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Cell Signaling Pathways in Vertebrate Lens Regeneration. Published in “New Perspectives in Regeneration” (E. Heber-Katz and D. L. Stocum, eds)

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Abstract

Certain vertebrates are capable of regenerating parts of the eye, including the lens. Depending on the species, two principal forms of *in vivo* lens regeneration have been described wherein the new lens arises from either the pigmented epithelium of the dorsal iris or the cornea epithelium. These forms of lens regeneration are triggered by retinal factors present in the eye. Studies have begun to illuminate the nature of the signals that support lens regeneration. This review describes evidence for the involvement of specific signaling pathways in lens regeneration, including the FGF, retinoic acid, TGF-beta, Wnt and hedgehog pathways.

1 Introduction: Phenomena of Vertebrate Lens Regeneration

Virtually all multicellular organisms possess the ability to repair damaged tissues. On the other hand, some animals can replace entire organs or even complex body parts. One remarkable example of these phenomena is that of complete lens regeneration, which takes place within the eyes of certain vertebrates, including newts and salamanders, a cobitid fish, as well as frogs in the genus *Xenopus* (examples are shown in Figure 1A–C).

One form of lens regeneration, first observed over 200 years ago, is that of Wolffian lens regeneration that takes place in some adult newts, salamanders and the cobitid fish *Misgurnus anguillicaudatus* (for examples see Figure 1A–B, reviewed by Henry 2003; Roddy and Tsonis 2008; Henry and Tsonis 2010). In these animals, a new lens arises from the dorsal edge of the iris (specifically the pigmented iris epithelium) once the original lens is removed. This process is diagrammed in Figure 2A–D. Wolffian lens regeneration takes place via transdifferentiation, which begins with dedifferentiation of the pigmented iris epithelial cells (PECs), followed by their re-differentiation and patterning to form the new lens. This process is triggered by signals provided by the neural retina once the original lens is removed (reviewed by Henry 2003; Roddy and Tsonis 2008). The presence of the lens plays a role in normally preventing Wolffian lens regeneration, though the mechanistic basis of this is not fully understood (Henry 2003; Roddy and Tsonis 2008; Henry and Tsonis 2010). *In vivo*, only the dorsal iris pigmented epithelium can support complete lens regeneration, although the initial changes associated with dedifferentiation (including depigmentation) do appear to be initiated in ventral iris tissues following lens removal (Roddy and Tsonis 2010).

A different form of lens regeneration occurs in species of frogs in the genus *Xenopus* (i.e., *X. laevis*, *X. tropicalis* and *X. borealis*, see Freeman 1963; Henry and Elkins 2001; Filoni et al. 2006, and reviewed by Henry 2003; Henry et al. 2008; Roddy and Tsonis 2008; Henry and Tsonis 2010). This process has also been documented in the salamander *Hynobius unnangso* (Ikeda, 1936a, b, 1939). In these cases, a new lens arises from cells of the inner or basal layer of the cornea epithelium, and takes place during larval stages. Cornea lens regeneration is diagrammed in Figure 3E–H. Although this process has historically been described as one involving transdifferentiation of cornea epithelial cells, new evidence suggests that this could involve the deployment of uncommitted, basal cornea epithelial stem cells (Perry and Henry 2012). As is the case in Wolffian lens regeneration, the retina provides the key signal(s) that trigger lens regeneration in the cornea (see Henry 2003). These factors are normally prevented from reaching the cornea by the presence of the lens and the inner cornea endothelium, which act as simple blocks to the diffusion of these key retinal factors. Interestingly, only the cornea and the pericorneal epithelium that immediately surrounds the eye are capable of responding to these signals (Freeman 1963). Other ectoderm, outside of these regions, cannot participate in lens regeneration, even if these tissues are implanted directly inside the eye where they can be continuously exposed to the retinal factors.

Together, these data reveal that the capacity to undergo lens regeneration is highly restricted to specific tissues (i.e., lens regeneration competent tissues). Furthermore, the retina provides a key source of paracrine signals that support the process of lens regeneration, which may be involved in initiating this process and/or supporting subsequent stages of lens differentiation, polarization (patterning) and growth. Once the lens begins to form, it may also provide autocrine signals that regulate its own formation. This review addresses the question as to what signaling pathways play important roles in regulating these processes of lens regeneration, and whether these may be shared amongst the different forms of lens regeneration found in these systems.

2 Fibroblast Growth Factor (FGF) Signaling

The FGF Signaling Pathway

The fibroblast growth factor (FGF) protein family members, historically identified through their affinity for heparin, are divided into seven evolutionarily conserved subfamilies (reviewed in Itoh 2007; Itoh and Ornitz 2011). With the exception of one subfamily (FGF11-14), FGFs bind and activate specific subsets of their corresponding receptor isoforms (FGFRs), causing autophosphorylation of FGFRs and downstream signaling (Ornitz et al. 1996; Zhang et al. 2006). The activated FGFRs signal through three main downstream pathways: the phospholipase C-gamma (PLC γ), phosphoinositide 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK) pathways (reviewed in Dailey et al. 2005; Mason 2007; Dorey and Amaya 2010). Of these three pathways, only the phosphoinositide 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK) pathways have been directly implicated in lens development and regeneration (see below, Figure 3A).

FGF Signaling in Lens Development

The FGF pathway has been identified to play important roles in embryonic lens development (reviewed in Donner et al. 2006; Robinson 2006; Gunhaga 2011). In early lens development, the FGF pathway participates in establishing lens-forming competence, bias, and specification. FGF signaling is essential for cell survival in the lens placode, as conditional deletion of FGFR1 and FGFR2 in the mouse lens placode increases apoptosis and prevents lens formation (Garcia et al. 2011). During later eye development, FGF is essential for inducing lens fiber development and establishing proper lens structure (Robinson 2006). A greater concentration of FGF is present in the vitreous humor than in the aqueous humor (Schulz et al. 1993), and normal lens polarity depends upon this FGF concentration gradient (Chamberlain and McAvoy 1997). For instance, perturbation of this gradient through lens-specific overexpression of FGFs in transgenic mice results in disorganized lens development (Stolen et al. 1997; Lovicu and Overbeek 1998; Robinson et al. 1998). In a fascinating recent study, FGF2 addition was required for inducing human embryonic stem cells to differentiate into lentoid bodies, mirroring the requirement of FGF signaling during embryonic lens development (Yang et al. 2010).

FGF Signaling in Wolffian Lens Regeneration

Members of the FGF family and their receptors are expressed in iris tissues during Wolffian regeneration. *fgf1* mRNA is present in the dedifferentiating dorsal iris and regenerating lens (Del Rio-Tsonis et al. 1997), and *fgf2* mRNA levels increase in both the dorsal and ventral iris regions following lens removal (Hayashi et al. 2004). As for the receptors, FGFR1, FGFR2, and FGFR3 are present in the dedifferentiating dorsal iris and early lens vesicle during lens regeneration (Del Rio-Tsonis et al. 1997; McDevitt et al. 1997; Del Rio-Tsonis et al. 1998). Interestingly, FGFR1 is present in the dorsal lens-forming iris but not in the regeneration incompetent ventral iris (Del Rio-Tsonis et al. 1998), and similarly, FGFR3 is present more in the dorsal iris than the ventral iris (McDevitt et al. 1997). The presence of these specific factors in the dorsal iris points to a potential role in lens regeneration.

In fact, the FGF pathway is necessary for Wolffian lens regeneration and proper morphology of the regenerated lens. Application of the small molecule inhibitor of tyrosine kinase activity (SU5402) inhibits FGFRs and lens regeneration, showing the necessity of FGFR function in this process (Figure 3A; Del Rio-Tsonis et al. 1998). Furthermore, the ligands for FGFR2 isoform IIIc appear to be necessary for lens regeneration, as daily injection of a competitive recombinant FGFR2 isoform IIIc (FGFR2/Fc) inhibited lens regeneration (Hayashi et al. 2004). In contrast, injection of soluble recombinant FGFR2(IIIb) did not inhibit regeneration. These results suggest that FGFR2(IIIc) receptor function itself may be essential for lens regeneration, and point to the importance of the ligand specificity of FGFR isoforms (Hayashi et al. 2004). Furthermore, FGFs appear to play a role in lens fiber differentiation and lens structure, similar to the case in development. Application of exogenous recombinant FGF1 or FGF4 after lens removal perturbs regenerated lens polarity and structure, and may result in the formation of multiple lenses (Del Rio-Tsonis et al. 1997; Yang et al. 2005).

Specific FGFs appear to be the key ligands sufficient for inducing Wolffian lens regeneration. An *in vitro* study of newt dorsal iris epithelial cell cultures noted that the addition of FGF2 or FGF4 to culture media was sufficient to induce lens development akin to Wolffian lens regeneration *in vivo*, whereas FGF8, FGF10, EGF, IGF1, and VEGF were not found to be sufficient (Hayashi et al. 2002). This effect by FGF2 and FGF4 was completely inhibited by the FGFR inhibitor SU5402; therefore, these FGFs are likely acting as the key ligand(s) for specific FGF receptors (Hayashi et al. 2002). Likewise, Hayashi et al. (2004) showed the sufficiency of FGF2 for *in vivo* Wolffian lens regeneration. In that study, the functions of members of the FGF family (1, 2, 4, 7, 8, 9, 10), as well as EGF, IGF, and VEGF were examined. Each growth factor was introduced into the anterior chamber of the eye without lens removal, and of those factors tested, only FGF2 was sufficient to induce secondary lens formation from the dorsal iris tissue. Overall, the results above suggest that the FGF signaling plays a key role in initiating Wolffian lens formation. Furthermore, a precise gradient of FGF is necessary for establishing proper lens polarity during subsequent stages of regeneration.

FGF Signaling in Cornea Lens Regeneration

fgf and *fgfr* expression have been observed in eye tissues during *Xenopus* cornea lens regeneration (Fukui and Henry 2011). Though many FGF family members are expressed, a few FGFs (*fgf1*, 8, and 9) are more highly expressed in the retina than in the cornea, and thus could be candidate signals originating from the neural retina that induce lens regeneration. In a microarray analysis comparing differences in expression between lenses, regenerating corneas, and sham operated corneas (where the inner cornea and lens were left intact), *fgf8b* expression was identified to be upregulated in regenerating corneas relative to lens and sham operated corneas (Day and Beck 2011). This raises the possibility of additional autocrine or paracrine FGF8 signaling within the cornea after it has received an initial signal from the neural retina. As for the receptors, *fgfr1*, *fgfr2*, and *fgfr3* mRNA are present in larval corneas, suggesting that any of these could act as the key receptor for induction of lens formation from corneas (Fukui and Henry 2011). Further, from a subtracted cDNA library enriched for genes expressed during lens regeneration, *fgfr3* expression was found to be upregulated in regenerating corneas (Henry et al. 2002; Malloch et al. 2009). In addition, FGFR2 expression has been correlated with lens-forming competence in *Xenopus* epidermal tissues (Arresta et al. 2005). Specifically, FGFR2 (*bek* isoform) was detected by immunohistochemistry in the cornea and pericorneal ectoderm, but not in regeneration incompetent flank epidermis (Arresta et al. 2005). Other downstream FGF pathway markers are also upregulated in regenerating corneas. In particular, *mek2* (a member of the MAPK pathway), *gbr2* (an upstream member of the MAPK and PI3K pathways), and *spry2* (a negative regulator of FGFRs) have been identified in the regenerating cornea (Malloch et al. 2009). Together, these expression data suggest that FGF1, 8, and/or 9 may interact with FGFR1, 2, and/or 3 in the cornea to induce lens regeneration.

There is also functional evidence that the FGF pathway is both necessary and sufficient for cornea-lens regeneration. Similar to observations made for Wolffian lens regeneration above, FGFR function also appears to be necessary for cornea lens regeneration, as addition of SU5402 inhibited lens regeneration (Figure 3A; Fukui and Henry 2011). Furthermore,

exogenous FGF1 appears to be sufficient for inducing lens cell formation within cultured *Xenopus* corneas (Bosco et al. 1994, 1997).

3 Retinoic Acid (RA) Signaling

The RA Signaling Pathway

Retinol (Vitamin A) is the biological source of retinoids in animals, which is delivered to a transmembrane protein located on the cell surface, STRA6 (Stimulated by retinoic acid, Figure 3B). In the cytosol, retinol is bound to Cytoplasmic Retinol Binding Protein (CRBP1), and is oxidized to retinaldehyde by a suite of dehydrogenases such as RDH10, and is subsequently oxidized to retinoic acid by tissue-specific Retinaldehyde Dehydrogenases RALDH1/2/3 (Moloktov et al. 2002a, b). RA is bound by Cellular Retinoic Acid Binding Protein (CRABP) and ultimately transported to the nucleus where it is released. RA exerts its influence by binding to nuclear Retinoic Acid Receptors (RAR α / β / γ), which dimerize with other RARs or Retinoid X Receptors (RXR α / β / γ). These complexes, in turn, bind to specific genomic elements known as Retinoic Acid Response Elements (RAREs), where they act as transcriptional activators or repressors. In the absence of ligand, the aporeceptors generally act to recruit chromatin condensing histone deacetylases, and when RA is bound to the receptors the chromatin is remodeled to enable transcription, via the recruitment of histone acetyl transferases (reviewed by Niederreither and Dolle 2008). RA may also form a complex with CRABP and exit the cell, enabling RA signaling to act in both autocrine and paracrine fashions (reviewed by Cvekl and Wang 2009).

RA Signaling in Lens Development

Retinoic Acid signaling plays important roles in the development of various eye tissues, including the retina, lens, and cornea (Kastner et al. 1994; Enwright and Grainger 2000; Wagner et al. 2000; see also review by Cvekl and Wang 2009). For instance, proper eye morphogenesis itself depends on RA (Hyatt et al. 1996; Molotkov et al. 2006), and studies using the mouse have shown that retinoic acid induces crystallin expression in lens cells (Gopal-Srivastava et al. 1998).

RA Signaling in Wolffian Lens Regeneration

RA has been shown to play a necessary role in Wolffian lens regeneration through the use of small molecule inhibitors such as disulfiram, which inhibits RA-synthesis enzymes (retinaldehyde dehydrogenases), as well as inhibitors of RAR nuclear receptors (Figure 3B). While there is almost certainly a redundancy of function of RAR receptor isoforms within the regenerating lens, RAR α was specifically implicated when the use of a RAR α specific inhibitor, AGN194301, stunted the regenerative capacity of the dorsal iris (Figure 3B, Tsonis et al. 2000; Tsonis et al. 2002). In many cases there was a failure to form a dedifferentiated lens vesicle from the dorsal iris, suggesting a role for RA in the earliest events of regeneration. Furthermore, it is interesting to note that the use of a pan-RAR antagonist (AGN193109, Figure 3B) resulted in some cases of ectopic lens formation, including one case of lens regeneration from the cornea, reminiscent of that which takes place in *Xenopus*. This effect was not seen when the RAR α specific antagonist was used, suggesting that whatever function RAR α might have within the regenerating tissue, it does

not involve defining the site of lens formation. *rara* ($RAR\alpha$) is not detected by *in situ* analysis in unoperated lenses, but it is expressed in the lens epithelial cells and the fiber cells within the regenerating lens, as well as the dedifferentiated lens vesicle that initially buds from the dorsal iris at the start of regeneration (Tsonis et al., 2002). The same expression pattern is true for $RAR\delta$ ($RAR\gamma$), but its functional role has not been specifically investigated in the regenerating lens (Tsonis et al., 2000). Work is needed to reveal what cellular or molecular mechanisms the various RA nuclear receptors control in the context of vertebrate lens regeneration.

Grogg et al. (2005) demonstrated that the normally lens-incompetent ventral iris could be induced to regenerate lenses when transfected with a cDNA expression construct encoding the transcription factor *six-3*, but only when these samples were co-treated with retinoic acid. These treatments forced the ventral iris to adopt patterns of gene expression that are seen only in the dorsal iris, and the authors suggest that it is this “dorsalization” that renders the ventral iris capable of transdifferentiation. Since retinoic acid was necessary for this transformation to take place, it can be reasoned that gene regulation by retinoic acid signaling is critical for conferring lens-competence upon lens-incompetent tissue. Taken together with the earlier studies described above, it appears that retinoic acid is important in all phases of Wolffian regeneration: acquiring/maintaining lens competence, dedifferentiation of the iris, and terminal differentiation of lens fiber cells. Investigations of which gene loci are under the control of RA-mediated chromatin remodeling within the regenerating tissue will cast light upon currently unknown genes or regulatory regions that are a critical part of lens regeneration.

RA Signaling in Cornea Lens Regeneration

The role of retinoic acid in *Xenopus* cornea lens regeneration is less understood. Malloch et al. (2009) identified *rxrg* (encoding $RXR\gamma$) from the subtracted cDNA library, but there is a lack of data regarding either function or expression of any RA nuclear receptors within the larval eye.

4 Transforming Growth Factor β (TGF β) Signaling

The TGF β /BMP Signaling Pathways

Signaling via the Transforming Growth Factor β (TGF β) superfamily of proteins is one of the major pathways of signal transduction in cells, and plays key roles in cell renewal and differentiation, as well as in the formation of complex tissue patterns and animal body plans. The superfamily encompasses many signaling pathways, including TGF β , Bone Morphogenetic Protein (BMP), Growth Differentiation Factor (GDF), Activin, Anti-Muellerian Hormone (AMH), and others. Signaling begins at the cell surface when a ligand, such as BMP, binds and activates Type I and Type II receptors (Figure 3C). This leads to the subsequent phosphorylation of the transducers of the signal, the Smad proteins. Receptor smads (RSmads, e.g., Smads 1/2/3/5/8) form a complex with Co-Smad (Smad4), which then translocate to the nucleus to effect transcriptional changes. Other Inhibitory Smads (ISmads, e.g., Smads 6/7) may act to negatively regulate these pathways (reviewed by Massague et al. 2005; Moustakas and Heldin 2009).

TGF β /BMP Signaling in Lens Development

BMP signaling has a well established role in eye development, including establishing lens competence, lens placode development and induction (Luo et al. 1995; Furuta and Hogan, 1998; Wawersik et al. 1999), and the differentiation of lens fiber cells (Belecky-Adams et al. 2002; Faber et al. 2002). The TGF β ligand is found in the aqueous humor of many animals, including humans (Jampel et al. 1990; Cousins et al. 1991; Granstein et al. 1990) and is known to inhibit lens epithelial cell proliferation *in vitro* (Kurosaka and Nagamoto 1994). It is also important for the repair of wounded lens epithelia (Saika et al. 2001; Saika et al. 2004), but can induce cataract-like effects in explanted lenses (Liu et al. 1994).

TGF β /BMP Signaling in Wolffian Lens Regeneration

Amongst the many members of the superfamily, only BMP signaling has been functionally characterized in the context of lens regeneration. A very striking involvement of BMP signaling in Wolffian regeneration was established when Grogg et al. (2005) enabled the ventral iris to transdifferentiate by treating the eyes with BMP inhibitors (Figure 3C). Specifically, treatment with either Chordin or a BMPRII competitor was sufficient to enable lens regeneration in the ventral iris, even in the absence of *six3* and retinoic acid, as mentioned earlier. Moreover, treating the normally lens-competent dorsal iris with BMP activators, such as BMP-4 and BMP-7, significantly diminished their capacity to regenerate. These results are beautifully consistent with what is known about the role of BMPs in establishing a ventral identity (DeRobertis et al. 2004), and the authors suggest that the ventralizing action of active BMP signaling within the ventral iris prohibits transdifferentiation. While BMP inhibition drove the lens-incompetent ventral iris of the newt to transdifferentiate, it did not have this effect on a related salamander (the axolotl), which is normally not capable of lens regeneration (Grogg et al. 2005). This suggests that the barrier to lens regeneration imposed by ventralizing factors may be a situation unique to the newt. A detailed understanding of the molecular differences in how the newt and axolotl eye structures develop and maintain their irides will provide insight as to why BMPs have different effects on the same tissue in related species. Our current understanding of TGF β superfamily activity is further complicated by a recent study, where several members of the BMP and TGF β pathways were identified amongst genes expressed in the dorsal iris during the dedifferentiation stage of Wolffian regeneration (Maki et al. 2010).

TGF β /BMP in Cornea Lens Regeneration

BMP-5 and a protein known to inhibit BMP signaling, Sclerostin domain-containing protein 1 (SOSTDC1), were both implicated in *Xenopus* cornea-lens regeneration when cDNAs encoding these proteins were identified in a subtracted cDNA library representing genes upregulated in the course of regeneration (Henry et al. 2002; Malloch et al. 2009). The same study implicated the TGF β type III receptor (encoded by *tgfr3*), also known as Betaglycan. This member of the TGF β pathway is a ligand-presenting proteoglycan and can have stimulatory effects on TGF β signaling (Blobe et al. 2001; Santander et al. 2006).

In the case of cornea-lens regeneration in *Xenopus*, BMP inhibition has the diametrically opposite consequence on regeneration as compared to the newt. When BMP signaling was inhibited in the larval *Xenopus* cornea, it drastically lost its ability to regenerate lenses (Day

and Beck, 2011). Through the use of transgenic animals that inducibly express the BMP signaling inhibitor, Noggin, Day and Beck demonstrated that sustained Noggin activity for the first 48 hours of regeneration greatly reduced successful regeneration (Figure 3C). It was shown further that the first stage of lens regeneration (Freeman Stage 1, see Freeman 1963), is not affected by Noggin expression, as the cornea epithelial cells still transform from a squamous to a cuboidal configuration as they do in wildtype regenerating animals, but subsequently these reverted to a squamous state rather than develop a differentiated lens. In light of these findings, it is possible that BMP signaling governs later events specific to the transformation/differentiation of lens cell fates, similar to in those in the newt, but direct molecular evidence of this in *Xenopus* is currently lacking.

5 Wnt Signaling

The Wnt Signaling Pathways

Wnt signaling is typically divided into the canonical Wnt/ β -catenin signaling pathway and the non-canonical Wnt/Planar Cell Polarity (PCP) and Wnt/Calcium pathways (Figure 3D). The canonical Wnt/ β -catenin pathway is centered on the regulation of β -catenin (reviewed in Logan and Nusse 2004; Clevers 2006; MacDonald et al. 2009). In the absence of a Wnt ligand, the β -catenin degradation complex, comprised of Glycogen synthase kinase 3 (GSK3), Casein kinase 1 (CK1), Adenomatous polyposis coli (APC), and the scaffolding protein Axin, form a complex to phosphorylate β -catenin, leading to its degradation via the proteasome. However, when Wnt binds to the Frizzled (FZD) receptor and the Low density lipoprotein receptor-related protein (LRP) co-receptor, signaling is activated. This ultimately inhibits the ability of the degradation complex to phosphorylate β -catenin, allowing it to accumulate and translocate into the nucleus. Once in the nucleus, β -catenin is able to activate transcription through T cell factor/Lymphoid enhancer factor (TCF/LEF) complexes.

Wnt signaling can also activate two non-canonical pathways (Figure 3D). In the Wnt/PCP pathway, Wnt signaling leads to the activation of either Rho or Rac. These small GTPases lead to cytoskeletal changes through Rho-associated protein kinase (ROCK) or gene transcription through the Jun N-terminal kinase (JNK) cascade (reviewed in Gao and Chen 2010). Alternatively, signaling may occur via the Wnt/Calcium pathway. Interaction between the ligand and receptor leads to an increase in the levels of intracellular calcium. This results in the activation of the calcium binding proteins, Calcium calmodulin-dependent protein kinase II (CAMKII) and Protein kinase C (PKC, reviewed in Gao and Chen 2010; De 2011). The latter pathway has not yet been implicated in processes of lens regeneration (see below).

Wnt Signaling in Lens Development

Wnt signaling is involved in many processes during eye organogenesis (reviewed by Fuhrmann, 2008), including the lens. In the early stages of vertebrate lens development, canonical Wnt signaling in the presumptive lens ectoderm needs to be inhibited in order for the initiation of lens formation (Smith et al. 2005; Kreslova et al. 2007; Machon 2010). However, during later stages of lens development, canonical Wnt signaling becomes

required for proper differentiation of the lens epithelium (Stump et al. 2003) and lens fiber cells (Chen et al. 2006). The expression patterns of various components and regulators of Wnt signaling during these processes have been well characterized and include most of the *wnt* ligands, the *fzd* receptors, as well as *dkks* and *sfrps* (Ang et al. 2004; Chen et al. 2004), the latter two being antagonists of the Wnt signaling pathway. More recently, the non-canonical Wnt/PCP pathway has emerged as a player in the organization of the lens through the elongation and orientation of lens fiber cells (Chen et al. 2008; Sugiyama et al. 2010, 2011).

Wnt Signaling in Wolffian Lens Regeneration

While much is known about the role that Wnt signaling plays during lens development, very little is known about the involvement of this signaling pathway during lens regeneration. The sole functional analysis of Wnt signaling during the process of Wolffian lens regeneration was carried out by Hayashi et al. (2006) in the newt. Using RT-PCR of iris tissue collected at various time points during regeneration, Hayashi and colleagues observed the expression of *wnt2b*, *wnt5a*, *fz2*, and *fz4*. Two of these genes, *wnt2b* and *fz4*, showed elevated expression levels in late lens regeneration, specifically in the dorsal iris (day 8 and day 12, respectively). Lens regeneration rates were dramatically reduced when cultured in the presence of FGF2 and the Wnt signaling antagonists DKK1 and SFRP1 (Figure 3D). When WNT3A (a canonical Wnt) was added with FGF2 in culture medium, larger lenses regenerated from the dorsal iris and limited lens regeneration was even observed from the ventral iris, which is not normally capable of regeneration. The findings of this study suggest that canonical Wnt signaling is necessary for lens regeneration from the dorsal iris and is sufficient for lens regeneration to occur in ventral irides that have already initiated the early steps of lens regeneration, which are triggered by FGF2. While a screen of genes expressed during the initiation of depigmentation and proliferation of dorsal and ventral irides did not produce any ESTs representing Wnt pathway members (Maki et al. 2010), the authors point out this could have been due to the particular stage of the irides collected or may have been a caveat of the cloning methods.

Wnt Signaling in Cornea Lens Regeneration

No functional studies have yet been undertaken to examine the role of Wnt signaling during cornea lens regeneration in *Xenopus*. However, Wnt signaling has been implicated in two screens designed to characterize gene expression during this process. As mentioned above, Henry et al. (2002) created a subtracted cDNA library of genes expressed during the process of lens regeneration in *X. laevis* using control corneas and corneas in the first four days of lens regeneration. From this study several ESTs were identified as members of the Wnt signaling pathway, including the ligand *wnt7b*, as well as the Wnt antagonists secreted frizzled-related proteins 3 and 5 (*sfrp3* and *sfrp5*; Malloch et al. 2009). These findings suggest some involvement of Wnt signaling during the process of cornea lens regeneration. The presence of these ESTs is also consistent with what is known about the expression of these genes during vertebrate lens development in mice (see above). For instance, *sfrp3* is expressed in the lens epithelium (Stump et al. 2003; Chen et al. 2004), *wnt7b* and *sfrp5* are expressed in the lens primordia, epithelium, and fiber cells (Ang et al. 2003; Chen et al. 2004).

In a more recent microarray study using a *X. laevis* Affymetrix GeneChip, Day and Beck (2011) identified the differential expression of numerous components of the Wnt signaling pathway during cornea lens regeneration. Furthermore, qRT-PCR analysis of two of these genes, *Fzd7* and *Wnt7a*, revealed significant changes (upregulation and downregulation, respectively) in expression take place during regeneration when compared to sham operated control corneas at three days following surgery. Taken together, the presence of Wnt signaling transcripts in these two studies suggests that Wnt signaling is likely involved in cornea lens regeneration. How closely related these processes may be to those taking place during vertebrate lens formation or Wolffian lens regeneration has yet to be determined.

6 Hedgehog Signaling

The Hedgehog (HH) Signaling Pathway

Hedgehog proteins are secreted signaling molecules that are present in the form of three homologs in vertebrates: Sonic hedgehog (SHH), Indian hedgehog (IHH), and Desert hedgehog (DHH) (Ingham and McMahon 2001). Hedgehog signaling occurs through Patched (PTC), which is a 12-transmembrane receptor that mediates Smoothened activity (Figure 3E). Smoothened is characterized as a heptahelical transmembrane protein that is a functionally divergent member of the G-protein coupled receptor (GPCR) superfamily (Goodrich et al. 1996; Johnson et al. 1996; Stone et al. 1996). Under conditions where hedgehog is limited, Smoothened activity is normally inhibited by PTC. When hedgehog is present, Patched-mediated repression of Smoothened is overcome and Smoothened positively regulates Hedgehog pathway activation. This results in transduction of the hedgehog signal into the cell and attenuates the processing of GLI proteins (van den Heuvel and Ingham 1996; Ingham et al. 2011). Zinc-finger motif transcription factors GLI1, GLI2 and GLI3 are activated and translocated to the nucleus where their accumulation controls transcription of hedgehog target genes (Sasaki et al. 1999). Cell surface proteins also mediate Hedgehog signaling, such as Hedgehog interacting protein (HHIP) and GAS1, which negatively or positively modulate the pathway, respectively (Chuang and McMahon 1999; Allen et al. 2007; Martinelli and Fan, 2007).

HH Signaling in Lens Development

The Hedgehog signaling pathway significantly influences vertebrate embryonic development and is vital for proper eye morphogenesis. More specifically, SHH regulates the spatial expression of *Pax-6* in the ventral forebrain, which is instrumental in establishing the midline and proper formation of the two eyes (Ekker et al. 1995; Macdonald et al. 1995). Further, *Shh* expression in the ganglion cell layer of the developing retina is important for proper retinal organization and differentiation of photoreceptors (Perron et al. 2003). Based on the important role hedgehog signaling plays during embryonic eye development, it would seem likely that this pathway would be involved during lens regeneration.

Hh Signaling in Wolffian Lens Regeneration

One study has explored Hedgehog signaling during newt lens regeneration (Tsonis et al. 2004). In this study, expression data was presented for members of the hedgehog pathway in embryonic lenses, and in adult and regenerating lenses. During development, newt

embryonic lenses express *Shh*, *Ihh*, *Ptc1* and *Ptc2*. Yet, once the adult lens has formed, only one hedgehog related gene (*Ptc1*) is detected in the lens epithelium. However, during the process of lens regeneration, members of the HH pathway (*Shh*, *Ihh*, *Ptc1* and *Ptc2*) are expressed again in the regenerating lens epithelium and lens fibers.

Tsonis et al. (2004) explored the necessity of the Hedgehog pathway with two known hedgehog inhibitors. The first experiment involved the use of a synthetic cyclopamine inhibitor, KAAD, which interferes with the activation of Smoothened and prevents GLI translocation to the nucleus (Figure 3E). In the presence of KAAD, 28.6% of lens regenerates were of smaller size in comparison to a normal regenerating lens. Perturbation of the Hedgehog signaling pathway was also accomplished through the introduction of mammalian cells transiently expressing the Hedgehog interacting protein (HHIP, Figure 3E). Results were similar to those seen for KAAD, and the authors reported that lenses were smaller in 33.3% of cases when compared to normal regenerating lenses. Inhibition of *Ptc1* was demonstrated through semi-quantitative PCR and corroborated the effects of KAAD and HHIP. Further, inhibition of the hedgehog pathway during lens regeneration affected cell proliferation and lens fiber differentiation. Eyes undergoing the process of regeneration in the presence of KAAD had lower rates of cell proliferation, indicating that the Hedgehog pathway regulates cell division during this process. An assay for lens fiber differentiation also showed that those eyes most affected by KAAD did not synthesize β -crystallin, which suggests the Hedgehog pathway is important for proper lens fiber differentiation during regeneration.

In order to better understand the dedifferentiation process (i.e., the earliest events during lens regeneration in newts), Maki et al. (2010) examined dorsal and ventral iris expression profiles early during newt lens regeneration at 8 days after lens removal. While other signaling pathway members were present in their ESTs (e.g., $TGF\beta$ and BMPs), Hedgehog pathway members were not present. However, this absence could best be explained by the stage of irides examined and the fact that pathways important for later lens cell differentiation may not be active this early in the regenerative process (Maki et al. 2010).

Hh Signaling in Cornea Lens Regeneration

No studies have been undertaken to explore the presence or absence of Hedgehog signaling during the process of cornea-lens regeneration in *Xenopus*. The only implication for Hh signaling in *Xenopus* comes from subtracted cornea cDNA library screens during lens regeneration (Henry et al. 2002; Malloch et al. 2009). In those studies a member of the hedgehog pathway, *Ptc1*, was found to be upregulated in the cornea within the first four days of regeneration. While no other members of the Hh family were uncovered during this screen, it is possible that this signaling pathway may participate in the process of lens regeneration in *Xenopus* and this should be explored in future studies.

7 Evidence for Involvement of Other Signaling Pathways in Lens Regeneration

As previously mentioned, a small number of EST surveys have been undertaken to characterize gene expression during the process of lens regeneration in newts (Makarev et al. 2007; Maki et al. 2010) and *Xenopus laevis* (Henry et al. 2002; Malloch et al. 2009; Day and Beck 2011). In addition to the pathways discussed above, those studies implicate additional signaling pathways in these processes of lens regeneration.

Studies of Gene Expression During Wolffian Lens Regeneration

The study by Maki et al. (2010) examined gene expression during Wolffian lens regeneration in the newt *Cynops pyrrhogaster*. A cDNA library was constructed using mRNA isolated from both dorsal and ventral iris tissues collected eight days after lens removal, when the pigmented epithelial cells were undergoing the process of de-differentiation (e.g., depigmentation and proliferation). Aside from the expression of certain elements of pathways described above (i.e., the BMP/TGF-beta pathways), they did not observe significant expression of elements from other signaling pathways.

Though more limited, an earlier study by Makarev et al. (2007) also examined gene expression in dorsal vs. ventral iris tissues collected eight days following lens removal in the newt *Notophthalmus viridescens*. That study used a small microarray representing 373 genes previously found to be expressed in regenerating newt limb blastemas (Atkinson et al. 2006). No evidence for specific signaling pathways was reported for Wolffian lens regeneration in that study.

A proteomic analysis was undertaken by Roddy et al. (2008) to compare protein expression changes in dorsal vs. ventral iris tissues following removal of the lens in the newt *N. viridescens*. They also compared expression for selected proteins in dorsal iris tissues of the axolotl (*Ambystoma mexicanum*), which is unable to regenerate lenses. Overall, a great deal of similarity was found in the expression of numerous proteins in newt dorsal vs. ventral iris tissues, suggesting that both respond similarly to initiate the process of lens regeneration, immediately following removal of the lens. There were, however, some interesting differences. For instance, Epidermal growth factor receptor (EGFR) was found to be significantly up-regulated in dorsal iris tissues of the newt when compared to ventral iris tissues, and EGFR was not found to be expressed in the dorsal iris of the axolotl. Correspondingly, a negative regulator of EGFR signaling, Suppressor of cytokine signaling 4, was found to be up-regulated in newt ventral iris tissues and in the dorsal iris of the axolotl, suggesting that EGFR signaling may play an important role in permitting lens regeneration to take place in the dorsal iris of the newt. Roddy et al. (2008) also detected significant increases in the expression of Protein-serine kinase C α , Calcium/calmodulin-dependent protein serine kinase 2 α , MAP kinase phosphatase 1 and p21 activated serine kinase 3 in dorsal iris tissue of the newt following lens removal.

Studies of Gene Expression During Cornea Lens Regeneration

In the case of cornea lens regeneration in *Xenopus*, Day and Beck (2011) carried out a microarray analysis to characterize gene expression three days following lens removal (using an Affymetrix GeneChip). As described above, that analyses implicated the involvement of the Wnt signaling pathways in lens regeneration. Evidence for other specific signaling pathways was not described. An earlier study of Malloch et al. (2009) revealed that elements of other signaling pathways are expressed following lens removal in *Xenopus*. These included a number genes encoding proteins involved in Rho/Ras signal transduction, including: Rho-related GTP-binding protein RhoB, Rho GDP-dissociation inhibitor 2, Rho GTPase-activating protein 10, Rho-related BTB domain-containing protein 1, and Ras-related protein Rab-4B. In addition, components of the MAPK/ERK signal transduction pathway involved in transducing a number of different signal pathways (including receptor tyrosine kinases such as FGF and EGF receptors) were also expressed (e.g., MAP kinase signal-integrating kinase 2, MAPK activator 2, RAS guanyl-releasing protein and UPF0485 or putative MAPK-activating protein PM18/20/22). Also detected was a mediator of delta-notch signaling (*Mindbomb*).

8 Summary

These studies indicate that lens regeneration is a complex process that appears to involve a number of different signaling pathways that are deployed in many different contexts, including the establishment of lens regeneration competence in specific tissues, the initiation of lens regeneration, and subsequent processes related to lens cell proliferation, patterning and differentiation. Furthermore, there appear to be some interesting differences in the deployment of specific signaling pathways in Wolffian vs. cornea lens regeneration in these animals.

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Figure 1.

Examples of various animals capable of regenerating the lens of the eye. A). The newt *Cynops pyrrhogaster* (the Japanese Fire Belly Newt). B). The cobitid fish, *Misgurnus anguillicaudatus* (the Japanese Weather Loach) C). The tadpole larvae of the frog *Xenopus laevis* (the South African Clawed Frog). See text for further details. Scale bar equals 7mm for A, 15mm for B and 6mm for C.

Figure 2.

Diagrams illustrating the two main processes of lens regeneration. A–D). Steps involved in Wolffian lens regeneration that take place in certain newts and salamanders. This process takes place within the dorsal iris of the adult eye following removal of the lens (B) (i.e., the new lens is derived from pigmented iris epithelial cells that undergo transdifferentiation). Dashed arrows in B and C indicate key retinal signals that support the process of lens regeneration. E–H). Steps involved in cornea lens regeneration that takes place in frogs of the genus *Xenopus* and the salamander *Hynobius unnangso*. The process takes place within cells of the basal layer of the larval cornea epithelium following removal of the lens and perforation of the cornea endothelium (F). Dashed arrows in F and G indicate key retinal signals that support the process of lens regeneration.

Figure 3.

Highly simplified summary diagrams of various signaling pathways known to play roles during vertebrate lens regeneration. Various pathways and pathway members are as labeled. Not all known elements are included here for the sake of simplicity. Specific activators and inhibitors used to manipulate these pathways are also included, and specific synthetic agents are enclosed in ovals. See text for further details.