wound-healing response leading to signals that promote residual lens cell proliferation, increased extracellular matrix deposition, lens cell differentiation, and capsular contraction (Wormstone, Wang et al. 2009). Unsuccessful attempts at lens fiber differentiation from the remaining lens epithelial cells result in the formation Soemmerring’s ring and Elschnig’s pearls. Proliferating lens epithelial cells result in Soemmerring’s ring formation in the peripheral regions of the lens. Clusters of proliferating lens epithelial cells, on the other hand, lead to the development of Elschnig’s pearls that may migrate on the posterior capsule and render the capsule opaque and significantly decrease vision following cataract surgery. In addition, Soemmerring’s rings and Elschnig’s pearls may alter the function of the implanted IOL by causing its decentration (Saika 2004). Also, implanted IOLs can lose their accommodative powers following cataract surgery due to the formation Soemmerring’ ring and Elschnig’s Pearls (Dewey 2006).
C. Role of Growth Factors in Phenotypic Changes of Residual Lens Epithelial Cells

Certain cells, such as epithelial cells, have an inherent plasticity in that their morphology and phenotype can be modulated by various growth factors and extracellular stimuli.

*Heterogeneous Expression Pattern of TGFbeta Isoforms in Mammalian Crystallin Lens*

*Tgfβ* is a multifunctional cytokine, with three isoforms (namely TGFβ1, TGFβ2, and TGFβ3) that have been found to be present in mammalian tissues. The heterogeneous expression patterns of *Tgfβ* isoforms in the human and mouse crystallin lens have been identified (Saika, Kono-Saika et al. 2004). Human and mouse lens epithelial cells express *Tgfβ1* and *Tgfβ2* in the equatorial region (Saika, Kono-Saika et al. 2004). *Tgfβ2* is also found to be expressed at much higher levels than the other two *Tgfβ* isoforms in the aqueous and vitreous humors that bathe the lens (Connor, Roberts et al. 1989; Jampel, Roche et al. 1990; Tripathi, Li et al. 1994; Allen, Davidson et al. 1998; Wallentin, Wickstrom et al. 1998; Picht, Welge-Luessen et al. 2001). Furthermore, a mouse embryo lacking *Tgfβ2* reveals multiple defects in the development of ocular structures (i.e., thin corneal stroma, absent corneal endothelium, immature retina, etc.) (Saika, Liu et al. 2001). Nonetheless, these abnormalities have been found to be rescued in *Tgfβ2*-null mice that over-express *Tgfβ1* using an α-crystallin promoter (Zhao and Overbeek 2001). Conversely, a mouse embryo that lacks *Tgfβ1* or *Tgfβ3* does not present any eye abnormalities (Saika, Liu et al. 2001).
TGFβ Implication in Post-Operative Cataract Complications

The role of TGFβ has been closely associated with cell proliferation, migration and extracellular matrix synthesis (Moustakas, Pardali et al. 2002). Using TGFβ, it was possible to replicate in vitro the observations associated with post-operative cataract surgery, such as increased expression levels of α-SMA, and capsule wrinkling and contraction (Wormstone, Tamiya et al. 2002). TGFβ enhances extracellular matrix production (Lee and Joo 1999) and suppresses the proliferation of lens cells. Moreover, TGFβ promotes a wound-healing response as evidenced by a study in which the introduction of TGFβ antibodies successfully suppressed the wound-healing response in mice (Saika, Okada et al. 2001). Furthermore, in vitro studies employing human lens cell lines (Dawes, Angell et al. 2007; Dawes, Eldred et al. 2008) or human capsular bag cultures (Wormstone, Tamiya et al. 2002) have implicated TGFβ2 and not TGFβ1 in lens cell transdifferentiation and extracellular matrix production. Compared to TGFβ1, TGFβ2 is more capable at inducing transdifferentiation and to a lesser extent at inducing matrix contraction (Dawes, Eldred et al. 2008), and is more influential to Smad translocation (Wormstone, Tamiya et al. 2002; Dawes, Angell et al. 2007). It must be noted, however, that despite the implication of TGFβ in lens cell transdifferentiation and matrix contraction, these two processes are driven by two different signaling pathways. This was demonstrated by a study in which the inhibition of fibronectin-fibronectin receptor and the suppression of TGFβ-induced α-SMA expression could not prevent matrix contraction (Dawes, Eldred et al. 2008).

Even more interesting is the long-term effect of TGFβ as demonstrated in an in vitro study by Wormstone and coworkers. TGFβ in culture with lens epithelial cells for a
period of two days generates signals that result in the transdifferentiation of the lens cells as well as matrix contraction 28 days later. These results suggest that following lens injury, TGFβ levels are likely to peak in the eye within the first week and still retain their capabilities to modulate lens cell behavior and induce capsular opacification in the long run (Wormstone, Anderson et al. 2006).

Cell cultures have been adopted as common simplistic models for the study of lens cell behavior in correlation with the activity of TGFβ. In one study, the expression patterns of Tgfβ1, Tgfβ2, and Tgfβ3 in the rat eye were determined, in addition to the growth factors’ potential in inducing cataractous changes in the lens. All three isoforms of Tgfβ were detected in the normal rate lens, and are thought to be up-regulated during injury, a condition that is likely to contribute to cataract development (Gordon-Thomson, de Iongh et al. 1998).

TGFβ is also thought to induce lens fiber maturation. In a study where TGFβ type II receptor was blocked, abnormal denucleation of the maturing lens fibers was observed (de Iongh, Gordon-Thomson et al. 2001; de Iongh, Lovicu et al. 2001). In particular, it is believed that it is TGFβ1 or TGFβ3 that regulate lens fiber maturation since a Tgfβ2-null mouse embryo presents minimal morphological lens defects [as reviewed in (Saika 2004)].

In vivo models have implicated TGFβ in PCO formation through the induction of EMT (Hales, Schulz et al. 1994; Liu, Hales et al. 1994; Hay 1995; Hay and Zuk 1995; Saika, Kawashima et al. 1998; Srinivasan, Lovicu et al. 1998; Saika, Okada et al. 2001; Savagner 2001; Lovicu, Schulz et al. 2002; Moustakas, Pardali et al. 2002; Saika, Miyamoto et al. 2002; Wormstone 2002). Following cataract surgery, inflammatory cells
in the anterior chamber of the eye were found to produce TGFβ to which lens epithelial cells at the site of injury respond to by transdifferentiating into myofibroblast cells expressing α-SMA (Hales, Schulz et al. 1994; Saika, Kawashima et al. 1998; de Iongh, Wederell et al. 2005). The implication of TGFβ in PCO formation was further exploited with experiments using transgenic mice. Transgenic mice overexpressing Tgfβ1 in lens cells led to the development of cataractous changes marked by EMT and the accumulation of fibrous/collagenous extracellular matrix components (Srinivasan, Lovicu et al. 1998). These findings further demonstrate the modulation of lens epithelial cell behavior by TGFβ.

Nevertheless, two in vivo studies on rodents by Lois and coworkers in 2005 put in question the role of TGFβ2 treatment in promoting PCO (Lois, Taylor et al. 2005; Lois, Taylor et al. 2005). This skepticism was, however, met by a plausible explanation by Wormstone and coworkers in 2009 (Wormstone, Wang et al. 2009) that stated that the reason why the treatment of rodent eyes with TGFβ failed to induce PCO following cataract surgery is because of the pre-existing active TGFβ isoforms that have been shown to be present in human and rodent eyes following injury (Ohta, Yamagami et al. 2000; Schlotzer-Schrehardt, Zenkel et al. 2001).

**TGFbeta and Smad Signaling**

TGFβ signals through Smad proteins, the downstream mediators of TGFβ (Ten Dijke, Goumans et al. 2002). Presence of TGFβ, as reported by Saika and coworkers in 2002, regulates Smad signaling proteins in the nuclei of lens cells in the capsular bag (Saika, Miyamoto et al. 2002). In particular, TGFβ2 is thought to mediate the activation
of Smad3 in lens epithelial cells 12 hours following lens injury, as evidenced by the inhibition of Smad3/4 signaling when TGFβ2 neutralizing antibody is administered in mice (Saika, Okada et al. 2001). A body of evidence also implicates signaling via Smad3 in injury-induced EMT in vivo. Smad3-null mice with normal ocular tissues were generated and used to study lens epithelial cell behavior in vivo during lens capsular injury. Results revealed that ablation of Smad3 signaling completely blocks injury-induced EMT, as demonstrated by the loss of expression of the EMT markers snail (Nieto 2002), α-SMA, actin, lumican and type I collagen (Saika, Kono-Saika et al. 2004). These findings strongly suggest that TGFβ induces EMT by Smad signaling.

Collectively, these results suggest that post-operative cataract complications may be prevented/managed by employing approaches that focus on blocking signaling through Smad proteins which could reduce capsular fibrosis, or by activating Wnt pathway which is thought to maintain the normal epithelial phenotype (Stump, Ang et al. 2003).

**TGFbeta and FGF2 Interplay**

The role of TGFβ extends from modulating its own expression (Van Obberghen-Schilling, Roche et al. 1988) to the modulation of the expression of a number of other growth factors such as CTGF (Wunderlich, Pech et al. 2000; Wormstone, Tamiya et al. 2004), and FGFs.

In particular, the balance between the expression of FGF2 and TGFβ has been studied, and found to dramatically change during the healing period that follows cataract extraction (Wallentin, Wickstrom et al. 1998). FGF2 has been reported to interact with TGFβ in inducing anterior sub-capsular cataract formation in rodents (Cerra, Mansfield et
FGF2 and TGFβ have also been found to lead to increased levels of tissue fibrosis (Wormstone, Wang et al. 2009). In addition to their importance for their synergistic effect (Wormstone, Wang et al. 2009), FGF2 and TGFβ have also been studied for their opposing effects (Ueda, Chamberlain et al. 2000; Mansfield, Cerra et al. 2004). Results indicate that where members of the TGFβ family mediate lens fiber maturation (de Iongh, Gordon-Thomson et al. 2001; de Iongh, Lovicu et al. 2001; Saika 2004), members of the FGF family induce lens cell proliferation. Moreover, in instances where TGFβ proteins have been found to promote EMT and mediate capsular fibrosis in response to injury (Kurosaka and Nagamoto 1994; Tanaka, Saika et al. 2004), FGFs have been shown to accelerate lens epithelial cell proliferation and lenticular structure formation (i.e., Soemmerring’s ring and Elschnig pearls). The latter findings were further verified by a study in which a neutralizing antibody against TGFβ2 was administered to mice and led to the induction of lens cell proliferation, suggesting that TGFβ2 is a potent inhibitor of lens cell proliferation. This study also revealed that while injured lenses down-regulate TGFβ2, they up-regulate FGF2 by up to 10 fold during healing intervals following injury. That, and the loss of FGF2 was found to decrease the proliferating potential of lens epithelial cells in mice by up to approximately 50%, further indicating that endogenous FGF2 is a potent inducer of lens cell proliferation in injured lenses (Tanaka, Saika et al. 2004).

**EGF, HGF and Thrombin Roles in PCO Formation**

Other growth factors have also been studied for their implication in PCO formation. These factors include but are not limited to EGF (Maidment, Duncan et al.
2004), HGF through activation of ERK1/2 and JNK/SAPK (Choi, Park et al. 2004), and thrombin (James, Collison et al. 2005). These factors have been associated with the increased proliferating activity of residual lens cell following injury.

**Extracellular Matrix Components**

As previously mentioned (in Chapter I: FGF and heparan sulphate proteoglycans), the proper function of growth factors require their binding to matrix components, such as the proteoglycans heparan sulphate. These molecules facilitate the interaction of the growth factors with their corresponding receptors [i.e., FGF (Yayon, Klagsbrun et al. 1991; Aviezer, Hecht et al. 1994) and HGF (Zarnegar and Michalopoulos 1995)]. In addition to serving as structural molecules, extracellular matrix components have also been shown to actively participate in the modulation of cellular behavior. For example, Fibronectin ED-A enhances the conversion of fibroblasts to myofibroblasts, whereas vitronectin reverses this process (Serini, Bochaton-Piallat et al. 1998; Serini and Gabbiani 1999; Scaffidi, Moodley et al. 2001). Decorin and collagen type IV bind and immobilize TGFβ (Paralkar, Vukicevic et al. 1991). In addition, the matrix molecules lumican (Saika, Miyamoto et al. 2003), vitronectin and fibronectin (Saika, Kobata et al. 1993; Taliana, Evans et al. 2006) have been closely associated with PCO formation by displaying increased levels in conjunction with an increased expression of α-SMA following cataract surgery. Conversely, SPARC, a basement membrane protein that has been found to be selectively expressed by the endothelium in response to injury (Goldblum, Ding et al. 1994), decreases PCO incidence rate. This is achieved by reducing the adhesiveness and attachment of lens epithelial cells through (1) a reduction in the secretion and deposition
of lamin 1 and paxillin, (2) inhibition of α6 integrin heterodimer formation (Weaver, Sage et al. 2006), and (3) through delaying the TGFβ-induced expression of fibronectin and α-SMA (Sawhney 2002; Gotoh, Perdue et al. 2007).

Matrix Metalloproteinases (MMPs):

A class of proteins with catalytic powers, the matrix metalloproteinases (MMPs), are capable of degrading extracellular proteins and have been implicated in physiological as well as pathological functions (Vaughan-Thomas, Gilbert et al. 2000; Wong, Daniels et al. 2004). Two MMPs in particular, MMP2 and MMP9 (also known as gelatinase A and gelatinase B), were found to be up-regulated in lens cells following stress-causing situations, such as cataract (Tamiya, Wormstone et al. 2000), cataract surgery (Wormstone, Tamiya et al. 2002) or primary lens cells cultures (Hodgkinson, Duncan et al. 2007). Furthermore, a study by Dwivedi and coworkers in 2006 suggested that MMP2 and MMP9 are implicated in the development of anterior subcapsular cataracts by mediating TGFβ-induced EMT (Dwivedi, Pino et al. 2006).

D. IOL Design and Biocompatibility

Current cataract treatments require that part of the anterior and the complete posterior lens capsules remain intact in the eye following the extracapsular extraction of cataract. An IOL can then be inserted in the remaining empty capsular bag (Bruck 1993). IOLs in various shapes have been developed using novel design and construction techniques. In addition, a number of materials have been used to fabricate IOLs; these include but are not limited to acrylic, silicone, poly(methyl methacrylate) (PMMA), and hydrogel (Frezzotti and Caporossi 1990; Nagata and Watanabe 1996; Ursell, Spalton et
al. 1998; Hollick, Spalton et al. 1999; Hayashi, Hayashi et al. 2001; Scaramuzza, Fernando et al. 2001; Joseph and Dua 2002; Kim and Lee 2003; Prosdocimo, Tassinari et al. 2003; Wejde, Kugelberg et al. 2003; Hayashi and Hayashi 2004; Findl, Menapace et al. 2005; Nishi 2005; Buehl, Menapace et al. 2007; Nishi, Yamamoto et al. 2007). Implantation studies were then used to evaluate the suitability of each IOL based on the presence or absence of PCO and its severity, and the extent to which the patient’s vision is impaired following cataract surgery and the implantation of an IOL. For example, IOLs with sharp edges have been shown to retard cell growth onto the posterior capsule (Nishi 2005; Nishi, Yamamoto et al. 2007). Nevertheless, PCO still develops in patients with such IOLs and YAG capsulotomy is required to correct vision (Scaramuzza, Fernando et al. 2001; Nishi 2005; Elgohary, Hollick et al. 2006). Foldable IOLs have also been designed, with silicone being the first material used to make them, to fit through the small incision generated by phacoemulsification procedures (Pandey, Ram et al. 1999).

IOLs and Accommodation

Accommodation is the ability of the eye to focus images of objects with varying distances. While currently used IOLs have the ability to restore refractive powers, they present limited accommodative abilities (Hancox, Spalton et al. 2007). Studies also predict that the fabrication and implantation of accommodating IOLs are, to a certain extent, destined for failure because the stages leading to PCO are likely to affect the stability of the implanted IOLs and decrease their accommodative abilities (Wormstone, Wang et al. 2009).
**IOLs and Biocompatibility**

The biocompatibility of an IOL is defined by the type of interaction it holds with the neighboring cells. The surface properties of inserted IOLs have been found capable of modulating cellular behavior in lenses following cataract surgery (Coombes and Seward 1999; Trivedi, Werner et al. 2002; Tehrani, Dick et al. 2004), thus, rendering the cellular behavior a suitable index for IOLs biocompatibility (Saika 2004).

IOL surfaces are also often subjected to the deposition of a thin layer of matrix molecules (i.e., fibronectin and collagen types), as revealed by immunohistochemistry studies (Saika, Tamura et al. 1992; Saika, Kobata et al. 1993; Saika, Yamanaka et al. 1995; Saika, Ohmi et al. 1997; Saika, Tanaka et al. 1997; Linnola, Werner et al. 2000; Linnola, Werner et al. 2000). Interestingly though, the IOLs are usually not encapsulated by fibrotic or granulation tissues (formed by macrophages, fibroblasts and matrix components), as in the case of foreign body encapsulation. This is due to the fact that the lens is an avascular tissue with a sparser supply of cellular components (i.e., macrophages usually supplied from blood) which could in part explain why matrix depositions fail to encapsulate the IOLs (Saika 2004).
CHAPTER III
LENS REGENERATION

One approach to avoiding the complications associated with cataract surgery is to promote regeneration of the lens. In other words, induce the organism to regenerate a lens following extracapsular cataract extraction or whole intact lens removal. Successful lens regeneration can then be assessed by the restoration of the refractive power of the lens and optical clarity. Lens regeneration was first observed in the adult newt by Colucci in 1891 and, independently, by Wolff in 1895, after whom this process was termed “Wolffian regeneration.” Since then, several amphibians as well as some mammals have been shown capable of regenerating a lens following its removal. Lens regeneration typically follows one of two pathways: (1) transdifferentiation of pigmented epithelial cells or cells of the inner corneal epithelium, as seen in the newt and *Xenopus laevis*, respectively, and (2) proliferation and differentiation of lens epithelial cells as in the case of rabbits and rodents. In the context of amphibian lens regeneration, non-lens tissue give rise to lens tissue, a process otherwise termed transdifferentiation. In the following sections, a brief overview of the regeneration of the lens in amphibians and some mammals is presented. Also, an in-depth review of lens regeneration in mice is given at the end of this chapter.
A. Lens Regeneration in Amphibians

Lens regeneration during adulthood is a remarkable process that have been reported in some urodeles (Stone 1967). In urode amphibians, the induction and regeneration of the lens occur through two different pathways. During development, the lens is induced from the mutual interaction of the ectoderm and the optic vesicle, whereas following lentectomy, a new lens is regenerated by the process of transdifferentiation of iris or corneal cells (Del Rio-Tsonis, Jung et al. 1997). That is not to say that both pathways do not share common regulatory players, because the gene *Pax6* for example, which has been found to be expressed during lens development, and to also play a role during lens regeneration (Del Rio-Tsonis, Washabaugh et al. 1995).

*Newts*

The newt is undoubtedly the most powerful model for lens regeneration. This urodele amphibian has the exceptional ability to regenerate whole body organs, such as the lens following its removal (Del Rio-Tsonis and Tsonis 2003), and this ability is not altered by the repetitive removal of the lens in ageing newts (Eguchi, Eguchi et al. 2011). Lens regeneration is achieved when terminally differentiated pigmented epithelial cells of the dorsal iris of the eye, and never the ventral iris, dedifferentiate by shedding their pigment, proliferate and transdifferentiate into lens epithelial cells that, in turn, differentiate into lens fibers (Eguchi and Kodama 1993). Following lentectomy, the dorsal iris-pigmented epithelium starts to dedifferentiation, and by 10 days the beginning of a lens vesicle is formed. By day 15, differentiation of the primary and secondary lens fibers ensues, and lens regeneration is considered complete at about 25 days (Del Rio-
In the newt, an exact replica of the lost lens is formed by recapping the process of pattern formation.

Studies have yet again demonstrated the key role of the Pax6 gene (Del Rio-Tsonis, Washabaugh et al. 1995) and FGFs (Del Rio-Tsonis, Jung et al. 1997) in lens regeneration in the newt. Other important factors also studied for their important function during lens regeneration in the newt include Hox genes (Jung, Del Rio-Tsonis et al. 1998), Proxl (Del Rio-Tsonis, Tomarev et al. 1999), retinoic acid receptors (RARs) (Tsonis, Tsavaris et al. 2002), cyclin dependent kinases (CDKs) (Tsonis, Madhavan et al. 2004), complement components (Kimura, Madhavan et al. 2003), and hedgehog pathway (Tsonis, Vergara et al. 2004).

Most of the studies in the newt have focused on lens induction from the dorsal iris. However, it is thought that the same key regulatory molecules that lead to the regeneration of the lens from the dorsal iris ought to also do so when induced in the ventral iris, seeing that lens induction mechanisms are highly conserved in the vertebrate eye (Tsonis 2006). As a result, a number of studies have identified key factors that play role in lens regeneration in the newt, and have attempted to manipulate their expression in the dorsal iris. In one study, lens regeneration was successfully induced from the ventral iris by inhibiting the BMP pathways and transfecting the iris with Six3 in combination with its treatment with retinoic acid (Grogg, Call et al. 2005; Tsonis 2006).

Frogs

Other amphibians, such as the tadpole frogs, are also capable of lens regeneration by transdifferentiation. Early experiments with Hynobius unnangso by Lkeda (1936,
1939) demonstrated that corneal tissue is the source of the regenerated lens in this species and that the capability for lens regeneration can take place only during a short period of embryonic life after lens induction. In 1963, Freeman experimented with a different species, *Xenopus laevis*, and verified that lens is regenerated by the transdifferentiation of the inner corneal epithelium, a process that he also found to limited to a short period of time before metamorphosis. In addition, Freeman studied lens regeneration in the absence of the optic cup, and found that regeneration of the lens failed to occur with eye-cup removal (Freeman 1963). Subsequent series of *in vivo* (Waggoner 1973; Reeve and Wild 1977) and *in vitro* (Campbell and Jones 1968) experiments studying the importance of the eye environment on lens regeneration in tadpole frogs revealed some discrepancies in the reported results. To set the speculations to rest, Reeve and Wild reported in 1978 that lens regeneration in *Xenopus laevis* required the wounding of the inner cornea and that a cut made in the outer cornea alone does lead to lens regeneration. Also, in 57% of the cases, lens regeneration was not inhibited by the re-implantation of the host lens, suggesting that the presence of the host lens poses no inhibitory effect on the regeneration of the lens from the cornea. The regenerates also showed lens crystallin expression, however, they were of abnormal shape probably due to the space restriction posed by the presence of the host lens (Reeve and Wild 1978). These observations agreed with a study by Eguchi in 1961 in which the lens regenerated in *Triturus* larva in the presence of the host lens, but only if the lens and the dorsal iris did not come in contact (Eguchi 1961).
B. Lens Regeneration in Mammals

Mammals possess the ability to regenerate the lens if the lens capsule is left behind following the removal of the lens capsule contents. Regeneration of the ocular lens was first reported in New Zealand albino (NZA) rabbits (Cocteau and D'Etoille 1827; Stewart and Espinasse 1959; Stewart 1962; Pettit 1963; Agarwal, Angra et al. 1964; Gwon, Enomoto et al. 1989; Gwon, Gruber et al. 1990; Gwon, Gruber et al. 1993). Despite the limited surgical techniques (operating without anesthesia or suture) and the unsophisticated tools at the time (i.e., curette and bent needles), Cocteau and Leroy-d’Etoille demonstrated in 1827 lens regeneration for the first time in NZA rabbits. The lens contents but not the capsule were removed and the lenses regenerated 6 months later (Cocteau and D'Etoille 1827). These findings were met with a lot of skepticism mainly due to the inability to reproduce the results because of the primitive surgical tools at the time that led to varying capsulotomy sizes and variable amount of tissue left behind, a damaged residual capsule, and resultant destructive inflammation that hindered regeneration [as reviewed in (Gwon 2006)]. Following the work of Cocteau and d'Etoille, a number of researchers attempted lens regeneration in rabbits (Day 1828; Mayer 1832; Middlemore 1832; Loewenhardt 1841; Valentin 1844; Milliot 1872; Randolph 1900; Agarwal, Angra et al. 1964), cats (Middlemore 1832; Milliot 1872; Gwon, Gruber et al. 1993), dogs (Middlemore 1832; Milliot 1872), sheep, guinea pigs (Milliot 1872), and primates (Agarwal, Angra et al. 1964). Lens regeneration in rabbits was reported with varying success rates: Backhausen reported 15 failed lens regeneration in rabbits following extraction (as reviewed in (Gwon 2006)), Mayer observed the formation of new lens material in 7 out of 9 experimental rabbits (Mayer 1832), Textor reported the
regeneration of the lens in only 3 of the 8 operated rabbit eyes [as reviewed in (Gwon 2006)], Randolph reported regeneration of the lens in 8 out of 20 rabbits (Randolph 1900), Milliot achieved regeneration of the lens in 5 of the 17 dogs with operated eyes and in 1 of 4 cats (Milliot 1872), and Agarwal reported the regeneration of the lens in 8 of 15 primates (Agarwal, Angra et al. 1964).

Since then, novel techniques have been developed that improved the conditions surrounding lens removal and its subsequent regeneration as well as the analysis of experimental results. More recently, lens regeneration studies have successfully extended to rodents (Lois, Dawson et al. 2003; Call, Grogg et al. 2004; Lois, Taylor et al. 2005). In the following sections, a brief overview of lens regeneration in rabbits and mice is given.

*Rabbits*

NZA rabbits were the first animal models to be reported to spontaneously regenerate their lens after the removal of the lens fibers but not the lens capsule (Gwon, Gruber et al. 1990). The early stages leading to the regeneration of the lens in rabbits begin as early as the sixth day after lens extraction. In the first week following extracapsular cataract surgery, the adherent lens epithelial cells in the extreme periphery of the residual lens capsule proliferate along the anterior and posterior capsules. By 2 to 4 weeks, elongation of the posterior lens cells is observed, and by 1 month the anterior migration of cell nuclei is detected. By 2 months postoperatively, cell differentiation was only observed in the equatorial region as in the normal adult lens (Gwon, Gruber et al. 1990). However as the lens matures, it becomes decidedly opaque and follows an irregular growth-like pattern. Irregularities in the regenerated lenses have been attributed
to adhesions and collapsing of the lens capsules at the lentectomy site, thus, suggesting that filling and sealing the empty capsular bag could greatly improve the shape of the regenerated lenses (Gwon, Gruber et al. 1993). In addition, the less than perfect growth of the regenerated lenses in rabbits could be attributed to the lack of key signals (i.e., FGF, TGFβ, and IGF) required for proper lens cell differentiation. Thus, supplying the necessary factors could provide a controlled environment that may be conductive for proper lens regeneration in mammals (Gwon 2006).

Furthermore, the protein content of regenerated lenses was measured by Metz and coworkers (Metz, Livingston et al. 1965). The protein level was found to rise steadily to reach normal levels by 6 months. The protein composition of normal and regenerated rabbit lenses is grossly the same (Gwon, Enomoto et al. 1989). In particular, all the major classes of crystallins (α-, β-, and γ-crystallin) are synthesized in the regenerating lens, although αA1- and βB3- crystallins are less prominent in the regenerated lenses. Also, a lower than normal levels of crystallins in the regenerated lens is indicative of incomplete differentiation of fiber cells and may account for the low optical density of regenerates (Gwon 2006).

Rodents

Case study: Mouse

A cross section of a normal adult mouse lens shows a thin posterior capsule that comes in direct contact with lens cortical fiber cells, and a thicker anterior capsule that provides points of attachment for the lens epithelial cells. The equatorial region of the lens is the site where lens cells elongation and differentiation occur.
Contrary to what has been observed with adult salamanders, mice do not regenerate their lens in the absence of a lens capsule. In a study by Call and coworkers, lens regeneration was reported in 45 total mice, of both sexes and belonging to three different strains (Balb/c, NZW and MRL/MpJ+/+) following the surgical removal of lens fibers but not the lens capsule. The study reported that the differentiation of lens epithelial cells can be detected, by histology and staining, as early as 2 days following lentectomy. Within a few days, the capsular bag is filled with lens fibers and at day 30 post-lentectomy, the regenerated lens is of about half the size of an intact lens and demonstrate well differentiated bow regions (Call, Grogg et al. 2004). However, it should be noted that the authors of this study ask that caution need be taken when comparing their study to other lens regeneration studies (as with those performed with rabbits) because of a lack of “functional studies” from their part. Conversely, no lens was regenerated in cases where mice had their lens capsule removed. Call and coworkers also noted the occurrence of EMT at the posterior capsule in the few days following lentectomy, that later diminished as regeneration progressed. This type of cellular behavior was attributed to a characteristic wound healing response following injury (Call, Grogg et al. 2004). Another study by the group of Tsonis looked at the gene expression during the early stages following cataract surgery in mice, the period during which EMT was thought to take place (Medvedovic, Tomlinson et al. 2006).

Lens regeneration experiments in rodents identified some of the major requirements that aid in the regeneration of the lens in mammals, such as age of the animal, inflammation, transplantation or implantation of ectodermal cells, vitamin A, and the integrity of the residual lens capsule, all of which are discussed in chapter IV. These
experiments also revealed that mammals possess a potential for lens regeneration stronger than what has previously been assumed.
CHAPTER IV

CONTRIBUTION OF CERTAIN FACTORS TO LENS REGENERATION IN MAMMALS

Research during the nineteenth century helped identify key factors necessary for promoting lens regeneration in mammals. In this chapter, the role of the age of the animal, inflammation, transplantation or implantation of ectodermal cells, vitamin A and the lens capsule integrity will be briefly discussed.

Age of the Animal

Lens has been shown to regenerate at a faster rate and to a fuller extent in younger animals. For example, the regenerated lens in an 18-month-old ram is two thirds the size of the normal lens versus a regenerated lens that is one fourth the size of the normal lens in 2-3-year-old rams (Milliot 1872). In humans, these observations are also observed in younger cataract surgery patients that tend to develop secondary cataract faster than older patients. Despite the fact that age is not an absolute requirement for lens regeneration, it still accounts for faster lens re-growth, a factor that might come handy in future clinical applications.
**Inflammation**

While severe inflammatory responses are typically viewed as detrimental (Agarwal, Angra et al. 1964; Angra, Agarwal et al. 1973), mixed views have been presented with regards to the role of mild inflammation in mammalian lens regeneration. Two separate studies by Middlemore (Middlemore 1832) and Stewart (Stewart 1962) stated that mild postoperative inflammation could actually mediate lens material growth. This observation might be partly explained by the fact that inflammatory mediators either stimulate or support the regenerative process (Gwon 2006).

**Transplantation or Implantation of Ectodermal Cells**

The closure of the lens pit to form the lens vesicle is accompanied by the trapping of few ectodermal cells inside the vesicle, that later disappear by cytolysis (Gwon 2006). These cytolyzing cells are thought to release products required for sustaining lens development, thus, suggesting that implantation of embryonic cytolyzed ectodermal cells into the empty capsular bag following cataract surgery could promote lens re-growth (Sicharulidze 1956; Chanturishvili 1958). Accelerated lens growth, and in some cases, improved lens shape and refractive power, have been reported in eyes that received embryonic ectodermal cells implants compared to eyes that received no implant (Sicharulidze 1956; Chanturishvili 1958; Stewart and Espinasse 1959; Agarwal, Angra et al. 1964; Angra, Agarwal et al. 1973). Nonetheless, few studies reported no significant difference between the regenerated lenses with or without ectodermal cellular implants and, some studies even revealed abnormal hair growth as a result of ectodermal cellular implants (Binder, Binder et al. 1962; Pettit 1963; Metz, Livingston et al. 1965). It is
likely that implanted embryonic ectodermal tissue provides the signals necessary for the
initiation of epithelial cell proliferation or early differentiation, but that as the cells are
cytolyzed, the signal is lost and regeneration slows down [as reviewed in (Gwon 2006)].

_Vitamin A_

Vitamin A, through the activity of retinoic acid, plays an important role during
embryonic eye development. Retinoic acid serves as a signaling molecule that enables the
interactions between the optic vesicle and the lens placode. Retinoic acid signaling has
also been found to promote the development of the ventral retina and optic nerve (Cvekl
and Wang 2009). Two different claims have been made with regards to the role of
vitamin A in mouse lens regeneration. Shekhawat and coworkers in 2001 found that
intraperitoneal injections of vitamin A palmitate on alternate days following complete
lens removal from mice induced the regeneration of lens in adult as well as young mice
35 days post surgery. Regeneration of the lens was achieved through the proliferation and
dedifferentiation of the pigment epithelial cells of the dorsal iris. These cells then
differentiated into lens fibers that gradually filled the lens vesicle (Shekhawat, Jangir et
al. 2001). However, the group of Tsonis stated in 2004 that every other day
intraperitoneal injections of retinol palmitate (50 IU mouse\(^{-1}\) in 50 µl solution) did not
lead to the regeneration of the lens in mice from the dorsal iris following whole lens
removal (Call, Grogg et al. 2004).


**Lens Capsule Structure, Composition, Functions, and Integrity**

The lens capsule is a strong, transparent, relatively thick modified basement membrane that completely encloses the ocular lens. It provides basal attachment for both lens epithelial and lens fiber cells (Danysh and Duncan 2009)

Regional lens capsule thicknesses:

During embryonic stages, the growth of the anterior, posterior and equatorial lens capsules is facilitated by molecules secreted by the lens epithelial as well as fiber cells (Parmigiani and McAvoy 1991). In an experiment by Haddad and Bennett in 1988, glycoproteins destined for the lens capsule, were first detected in the Golgi apparatus of the lens epithelial cells and in cluster of vesicles in the lens fibers. These molecules migrated to the adjacent lens capsule in rats by 14.5 months (Haddad and Bennett 1988). After birth, matrix molecules are continuously being deposited at the inner surface of the lens capsule by lens epithelial and fiber cells (Young and Ocumpaugh 1966; Haddad and Bennett 1988). However, matrix deposition does not lead to a uniformly thick lens capsule but rather to regions with different thicknesses. Studies on several mammalian models, such as mice (Danysh, Czymmek et al. 2008), rats (Young and Ocumpaugh 1966; Parmigiani and McAvoy 1989), rabbits (Ziebarth, Manns et al. 2005), and humans (Ziebarth, Manns et al. 2005; Barraquer, Michael et al. 2006) revealed regional thicknesses along the lens capsule. A study on prenatal rat lenses revealed a thicker posterior compared to the anterior lens capsule (Parmigiani and McAvoy 1989). A similar observation was noted in mouse embryos (Csato 1989). However, and in as little as 2 days postnatally, the anterior capsule is already thicker than the posterior capsule and
becomes as much as 7.5 times thicker than the posterior capsule by 600 days postnatally (Parmigiani and McAvoy 1989). The reason being that the anterior side of the lens capsule continues to grow throughout life as a result of lamellae of extracellular matrix being continuously deposited by lens epithelial cells and fiber cells. Another plausible reason accounting for the regional thicknesses in lens capsule is the very slow turnover rate of capsular components (Young and Ocumpaugh 1966; Fisher and Pettet 1972; Seland 1976). However, and to date, the mechanisms by which the lens capsule area expands to accommodate the matrix addition are not known (Danysh and Duncan 2009). Nevertheless, some labs noted that the physical, biochemical and biomechanical properties of the lens capsule change with age which may partly be the result of the increasing size of the lens capsule (Fisher 1969; Seland 1974; Krag and Andreassen 2003). In humans, the anterior capsule of young members have been found to be strong and highly extensible, whereas, in older human capsules, the capsule is thick and more brittle and has lower breaking strength (Krag, Olsen et al. 1997). Similarly, the posterior lens capsule was found to lose mechanical strength with age (Fisher 1969; Seland 1974; Krag and Andreassen 2003).

Interestingly, Danysh and coworkers in 2008 observed that the thickness of anterior capsule of intact mouse lenses varies even in animals of the same age but of different strains (Danysh, Czymmek et al. 2008).

Varying roles of the lens capsule:

The role of the lens capsule does not merely stop at sequestering the lens from the surrounding ocular tissues (Seland 1992; Krag and Andreassen 1996) but also for
providing a protective environment for the developing and later mature lens. In a study by Beyer et al. in 1984 on Rhesus monkeys, eyes with intact posterior capsules were less susceptible to the development of bacterial endophthalmitis (Beyer, Vogler et al. 1984). The lens capsule has also been shown to protect the developing fibers against viral diseases (i.e., rubella virus) (Karkinen-Jaaskelainen, Saxen et al. 1975). The lens capsule also plays a role in lens epithelial cells attachment, migration and proliferation. The capsule is composed of extracellular matrix molecules, four of which, laminin, collagen VI, nidogen, and perlecan are of particular interest because of their ability to self-assemble into a three-dimensional scaffold (Danysh and Duncan 2009) capable of mediating lens growth. Interaction of capsular molecules with epithelial and fiber cells membrane bound integrins (such as β1-integrin (Simirskii, Wang et al. 2007), α6-integrin and α3-integrin (De Arcangelis, Mark et al. 1999)) is necessary for the maintenance of lens epithelial cell phenotype at the anterior capsule. The capsule also comprises anchor points, such as the basal membrane complex (BMC) that was found to anchor and facilitate the migration of fiber cells across the lens capsule (Bassnett, Missey et al. 1999; Lu, Mohammed et al. 2008). The lens capsule also allows for the transmission of accommodative forces to the lens (Koretz and Handelman 1982) via the components of the zonules fibers in the equatorial region (Mir, Wheatley et al. 1998). In addition, the lens capsule is a source of essential growth factors (i.e., FGF1 and FGF2) necessary in promoting lens growth and lens cell survival (Vlodavsky, Bar-Shavit et al. 1991; Lovicu and McAvoy 1993). The growth factor release from the lens capsule is mediated by MMPs (Vu and Werb 2000; Tholozan, Gribbon et al. 2007; Wormstone, Wang et al. 2009). The lens capsule also allows for the passive exchange of metabolic substrates
(required for the lens cells metabolism) and waste. Being an avascular tissue, the lens fibers contained within the lens capsule derive the bulk of their energy from anaerobic glycolysis (Winkler and Riley 1991). As a result, macromolecules such as glucose, salts, water and gases (O2 and CO2) must traverse the capsular bag first before reaching the lens fibers and enabling metabolism (Friedenwald 1930; Fisher 1977). Studies have shown that the lens capsule selectively filters for molecules based on charges and size (Lee, Vroom et al. 2006; Danys and Duncan 2009). A study by Danys and coworkers on mouse lenses revealed that lens capsules permeability and binding affinity are influenced by the ionic interactions between the capsule matrix and the crossing proteins (Danys, Patel et al. 2010). Lastly, and as previously discussed, the lens capsule provides the source for the new lens material regenerated in mammals (Randolph 1900; Gwon, Gruber et al. 1990; Gwon, Jones et al. 1992; Gwon, Gruber et al. 1993). The presence of a relatively intact posterior capsule and a sound anterior capsule with no adherence between the two capsules is crucial, as evidenced by studies that showed that no new lens material if formed the sites of lesion in the residual capsules (Coulombre and Coulombre 1971; Gwon, Gruber et al. 1990). Also, a sound residual lens capsule provides the structural framework that dictates the form of the regenerated lens. Interestingly, the capsule retains its ability to regenerate through several successive rounds, as demonstrated by a study by Loewenhardt that showed that the removal of the regenerated lens (9 months after extraction of the original lens), a second lens and a third new lens were generated (Loewenhardt 1841).
Capsule integrity:

Regenerated lenses are believed to have improved shapes when the capsulotomy site is sealed (so as to eliminate lesions and opening in the anterior capsule) and the lens capsule bag is filled with gas or a polymer (so as to prevent adhesion between the capsule walls) (Gwon 2006). These findings were put to test by an experiment by Gwon and coworkers in 1993 where a collagen patch was used to seal the opening in the anterior capsule and air, Healon, or perfluoropropane were used to fill the empty capsular bag. Favorable results were obtained in which lenses regenerated faster and filled the entirety of the capsular bag by 5 weeks in sealed capsules. Capsules injected with air regenerated most regularly, whereas Healon failed to permanently prevent lens capsule adhesions, and perfluoropropane delayed regeneration due to its slow resorption time (Gwon, Gruber et al. 1993). Furthermore, a polymer implant has been used to fill the capsular bag, and in comparison with implant-free rabbit eyes, the eyes that received the polymer implant and a collagen patch showed no notable differences in the regenerated lenses (Gwon, Kuszak et al. 1999; Gwon 2006).
CHAPTER V
DNA AND RNA LEVELS, AND CRYSTALLIN COMPOSITION OF
REGENERATED LENSES

DNA and RNA Levels

Over the years, analysis of the regenerate composition has progressed from
dropping the lenses in boiling water or alcohol, to measuring overall protein and RNA
content, to biochemical analysis and the detection of specific molecules in the
regenerated lenses. Regenerated mammalian lenses were found to contain about twice as
much RNA (Metz, Livingston et al. 1965; Angra, Agarwal et al. 1973) and about four
times as much DNA as control lenses (Angra, Agarwal et al. 1973) [levels measured in
µg/100 mg wet weight] in the early stages of regeneration. The RNA content of
regenerating lenses was found to decline to a normal level by 4 to 6 months, whereas
DNA content remained higher than normal amounts of DNA all throughout regeneration
(Angra, Agarwal et al. 1973). This is partly due to the fact that regenerating lenses
contain a large number of actively dividing and growing cells (Gwon 2006).

Crystallin Composition

The major protein components of the vertebrate lens are α-, β-, and γ-crystallins.
Crystallins are mainly structural proteins. In mammals, α-crystallin is detected in all lens
cells, whereas β- and γ-crystallins are mostly expressed in the differentiating lens cells
A study on rat lenses revealed that β-crystallin appears before γ-crystallin as lens cells differentiate into lens fibers (McAvoy 1978; McAvoy 1978). In this study, a lens fiber-specific antibody against α-crystallin was used. α-crystallins are members of the family of small heat shock proteins (de Jong, Hendriks et al. 1989; Slingsby and Clout 1999; Bloemendal, de Jong et al. 2004) characterized by increased expression levels in response to stress (Dasgupta, Hohman et al. 1992; Head, Corbin et al. 1994; Andley, Song et al. 2000). α-crystallins function as molecular chaperones to protect against stress-induced cell aggregation (Horwitz 1992; Horwitz, Bova et al. 1999; Xi, Bai et al. 2003; Bloemendal, de Jong et al. 2004), as well as apoptosis in vivo (Xi, Bai et al. 2003) and in vitro (Andley, Song et al. 1998; Andley, Song et al. 2000). More specifically, targeted gene deletion revealed that αA-crystallin protects lens epithelial cells against apoptosis, and that αB-crystallin promotes lens epithelial cells stability in culture (Bai, Xi et al. 2003). While heat shock or other stresses seem to modulate the level of αB-crystallin, they not seem to alter the synthesis or accumulation of αA-crystallin (de Jong, Hoekman et al. 1986; Head, Corbin et al. 1994; Bloemendal, de Jong et al. 2004). Furthermore, α-crystallin seem to be predominantly expressed in rat lens epithelial cell explants cultured with FGF (Chamberlain and McAvoy 1997). Moreover, the responses of γ-crystallins, and to a lesser extent α- and β-crystallins, to FGF seem to decline with the age of the rat (Richardson and McAvoy 1990; Richardson, McAvoy et al. 1992; Richardson, Chamberlain et al. 1993).
CHAPTER VI

A BIODEGRADABLE HYDROGEL OF OLIGO(POLY(ETHYLENE GLYCOL)FUMARATE) CROSS-LINKED WITH POLY(ETHYLENE GLYCOL)-DIACRYLATE FOR GROWTH FACTOR DELIVERY AND ENCAPSULATION OF IRIS PIGMENTED EPITHELIAL TISSUE

The previous chapters established the importance of providing a suitable structural support, maintaining a lesion-free continuous anterior surface, and supplying the necessary growth factors in inducing the regeneration of a lens in mammals (Gwon, Gruber et al. 1993; Gwon, Kuszak et al. 1999; Gwon 2006). In this study, the efficacy of an implanted biodegradable hydrogel of oligo(poly(ethylene glycol)fumarate) cross-linked with poly(ethylene glycol)-diacrylate (OPF;PEG-DA) in delivering growth factors and supplying the cellular material necessary for the regeneration of a lens in mice in the absence of a lens capsule was tested. In general, hydrogels are fabricated by chemically or physically cross-linking water-soluble polymers. The resulting hydrogel matrices are either degradable (resorb by hydrolysis) or biodegradable (actively degraded by cells) [as reviewed in (Whitaker, Quirk et al. 2001)]. Scaffold materials based on poly(α-hydroxyacid)s – such as poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) – have been found to be by far the most commonly used matrices for tissue engineering applications, such as for growth factor delivery. Briefly, a growth factor is a polypeptide transiently synthesized and stored as inactive or partially active precursor that requires
proteolytic activation or binding to matrix molecules or specific cell surface receptors for stabilization or activation. As a result, most cellular responses to growth factors require new mRNA and protein synthesis. Growth factors fulfill a number of functions in the organism, from modulation of cellular activities (i.e., stimulation or inhibition of cellular proliferation, differentiation, migration, and/or adhesion) and gene expression, to the regulation of the secretion of other growth factors. Growth factors exhibit their effect in a concentration-dependent manner. In addition, many cell types can produce the same growth factor and the same growth factor can act on different types of cells with the same or different effect. Nevertheless, growth factors have short half-lives, a relatively large size, slow tissue penetration rates, as well as a potential toxicity when delivered at a systemic level causing the tissue to respond after a long time, all properties of which obviates the conventional routes of growth factors administration. Hence, one proposed way of enhancing the in situ delivery of growth factors is to facilitate their sustained release over an extended time period by their incorporation into a polymer carrier. Proteins in general, and growth factors in particular can be entrapped within these materials either directly at time of fabrication (Lo, Kadiyala et al. 1996; Whang, Tsai et al. 1998) by for example polymerizing the hydrogel in the presence of the active molecule, or after fabrication (Fournier and Doillon 1996; Tabata, Nagano et al. 1999). Depending on the molecular weight of the incorporated protein, release from these hydrogel networks may be controlled by either diffusive or degradative processes (Whitaker, Quirk et al. 2001). Poly(α-hydroxyacid)s are particularly useful in tissue engineering applications because their resorption results in a natural replacement tissue without the long-term complications associated with foreign implants (Mikos, McIntire et
al. 1998; Anderson and Langone 1999). As the hydrogel biodegrade, the growth factor would is released in situ to induce tissue regeneration. The duration of drug release from a polymer matrix can be regulated by the drug loading, type of polymer used, and the processing conditions (Babensee, McIntire et al. 2000).

In this study, bFGF was directly incorporated into an OPF;PEG-DA hydrogel at time of fabrication and implanted in the empty eye cup of mice. In some instance, the hydrogel was also used to encapsulate and deliver pigmented iris tissue. A similar experimental set up was recently performed in an experiment using newts. A hydrogel encapsulating iris pigmented epithelial cells was implanted in lentectomized newts and the presence of this hydrogel matrix did not adversely affect the process of lens regeneration in these animals (Zhang, Park et al. 2010). Although lens regeneration from the iris pigmented epithelium can be achieved in only a limited number of amphibian and teleostean species in vivo, pigment epithelial cells of avian embryos and even of human fetuses have been shown capable of transdifferentiation into lens phenotypes when dissociated and cultured in vitro (Eguchi and Okada 1973; Yasuda, Okada et al. 1978). These results suggest that vertebrate pigment epithelial cells have retained their ability to transdifferentiate into lens phenotypes, a feature that was put to test in vivo in this study.
STATEMENT OF THE PROBLEM

When compared to amphibians, mammals have very limited regenerative abilities. Mice are capable of lens regeneration only if the lens capsule is left behind following lentectomy. The regeneration of the lens is achieved by the proliferation and differentiation of residual mouse lens epithelial (MLE) cells adherent to the remaining lens capsule. In contrast, urodele amphibians (commonly called salamanders), as best reflected by the newt, have the ability to regenerate a lens from non-lens tissue following the organ’s complete removal. In newts, lens regeneration is achieved by the transdifferentiation of dorsal pigmented epithelial cells (PECs) to lens cells. Furthermore, aggregated PECs in vitro, when implanted into the limb blastema of the newt in vivo have been shown to successfully regenerated a lens of correct structure and polarity (Ito, Hayashi et al. 1999). The ability of PECs to transdifferentiate in vitro is not restricted to lower vertebrates. Aged human PECs have also been shown capable of transdifferentiation in vitro (Eguchi 1988). These findings suggest that transdifferentiation of PECs is widely conserved in vertebrates, and this potential might be dormant in certain species and require activation in vivo.

Having determined from literature (1) the transdifferentiation potential of iris PECs, (2) the role of growth factors (i.e., FGF) in the spontaneous regeneration of the lens in lower vertebrates, and (3) the importance of scaffolds in providing the necessary structural framework needed for the regeneration of a suitable shaped lens, the hypothesis
that an iris pigmented epithelial tissue encapsulated by a hydrogel bead combined with bFGF and implanted in lentectomized mice eyes can be coaxied into regenerating a lens in vivo was put to test in this study.

Also, the mechanisms (elongation, fiber-formation, nuclei extrusion, and crystallin synthesis) by which MLE cells lead to the formation of lenses in mammals in vivo were mimicked by a cell culture system in vitro in which lentoids were generated. To shed light on this process, a primary MLE cell line was generated and cultured in regular as well as serum-starved medium on aligned Poly-ε-caprolactone (PCL) nanofibers. The ability of the aligned PCL nanofibers to induce MLE cell differentiation and lens fiber alignment was also assessed.
MATERIALS AND METHODS

Project I: Mouse Surgeries

Animals: Adult (8-16 weeks of age) female C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The mice were anesthetized, and some were lentectomized by cutting the cornea and removing the whole lens (lens fibers and lens capsule), and others had their whole lens removed and a hydrogel with or without lens iris tissue implanted. Mice were also used for lens iris tissue harvest.

Anesthesia: A Ketamine hydrochloride (80 mg/mL) / Xylazine hydrochloride (12 mg/mL) solution (K113-10ML) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The anesthetic was diluted to a final working concentration of Ketamine hydrochloride (4.8 mg/mL) / Xylazine hydrochloride (0.72 mg/mL) in sterile 0.9% sodium hydrochloride (Hospira, Lake Forest, IL, USA). The anesthetic was administered to the mice on a 19.79 µL Ketamine hydrochloride/Xylazine hydrochloride/g mouse basis.

Analgesia: Buprenorphine hydrochloride, 50 mg, was obtained from Sigma-Aldrich (St. Louis, MO, USA). The analgesic was dissolved in sterile 0.9% sodium hydrochloride (Hospira, Lake Forest, IL, USA). The drug was administered to the mice on a 12.5 µL Buprenorphine/g mouse basis.

Pupillary Dilation: AK-Dilate™ Phenylephrine Hydrochloride Ophthalmic Solution, USP, sterile 2.5% (Akorn, Lake Forest, IL, USA) is a vasoconstrictor and
mydriatic recommended for use in ophthalmology. Two to three drops of this drug per eye were used to dilate the pupil of the mouse eye prior to ocular surgery.

**FGF:** Recombinant Murine FGF-basic, sterile (PetroTech, Rocky Hill, NJ, USA) was diluted in sterile 1X PBS (Hyclone Laboratories, Logan, Utah, USA) to a final concentration of 30 ng/mL.

**N-2 Supplement:** N-2 supplement, sterile 100X (Invitrogen, Grand Island, NY, USA) was diluted in sterile 1X PBS (Hyclone Laboratories, Logan, Utah, USA) to a final working concentration of 10X.

**Vetbond:** 3M Vetbond™, sterile (Animal Care Products, St. Paul, MN, USA) is an n-butyl cyanoacrylate tissue adhesive that has been successfully tested for corneal closure of mice (Hayakawa and Kawasaki 2010) and rabbits (Ollivier, Delverdier et al. 2001). After application, these two studies reported that Vetbond displayed a good degree of adhesion efficacy, low toxicity, and no interference with corneal healing (Ollivier, Delverdier et al. 2001; Hayakawa and Kawasaki 2010). Vetbond™ was used to seal the corneal cut after removal of the lens.

**Lens Iris Tissue Harvest for Hydrogel Encapsulation:** For tissue harvest, the mice were euthanized, after loss of consciousness, by administering a lethal dose of carbon dioxide under monitoring of cessation of heartbeat and respiration. Following euthanasia, the eyeballs were dissected out under the microscope and dropped in sterile 1X PBS (Hyclone Laboratories, Logan, Utah, USA). The eyeballs were cut open so that to separate the anterior and posterior sides, and the lens was removed with a pair of forceps. The lens iris tissue was then detached from the cornea, collected and transferred to a second dish with sterile 1X PBS. The iris tissue from each eye was, subsequently, cut
into approximately 5 pieces, stained with vital dye CFDS-SE and encapsulated in a hydrogel bead for implantation.

**CFDA-SE staining:** Two hours prior to encapsulating, the pigmented iris tissue pieces were stained with vital dye CFDA-SE (Invitrogen, Grand Island, NY, USA). Briefly, one vial CFDA-SE (Invitrogen, Eugene, Oregon, USA) containing 500 µg was diluted in 90 µL Dimethyl-Sulfoxide. The resulting stock solution was further diluted 1:5000 in 1XPBS prior to incubating with pigmented iris tissue. Following a 15 minute incubation at 37 °C in the CO2 incubator, the CFDA-SE/1XPBS dye solution was removed and replaced by sterile 1X PBS.

**Hydrogel:** Hydrogel beads (see Figure 1), 1 mm in diameter, were prepared by combining 0.05 g of oligo(poly(ethylene glycol) fumarate) (OPF) kindly provided by Dr. A. Mikos (Rice University, Houston, TX, USA) with 0.005 g of the crosslinker poly(ethylene glycol)-diacrylate (PEG-DA; Glycosan BioSystems, Alameda, CA, USA), and adding 222 µL of FGF in 1X PBS and 1X N-2 supplement for 20 – 60 minutes at room temperature, or until the solution is clear. Two solutions, 0.3 M ammonium presulfate (APS; Sigma-Aldrich, St. Louis, MO, USA) and 0.3 M N, N, N’, N’-tetramethylenediamine (TEMED, Sigma-Aldrich, St. Louis, MO, USA) were prepared in sterile 1X PBS (Hyclone Laboratories, Logan, UT, USA). Crosslinking of the polymers was initiated by adding 23.4 µL of the 3M APS solution and 23.4 µL of the 3M TEMED solution. The resulting solution was vortexed following each solution addition, and then injected into a silicone mold (see Figure 1) kindly provided by Dr. A. Mikos (Rice University, Houston, TX, USA). The mold was closed and left in a petri dish at room temperature for 8 minutes for the gel to firm.
For the surgeries that required the encapsulation of the lens iris tissue, iris pieces were inserted into the mold prior to closing and left at room temperature for 8 minutes for the lens iris tissue pieces to be encapsulated in the gel beads as they firm.

**Figure 1: Silicone Mold and Hydrogel Beads.** Silicone mold in closed (A) and open (B) positions. Arrows point to the 1 mm mold for individual bead formation. Hydrogel beads after fabrication (C); each unit in the ruler represents 1 mm

**Surgeries:** Prior to operating, C57BL/6J mice were anesthetized with Ketamine in combination with Xylazine by administering half of the anesthesia subcutaneously in the back of the animal and the other half intraperitoneally. For analgesia, Buprenorphine was injected subcutaneously, underneath the skin between the animal’s ears. Prior to the administration of Buprenorphine, one to two drops of AK-Dilate™ Phenylephrine Hydrochloride Ophthalmic Solution was applied to both eyes, to dilate the mouse’s pupils. The mice were placed under the microscope and a longitudinal incision was made in the cornea of the left eye using a sharp blade (see Figure 2). The whole lens (lens capsule content and lens capsule) was extracted intact through the incision with fine
forceps by applying pressure to the eye. The right, non-surgical eye was left intact. The mice were then divided into three groups. Mice in group 1 served as control, they received no implant and had their corneal incision sealed by applying a drop of the tissue adhesive Vetbond™ after having dried the tissue surface with a cellulose spear. Animals in group 2 had a hydrogel-carrying-FGF bead inserted in their left eye in place of the lens with their cornea sealed with a drop of Vetbond™. Lastly, mice in group 3 received a hydrogel-carrying-FGF bead encapsulating a piece of lens iris tissue implanted into their eye cavity followed by the closure of their cornea with Vetbond™. Immediately following surgeries, the mice were then placed in a recovery cage located on top of a heating pad to assure maintenance of correct body temperature following anesthesia, until completely awake by which time they were moved into a new cage and given fresh water and food supply.

**Euthanasia:** 50 days post-surgeries, the mice were euthanized, after loss of consciousness, by administering a lethal dose of carbon dioxide under monitoring of cessation of heartbeat and respiration. Following euthanasia, the eyeballs were dissected out under the microscope and prepared for histology (see Figure 3) by fixing in 4% paraformaldehyde (PFA) at 4 °C overnight. 4% PFA was diluted in 1X PBS (Hyclone Laboratories, Logan, Utah, USA) from a 40% stock PFA solution (Electron Microscopy Sciences, Hatfield, PA).

**Histology:** From 4% PFA, the eyes were washed with cold 1X PBS for 30 minutes at 4 °C, cold 0.85% saline solution for 30 minutes at 4 °C, and a 1:1 saline-ethanol solution for 30 minutes at room temperature. The eyes were then dehydrated at room temperature with 70% ethanol twice 15 minutes each, 80%, 90% and 95% for 30
minutes each, and 100% twice 30 minutes each time. The eyes were then left in 100% ethanol (Fisher Scientific, Fair Lawn, NJ, USA) at 4 °C overnight. Subsequently, the eyes were washed with xylene (Fisher Scientific, Fair Lawn, NJ, USA) twice for 30 minutes at room temperature, and a 1:1 xylene-paraffin mixture for 45 minutes at 60 °C. Lastly, the tissues were soaked in paraffin (Fisher Scientific, Houston, TX, USA) three times for 30 minutes at 60 °C before being embedded in paraffin in the embedding molds. Sections of eye tissues, 15 micrometer thick, were prepared and collected on gelatinized slides for immunohistochemistry studies.

Figure 2: Schematic Illustrating the Different Steps of the Surgical Procedure Performed on Mice
**Antibodies:** A lens fiber-specific antibody for alpha-crystallin, kindly provided by Dr. M. Robinson (Miami University, Oxford, OH, USA) was used. The primary antibody was used at a dilution of 1:100 in 10% normal goat serum (abcam, Cambridge, MA, USA) prepared in 1X PBS.

The secondary antibody used was a Cy<sup>TM3</sup>-conjugated AffiniPure F(ab')<sub>2</sub> FragmentDonkey Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) diluted in 0.2% Triton X-100 (Sigma Chemical Company, St. Louis, MO, USA) in 1XPBS.

For nuclear staining, 4’,6-diamidino-2-phenylindole or DAPI (MP Biomedicals, LLC, Solon, OH, USA) is the fluorescent staining that was used for nuclear labeling. DAPI was used at a 1:1000 dilution in 0.2% Triton X-100 in 1XPBS.

**Immunohistochemistry:** Slides with paraffin sections were deparaffinized in xylene twice for 15 minutes at room temperature, and rehydrated using an ethanol series of 100%, 95%, 80%, 70% and 30% solutions for one minute each, also at room temperature. The slides were then rinsed in dionized water for 1 minute, washed in 1X PBS for 15 minutes, 1X PBST (0.2% Triton X-100 in 1X PBS) for 15 minutes, followed once again by 1X PBS for 15 minutes, all steps at which incubation was at room temperature. Subsequently, the slides were blocked with 10% goat serum for 60 minutes at room temperature, and in primary antibody overnight at 4 °C. Control sections were incubated in 10% goat serum overnight at 4 °C.

After the primary incubation with antibodies, and prior to incubating in secondary antibody, the slides were washed in 1X PBS for 15 minutes, 1X PBST for 15 minutes, and again in 1X PBS for 15 minutes, each of the washed occurring at room temperature.
The secondary antibody and DAPI were added to the slides that were left to sit at room temperature for 2 hours. Subsequently, the slides were washed at room temperature in 1X PBS, 1X PBST, and 1X PBS, each for 15 minutes. Cover slips were then applied to the slides using DAKO fluorescent Mounting Medium (Dako, Carpinteria, CA, USA).

Sections were photographed using a BX51 microscope (Olympus, Center Valley, PA, USA) under fluorescence setting with a CCD camera (RTKE Spot, Diagnostics Instruments, Inc, Sterling Heights, MI, USA) and imaging software (Spot version 4.1, Diagnostics Instruments, Inc).

**Hematoxylin and Eosin Staining:** The paraffin was melted by heating the slides at 55-60 °C for 30 minutes. The slides were afterward washed at room temperature with xylene twice for 15 minutes each, and then hydrated through an ethanol series of 100%, 95%, 80%, 70% and 30% ethanol solutions for one minute each. Then the slides were rinsed with distilled water for 1 minute at room temperature prior to staining with hematoxylin for 2 minutes. The slides were rinsed with tap water for 1 minute, and distilled water for 2 minutes, and then dehydrated through an ethanol series of 30%, 70%, 80% and 95% ethanol solution each for 1 minute at room temperature. The slides were, subsequently, stained with eosin for 1 minute prior to washing with 95% ethanol for 1 minute and 100% ethanol for 2 minutes. Lastly, the slides were placed in xylene for 7 minutes at room temperature followed by the application of cover slips using Permount. The slides were left to dry overnight prior to observing using a brightfield microscope.
Figure 3: Schematic Summarizing all the Steps Leading to the Detection of α-Crystallin Expression in Surgical Mouse Eye Sections
Capsular bag culture: A radial incision was made at the border of the cornea and the sclera using a scalpel and scissors. The intact lens was removed through the incision using forceps. Lenses were then washed in Advanced MEM (Gibco, Grand Island, NY, USA) in combination with 1X antibiotics/antimycotics. Unwanted residual ocular tissues on the outer lens capsule were removed by incubating the lens capsule with 0.25% trypsin/EDTA (Cellgro, Mediatech Inc., Manassas, VA, USA) for 1-5 min. Following submersion in MEM supplemented with 20% FBS (MEM/20%FBS; Sigma-Aldrich, St. Louis, MO, USA) the posterior capsule was positioned on a ring support structure at the base of the dish. Opening of the anterior capsule was achieved by positioning the lens capsule with forceps and making three clockwise incisions that did not extend beyond the lens equator. The capsule was peeled from the lens fiber cell mass using small scissors and the posterior capsule was pinned with the exterior facing the bottom of a 3 mm culture dish and the anterior edge forming a cup-like structure (capsular bag) using six entomological pins (D1, Watkins and Doncaster, Kent, UK). Residual lens fibers were then removed by changing the medium. For immunohistochemistry, capsular bags were fixed with 2% PFA in 1XPBS and 0.1% Triton X-100.

MLE Cell Culture: For generation of a primary mouse lens epithelial cell (MLE) culture, the capsular bag was pinned with the MLE cells facing the bottom of the dish for one week. The lens capsular bag was removed and cells were further expanded. Mouse primary lens epithelial cells derived from capsular bag culture (at passages 10 to 14) were used for the study.
One day prior to plating cells for the experiment, cells were stained with vital dye CFDA-SE (Invitrogen, Grand Island, NY, USA). Briefly, one vial CFDA-SE (Invitrogen, Eugene, Oregon, USA) containing 500 µg was diluted in 90 µL Dimethyl-Sulfoxide. The resulting stock solution was further diluted 1:5000 in 1XPBS prior to addition to the cell culture flask. Following incubation of cells for 15 minutes at 37 °C in the CO2 incubator, the CFDA-SE/1XPBS dye solution was removed and replaced by MEM with combination with 20% FBS. For the non-matrix control, CFDA-SE stained cells were plated in 24-well plates on cover slips (proportional 20 000 cells/well).

For the nanofiber experimental group, cells were plated at proportional 5000 cells/well in 96-well plates with aligned nanofibers (Part#9602, Nanofiber Solutions, Columbus, OH, USA). Following complete cell adhesion (around 2h after plating), the media were replaced to MEM/20%FBS (media control) and MEM supplemented with 2% FBS (MEM/2%FBS, serum starvation group). For the non-matrix control, two samples were analyzed in the media control group and two samples were analyzed in the serum starvation group per day for a total of four days. For the nanomatrix group, six samples were analyzed in the media control group and six samples were analyzed in the serum starvation group per day also for a total of four days.

**Immunohistochemistry:** Capsular bag and MLE culture samples were stained with primary antibodies Aquaporin 0 (AQP0 H-44, sc-99059, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and β-crystallin (β-crys H-3, sc-48335, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 1:250 dilution over night followed by staining with secondary antibodies coupled to cy3 Jackson Immunoresearch at 1:600 dilution for 2 hours. Imaging was performed using a BX51 microscope (Olympus, Center
Valley, PA, USA) under fluorescence setting with a CCD camera (RTKE Spot, Diagnostics Instruments, Inc, Sterling Heights, MI, USA) and imaging software (Spot software version 4.1, Diagnostics Instruments, Inc.).
RESULTS

*Project I: Mouse Surgeries*

Lens-like regenerates (LLRs) were observed in one mouse from group 2 (where the mouse underwent lentectomy followed by the implantation of a hydrogel bead in combination with bFGF) (Figures 4, 5 and 6), and in one mouse from group 3 (where the mouse underwent lentectomy followed by the implantation of a hydrogel bead in combination with bFGF and encapsulating an iris tissue) (Figures 8, 9, 10 and 11). However, and as suspected, no LLR was observed in any of the lentectomized mice from group 1 that served as negative control (data not shown). At 7 weeks, LLRs were small in size when compared to lenses from intact eyes (Figure 15). Nonetheless, both LLRs and intact lenses are positive for α-crystallin, a lens cell specific protein (Figures 5 and 15).

Group 2: The LLRs (pink arrowhead) are positive for α-crystallin (Figures 5 and 6) while no background staining was observed in the negative control (Figure 7). Also, α-crystallin is expressed in a thin layer (white arrowhead) in the hollow area between the pigmented iris and the inner corneal epithelium, suggesting the presence of lens cells lining the iris stroma. DAPI staining is restricted to the thickened retina (yellow arrowhead), corneal epithelium, and to circular areas and the peripheries (grey arrowhead) of the LLRs (Figures 5 and 6).
Group 3: The LLRs (pink arrowhead) are positive for α-crystallin (Figures 9, 10, and 11), while no background staining was observed in the negative control (Figure 12). α-crystallin is also expressed in differentiated lens fibers (Figure 10, yellow arrowhead). CFDA-SE is present in the area surrounding the LLRs (Figures 9 and 10, green arrowheads). DAPI staining is present in the cornea and retina epithelium, and the cells of the LLRs (grey arrowhead) of the LLRs (Figures 9, 10, and 11).

It is important to note that most of the eyes without LLRs from groups 2 and 3 showed abnormal eye structures: detached retina (Figures 13 and 14, yellow arrowheads), thickened retina pigment epithelium (Figure 14, white arrowhead), and non-degraded tissue adhesive (Figure 14, green arrowhead) that failed to promote healing of the cornea and caused damage of the host iris.
Figure 4: Phase Contrast Images of Serial Sections from a Mouse from Group 2.

The micrographs are taken with a 4X objective. The anterior side of the eye is to the right of the image. Purple arrowhead: LLRs. c, f: asterisk (*) indicates that these eye sections were used for immunohistochemistry (Figures 5 and 6, respectively).
Figure 5: Immunohistochemistry for α-Crystallin of Eye Section (Figure 4c) of Mouse from Group 2. a, d, g: phase contrast images. b, e, h: α-crystallin (red) and DAPI (blue). c, f, i: α-crystallin (red) and DAPI (blue) over phase contrast. a, b, c: 4X, d, e, f: 20X, g, h, i: 40X objective lens. d, e, f: magnified area of orange box, g, h, i: magnified area of purple box. The anterior side of the eye is to the right of the micrographs. Arrowheads: white: α-crystallin positive cells in the iris stroma, yellow: thickened retina, pink: LLRs, grey: DAPI-stained cells in LLRs, green: lens capsule.
Figure 6: Immunohistochemistry for α-Crystallin of Eye Section (Figure 4f) of Mouse from Group 2. a, d: phase contrast images. b, e: α-crystallin (red) and DAPI (blue). c, f: α-crystallin (red) and DAPI (blue) over phase contrast. a, b, c: 4X, d, e, f: 20X objective lens. d, e, f: magnified area of orange box. The anterior side of the eye is to the right of the micrographs. Arrowheads: pink: LLRs, grey: DAPI-stained cells in LLRs, green: lens capsule.
**Figure 7: Negative Control for α-Crystallin Staining, Immunohistochemistry of Eye Section of Mouse from Group 2.** a, d: phase contrast images. b, e: α-crystallin (red) and DAPI (blue). c, f: α-crystallin (red) and DAPI (blue) over phase contrast. a, b, c: 4X, d, e, f: 10X objective lens. d, e, f: magnified area of orange box. The anterior side of the eye is to the right of the micrographs. Arrowheads: pink: LLRs.
Figure 8: Phase Contrast Images of Serial Sections from a Mouse from Group 3.

The micrographs are taken with a 4X objective. The anterior side of the eye is to the bottom of the image. Purple arrowhead: LLRs. a, b, g: asterisk (*) indicates that these eye sections were used for immunohistochemistry (Figures 9, 10 and 11, respectively).
Figure 9: Immunohistochemistry for α-Crystallin of Eye Section (Figure 8a) of Mouse from Group 3. a, d, g: phase contrast images. b, e, h: α-crystallin (red), CFDA-SE (green) and DAPI (blue). c, f, i: α-crystallin (red), CFDA-SE (green) and DAPI (blue) over phase contrast. a, b, c: 4X, d, e, f: 10X, g, h, i: 20X objective lens. d, e, f: magnified area of orange box, g, h, i: magnified area of purple box. The anterior side of the eye is to the bottom of the micrographs. Arrowheads: pink: LLRs, grey: DAPI-stained cells in LLRs, green: CFDA-SE-stained area.
Figure 10: Immunohistochemistry for α-Crystallin of Eye Section (Figure 8b) of Mouse from Group 3. a, d: phase contrast images. b, e: α-crystallin (red), CFDA-SE (green) and DAPI (blue). c, f: α-crystallin (red), CFDA-SE (green) and DAPI (blue) over phase contrast. a, b, c: 4X, d, e, f: 20X objective lens. d, e, f: magnified area of orange box. The anterior side of the eye is to the bottom of the micrographs. Arrowheads: pink: LLRs, grey: DAPI-stained cells in LLRs, green: CFDA-SE-stained area, yellow: onset of lens fiber differentiation.
Figure 11: Immunohistochemistry for α-Crystallin of Eye Section (Figure 8g) of Mouse from Group 3. a, d: phase contrast images. b, e: α-crystallin (red), CFDA-SE (green) and DAPI (blue). c, f: α-crystallin (red), CFDA-SE (green) and DAPI (blue) over phase contrast. a, b, c: 4X, d, e, f: 10X objective lens. d, e, f: magnified area of orange box. The anterior side of the eye is to the bottom of the micrographs. Arrowheads: pink: LLRs, grey: DAPI-stained cells in LLRs.
Figure 12: Negative Control for α-Crystallin Staining, Immunohistochemistry of Eye Section of Mouse from Group 3. a, d: phase contrast images. b, e: α-crystallin (red), CFDA-SE (green) and DAPI (blue). c, f: α-crystallin (red) and DAPI (blue) over phase contrast. a, b, c: 4X, d, e, f: 10X objective lens. d, e, f: magnified area of orange box. The anterior side of the eye is to the bottom of the micrographs. Arrowheads: pink: LLRs.
Figure 13: Immunohistochemistry for α-Crystallin of Eye Section of Mouse from Group 2. a, d, g: phase contrast images. b, e, h: α-crystallin (red), and DAPI (blue). c, f, i: α-crystallin (red) and DAPI (blue) over phase contrast. a, b, c, g, h, i: 4X, d, e, f: 20X objective lens. d, e, f: magnified area of orange box. g, h, i: negative control staining for α-crystallin. The anterior side of the eye is to the right of the micrographs. Arrowheads: yellow: detached retina.
Figure 14: Immunohistochemistry for α-Crystallin of Eye Section of Mouse from Group 3. a, c: phase contrast images. b, d: α-crystallin (red), CFDA-SE (green) and DAPI (blue) over phase contrast. a, b, c, d: 4X objective lens. c, d: negative control staining for α-crystallin. The anterior side of the eye is to the right of the micrographs. Arrowheads: yellow: detached retina, white: thickened retina pigmented epithelium, green: non-degraded tissue adhesive.
Figure 15: Immunohistochemistry for α-Crystallin of Eye Section of a Mouse Intact Eye. a, c, e: phase contrast images. b, d, f: α-crystallin (red) and DAPI (blue) over phase contrast. a, b, e, f: 4X, c, d: 20X objective lens. c: magnified area of orange box. e, f: negative control staining for α-crystallin. The anterior side of the eye is to the top (a, b, c, d), or right (e, f) of the micrographs.
MLE form lens-like structures under serum starvation conditions when cultured in regular culture plates:

Previously, mouse lens epithelial (MLE) cells were demonstrated to differentiate into lens fiber cells or lens-like structures (lentoids) under defined cell culture conditions. To confirm the differentiation potential of MLE cells under regular and serum-starved culture conditions, a primary MLE cell line was established through cell expansion from lens capsular explant culture. While cells in regular culture medium (MEM/20%FBS) showed the typical fibroblastic-like cell appearance, cells cultured in serum-deprived medium (MEM/2%FBS) developed lens-like structures starting from day 2 in culture (Figure 16). Conforming to the lens-fiber differentiation process in vivo, the lens-like structures demonstrated nuclear loss in vitro.

MLE cells grown in regular culture medium revealed the expression of aquaporin 0 within the cell nuclei (Figure 16). In contrast, cultures grown in serum starvation for two days demonstrated accumulation of MLE cells into a lens-like structures with decreased aquaporin 0 nuclear staining (Figure 16, white arrowheads), suggesting onset of lens fiber differentiation.

Accumulation of β-crystallin was observed within the lens-like structures starting at day two of serum deprivation in MEM/2%FBS (Figure 16, yellow arrowheads), also supporting theory of onset lens fiber differentiation.
MLE cells demonstrate alignment on PCL nanofibers when cultured in regular and serum-deprive medium:

In contrast to the one-hour usually required for MLE cells to adhere onto the surface of regular cell culture plates, MLE cells adhered rather rapidly (in about 5 minutes) onto plates with aligned PCL nanofibers. Following a one day culture on PCL nanofiber plates, cells demonstrated longitudinal elongation in both, MEM/20%FBS regular culture medium and in MEM/2%FBS serum deprived medium (Figure 17 a, b). While MLE cells cultured in regular culture medium with 20% FBS demonstrated more elongation at day 2 than cells cultured in serum deprivation (Figure 18 c, d), MLE cells cultured in 2%FBS serum deprived medium demonstrated preferential lentoid formation (arrowheads) (Figure 17d, f, white arrowheads). At day 3 of MLE cell culture on PCL nanofiber, cells in regular medium demonstrated further extension into a fiber-like appearance with loss of nuclei (Figure 17 e). In contrast, cells grown in 2% serum deprived medium showed further appearance of lentoids and increased cell death (Figure 17 f).

MLE cells differentiate and align on PCL nanofibers:

To assess the fiber-inducing potential of aligned PCL nanofibers on MLE cells cultured in regular 20% FBS medium and 2% serum-starved medium, MLE cells were stained with the differentiation markers aquaporin 0 (Figure 18) and β-crystallin (Figure 19).

Similar to cells plated on regular culture dishes, MLE cells cultured for one day on PCL nanofibers expressed aquaporin 0 staining within the cell nuclei in both, regular
culture medium and in 2% FBS serum-deprived medium (Figure 18 a, b). In contrast, cells grown for two days on PCL nanofibers demonstrated a more diffused expression of aquaporin 0 throughout the whole cell body in regular and in 2% FBS serum-deprived culture mediums (Figure 18 c, d). At three days in culture on PCL nanofibers, aquaporin 0 expression could be detected within the enhanced accumulation of fiber forming cells in regular and 2% serum deprived mediums (Figure 18 e, f, white arrows) and in lentoids in 2% serum-deprived medium (Figure 18 f, white arrowheads).

Staining for β-crystallin was negative in both, MLE cells cultured in regular medium and 2% FBS serum deprived medium on the first day of cell culture on PCL nanofibers (Figure 19 a, b), an observation similar to that of MLE cells cultured on regular cell culture plates. At the second and third day of MLE cell culture on PCL nanofibers, a slight accumulation of β-crystallin could be found in fiber forming cells in regular culture medium as well as in 2% serum deprived medium [Figure 19 c, d (for day 2), e, f (for day 3)]. In addition, a high accumulation of β-crystallin staining could be found in lentoids following two and three day cultivation in 2% serum deprived medium (Fig. 19 d, f, white arrowheads).
Figure 16: Immunocytochemistry for Aquaporin 0 and β-Crystallin of MLE Cells Grown in MEM/20%FBS Regular Culture Medium and MEM/2%FBS Serum-Deprived Medium on Regular Cell Culture Plate. a, d, g, j: MLE cells grown in MEM/20% FBS regular medium. b, e, h, k: MLE cells grown in MEM/2% serum deprived medium for 2 days. c, f, i, l: MLE cells grown in MEM/2%FBS serum deprived medium for 3 days.
medium for 2 days. a, b, c: AQ0 (red). d, e, f: AQ0 (red), CFDA-SE (green) and Hoechst (blue) merge (in purple). g, h, i: β-crys (red). j, k, l: β-crys (red), CFDA-SE (green) and Hoechst (blue) merge (in purple). a-l: 20X objective lens. Arrowheads: white: lentoid bodies.
Figure 17: CFDA-SE-Stained (Green) MLE Cells Grown on PCL Nanofibers in MEM/20%FBS Regular Medium and MEM/2%FBS Serum-Deprived Medium.

a, c, e,: MLE cells grown in MEM/20% FBS regular culture medium. b, d, f: MLE cells grown in MEM/2% serum-deprived medium. a, b: MLE cells cultured for 1 day. c, d: MLE cells cultured for 2 days. e, f: MLE cells cultured for 3 days. a-f: 20X objective lens. Arrowheads: white: lentoid bodies.
Figure 18: Immunocytochemistry for Aquaporin 0 of CFDA-SE-Stained (Green) MLE Cells Grown in MEM/20%FBS Regular Culture Medium and MEM/2%FBS
**Serum-Starved Medium on PCL Nanofibers.** a, c, e, g: MLE cells grown in MEM/20% FBS regular culture medium. b, d, f, h: MLE cells grown in MEM/2% serum-deprived medium. a, b: MLE cells cultured for 1 day. c, d: MLE cells cultured for 2 days. e, f: MLE cells cultured for 3 days. a-f: AQ0 (red). g,h: AQ0 (red), CFDA-SE (green), and Hoechst (yellow) overlay over phase contrast image of the PCL nanofibers at day 3. a-h: 20X objective lens. Arrowheads: white: lentoid bodies. Arrows: white: elongated lens fibers.
Figure 19: Immunocytochemistry for β-Crystallin of CFDA-SE-Stained (Green) MLE Cells Grown in MEM/20%FBS Regular Culture Medium and MEM/2%FBS
Serum-Starved Medium on PCL Nanofibers. a, c, e, g: MLE cells grown in MEM/20% FBS regular culture medium. b, d, f, h: MLE cells grown in MEM/2% serum-deprived medium. a, b: MLE cells cultured for 1 day. c, d: MLE cells cultured for 2 days. e, f: MLE cells cultured for 3 days. a-f: β-crys (red). g, h: β-crys (red), CFDA-SE (green), and Hoechst (yellow) overlay over phase contrast image of the PCL nanofibers at day 3. a-h: 20X objective lens. Arrowheads: white: lentoid bodies. Arrows: white: elongated lens fibers.
DISCUSSION

*Project I: Mouse Surgeries*

Mice have the ability to regenerate a lens only after part of the anterior and the whole posterior capsules are left behind following the removal of the lens fibers. In this study, mice from which whole intact lenses were surgically extracted (lentectomy) were used. Following lentectomy, some mice (group 1) were left untreated serving as negative control. After 50 days, mice from group 1 showed no regeneration of lens-like regenerates (LLRs), as evidenced by absence of α-crystallin staining. Interestingly, lentectomized mice treated with bFGF by the mean of an implanted hydrogel encapsulating (group 3, n = 14) or not (group 2, n = 11) a CFDA-SE-stained iris tissue revealed (group 1, n = 1 and group 2, n = 1) α-crystallin positive LLRs (group 2: Figures 5 and 6, group 3: Figures 9-11). The LLRs from mice in groups 2 and 3 are similar in shape and size, however, they are irregular in structure and do not resemble the morphology of an intact lens (Figure 15). Intact lenses exhibit a distinct lens cell arrangement in which lens fiber cells at the posterior side of the lens migrate and fill up the bulk of the lens and are covered on their anterior surface by a monolayer of lens epithelial cells. A similar pattern was also observed in the LLRs whereby a lens epithelium covered the exterior of differentiating lens fiber cells. In addition, the LLRs were completely surrounded by a lens capsule that in some cases, folded upon itself
(Figure 6), an aspect typically absent in intact lenses. Lens capsules were also found to be stained positive for CFDA-SE (Figures 9 and 10). It is worth noting that some of the complications at the time of surgery could have contributed to the failed regeneration of the lens in the remaining mice that underwent ocular surgery (from groups 2 and 3). For instance, some bleeding from the iris occurred during the making of the corneal incision. Also, retinal detachment was the result of whole intact lens removal using forceps, and iris tissue might have been caught in the tissue adhesive at the time of corneal closure (Figures 13 and 14).

This study is the first to utilize a biodegradable hydrogel of oligo(poly(ethylene glycol)fumarate) cross-linked with poly(ethylene glycol)-diacrylate in combination with bFGF in an attempt to induce lens regeneration in rodents. This hydrogel have been previously used in newts. The hydrogel encapsulating iris pigment epithelial cells was implanted in lentectomized newts and shown to not inhibit the process of iris pigment epithelial cells transdifferentiation to lens cells in newts, thus, allowing lens regeneration to occur (Zhang, Park et al. 2010).

In vivo and in vitro studies in newts and Xenopus also helped elucidate the important role of exogenous and endogenous FGF in lens regeneration. In newts, FGF triggers lens regeneration from the pigmented epithelial cells of the dorsal iris both in vivo and in vitro (Del Rio-Tsonis, Jung et al. 1997; McDevitt, Brahma et al. 1997; Del Rio-Tsonis, Trombley et al. 1998; Hayashi, Mizuno et al. 2002; Hayashi, Mizuno et al. 2004). In Xenopus, FGF has also been found to be necessary for early induction of lens regeneration from the cornea in tadpoles (Henry and Tsonis 2010). By utilizing hydrogel in combination with bFGF (group 2), this study is the first of its kind to demonstrate the
regeneration of $\alpha$-crystallin positive lens-like structures in the absence of lens capsules in lentectomized mice.

Furthermore, iris pigmented cells from human fetal eye tissues were shown to transdifferentiate into crystallin positive lentoid bodies in vitro (Yasuda, Okada et al. 1978). These findings clearly indicate that mammalian iris has the potential to transdifferentiate to lens as observed in lower vertebrates, such as the newt (Eguchi 1988); an ability that has not been reported in vivo. In this study, CFDA-SE-stained iris pigmented tissue pieces were encapsulated in the hydrogel in combination with bFGF prior to implantation in lentectomized mice (group 3), and found to lead to the formation of LLRs. The resulting LLRs were found be in close proximity to the host pigmented iris or to be in part (i.e., the lens capsule) CFDA-SE-positive stained, thus, suggesting that the iris tissue might be implicated in the process of regenerating the LLRs. The bFGF is believed to be the trigger for the initiation of lens regeneration in mice in vivo, as it has been reported in lower vertebrates.

Further investigation remains to be performed in order to (1) determine the exact structural components that make up the regenerates, and (2) to determine the exact source of the LLRs. For example, pigmented iris tissue can be harvested from GFP mice [of the same genetic background as the wild-type mice to assure biocompatibility] and encapsulated in a hydrogel bead with bFGF and implanted in lentectomized mice. By doing so, if the pigmented iris tissue were the source of the regenerated lens or lens-like structure, then the regenerate will be GFP positive (Figure 17).
When the underlying mechanisms of lens regeneration in mice are fully understood, they will help pave the way towards overcoming the limitation of lens regeneration in humans.

![Diagram](image)

**Figure 20:** Schematic Predicting the Possible Outcome of a Surgery in which the Implanted Pigmented GFP Iris Gives Rise to a Regenerated GFP-Positive Lens
In this study, MLE cells were cultured on aligned PCL nanofibers for one, two and three days, in regular MEM/20%FBS as well as serum-deprived medium (MEM/2%FBS). MLE cells adhered rather quickly onto the aligned nanofibers. Following a one day culture on PCL nanofiber, cells demonstrated longitudinal elongation and organized alignment in both MEM/20%FBS regular culture medium and in MEM/2%FBS serum-deprived medium. At day 2 in culture, the cells grown both in regular 20%FBS and 2% serum-deprived medium continued to elongate on the aligned PCL nanofibers, with preferential formation of lentoid-like bodies observed with cells cultured in MEM/2%FBS. At day 3, MLE cells in regular medium demonstrated further extension into a fiber-like appearance in combination with loss of nuclei, in contrast to cells grown in 2% serum-deprived medium which continued to aggregate into lentoid-like bodies. Increased cell death was also observed at day 3 in MLE cells cultured in 2% FBS serum-deprived medium (Figure 17).

Aquaporin 0 staining was first observed within the cell nuclei of MLE cells in both, regular culture medium and in 2% FBS serum-deprived medium. At day 2 in culture on PCL nanofibers, a more diffused expression of aquaporin 0 throughout the whole cell body of MLE cells in regular and in 2% FBS serum-deprived culture mediums was detected, suggesting onset of fiber differentiation. Aquaporin 0 was also detected in lentoid-like bodies at day 2 in MLE cells cultured in 2% FBS serum-deprived medium. At 3 days in culture, aquaporin 0 expression could be detected within the enhanced accumulation of fiber-forming cells in both culture mediums, and in the lentoid-like
bodies in MLE cells grown in 2% FBS serum-deprived medium (Figure 18). As for β-crystallin, staining was negative in both, regular medium and 2%FBS serum-deprived medium on the first day of MLE cell culture on PCL nanofibers. At the second and third day of culture, a slight accumulation of β-crystallin could be found in fiber forming cells in regular culture medium as well as in 2% serum deprived medium, and by day 3, a high accumulation of β-crystallin staining could be detected in the lentoid-like bodies in the 2% serum-deprived medium cultures (Figure 19).

Poly-ε-caprolactone (PCL) is a semi-crystallin polymer that has gained a lot of attention recently in tissue engineering applications because of its biocompatibility and biodegradability, its slow degradation rate, and its compatibility with a number of cell types. As a result, PCL has been proposed as a suitable material for scaffold fabrication, culture systems, and for tissue engineering applications (Li, Danielson et al. 2003). PCL has been either used alone, or coated with gelatin (Beachley and Wen 2009), collagen (Bender, Bennett et al. 2004; Chen, Ekaputra et al. 2008), starch (da Silva, Crawford et al. 2009), hyaluronic acid (Ekaputra, Prestwich et al. ; Li, Qian et al. 2012) or in combination with poly(lactide-co-glycolide) (PLGA) (Curran, Tang et al. 2009).

Recently, the material has been proposed for use in the form of a nanofibrous scaffold for the modulation of complex cellular types.

In contrast to some studies that used PCL nanofibers in combination with natural materials (i.e., collagen or gelatin) and found that human embryonic stem cells remain in their undifferentiated state (Gauthaman, Venugopal et al. 2009), this study suggests that PCL nanofibers are capable of inducing differentiation of MLE cells and the subsequent alignment of lens fiber cells.
CONCLUDING REMARKS

Over the years, many researchers have approached the problem of lens regeneration from embryological and developmental perspectives. To date, the regeneration of a mammalian lens has not been reported in vivo following the complete removal of the whole intact lens. However, with ample evidence of the conserved potential of mammalian iris pigmented epithelial cell to transdifferentiate in vitro, this study utilized a hydrogel to deliver growth factors in vivo as a mean to induce the transdifferentiation of iris pigmented epithelial cells to lens cells in situ. This approach led to the formation of α-crystallin positive LLRs with irregular shapes, a finding that requires further investigation to elucidate the cellular origin of these regenerates.

This study also demonstrated the ability of aligned PCL nanofibers to induce the differentiation of MLE cells cultured in regular 20%FBS and 2%FBS serum-deprived medium, as well as to align the resulting lens fibers.

In the absence of an intrinsic regenerative ability, the ultimate goal of regenerative medicine is to overcome the limitations of organ regeneration in humans. It is, thus, by the study of the mechanisms underlying the intrinsic regenerative capabilities of certain organs, in combination with tissue engineering applications that lie the indispensable tools towards overcoming the restricted organ and tissue regenerative potential in humans.
BIBLIOGRAPHY

Note: because of the difficulty in obtaining articles from nineteenth century, information from studies dated back to the nineteenth century were obtained through a review by Gwon (Gwon 2006).


Zhao, H., Y. Yang, et al. (2006). "Fibroblast growth factor receptor 1 (Fgfr1) is not essential for lens fiber differentiation in mice." Mol Vis 12: 15-25.


