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Molecular and Cellular Aspects of Amphibian Lens Regeneration

Jonathan J. Henry[1] and **Panagiotis A. Tsonis**[2]

[1]Department of Cell and Developmental Biology, University of Illinois, Urbana, IL 61801

[2]Department of Biology and Center for Tissue Regeneration and Engineering, University of Dayton, Dayton, OH, 45469-2320

Abstract

Lens regeneration among vertebrates is basically restricted to some amphibians. The most notable cases are the ones that occur in premetamorphic frogs and in adult newts. Frogs and newts regenerate their lens in very different ways. In frogs the lens is regenerated by transdifferentiation of the cornea and is limited only to a time before metamorphosis. On the other hand, regeneration in newts is mediated by transdifferentiation of the pigment epithelial cells of the dorsal iris and is possible in adult animals as well. Thus, the study of both systems could provide important information about the process. Molecular tools have been developed in frogs and recently also in newts. Thus, the process has been studied at the molecular and cellular levels. A synthesis describing both systems was long due. In this review we describe the process in both *Xenopus* and the newt. The known molecular mechanisms are described and compared.

1. Background

Among vertebrates the ability of regenerating the eye lens is restricted only to frogs and newts. Such regeneration is also remarkable because the lens is regenerated by transdifferentiation of terminally differentiated somatic cells. Regeneration of the lens in adult newts was observed first by Collucci in 1891 and independently by Wolff in 1895, after whom the process is often called Wolffian regeneration. The process of lens regeneration in frogs was characterized more recently by Freeman (1963). There are, however, some basic differences between regeneration in frogs and newts. Frogs can normally regenerate the lens during a certain period before metamorphosis and the source of the regenerated lens is the cornea. On the other hand, lens regeneration in newts occurs throughout their adult life and is mediated via transdifferentiation of the dorsal iris pigment epithelial cells (PECs). A few other organisms can apparently replace the lens via Wollfian lens regeneration, including cobitid fish (Sato, 1961; Mitashov, 1966). In this review we will outline and compare these processes mainly in both amphibians with a focus on the molecular and cellular aspects. In recent years, advances in molecular technology and manipulation in these animals has led to important understanding of the process and its relevance to regenerative biology in general, and positions these models as excellent systems in which to undertake further studies.

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Correspondence: j-henry@illinois.edu, panagiotis.tsonis@notes.udayton.edu.

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2. LENS REGENERATION IN *Xenopus*

2.1. Morphological events

Though not as extensive as the forms of regeneration displayed by certain urodele amphibians (e.g., newts and salamanders), some species of anurans (frogs) exhibit the capacity to regenerate certain structures such as the intestine, larval tail (including the spinal cord, and muscles), limb structures, and even parts of the eye (including the retina, and lens; reviewed by Henry, 2003; Slack et al., 2004, 2008; Callery, 2006; Tseng and Levin, 2008; Henry et al., 2008; Beck et al., 2009; Filoni, 2009). The most widely studied species are African frogs of the genus *Xenopus*. One species in particular, *Xenopus laevis,* has been used for over 60 years as an experimental model system in studies of development and regeneration (Kay and Peng, 1991; Tinsley and Kobel, 1996; Gurdon and Hopwood, 2000; Beck and Slack, 2001; Henry, 2003; Callery 2006; Henry et al., 2008). More recently, many researchers have adopted a related species, *X. tropicalis*. The latter has certain advantages that include a shorter generation time and *X. tropicalis* is a true diploid, in contrast to the allotetraploid condition found in *X. laevis*. These features and the smaller genome size favored the selection of *X. tropicalis* for genome sequencing, which was been completed by the U.S. Department of Energy's Joint Genome Institute (http://genome.jgi-psf.org/Xentr4/Xentra4.home.html). Given the recent advances in high-throughput sequencing technology, an effort is being mounted to sequence the genome of *X. laevis* (http://www.xenbase.org/common/), which will serve as an additional resource for molecular level studies, like those described below.

Species in the genus *Xenopus* are the only frogs known to regenerate lenses (reviewed by Henry, 2003; Filoni 2009). Lens regeneration in *X. laevis* was first described by Freeman (1963) and differs significantly from that of Wolffian lens regeneration, which has been well described for newts and salamanders (discussed below). Following removal of the original lens in *Xenopus* tadpoles, the inner layer of the outer corneal epithelium undergoes a series of transformations to re-form a new lens. Freeman sub-divided this process into 5 stages, which are illustrated in Figure 1. This process has been described as one of *transdifferentiation* (metaplasia) of the corneal epithelium (though see further discussion, below), and shares many similarities to that of embryonic lens formation, but there are also some interesting differences (Henry, 2003; discussed below). Lens formation typically occurs in the cornea epithelium that lies directly over the pupillary opening and requires secreted factors provided by the neural retina (Freeman, 1963; Filoni et al., 1981, 1982, 1983). Under normal circumstances, the physical presence of the inner cornea endothelium (derived from neural crest) and the lens prevent these factors from reaching the cornea epithelium (Freeman, 1963; Filoni et al., 1997; Henry and Elkins, 2001). Removal of either barrier alone will not initiate lens regeneration (Bosco et al., 1980; Filoni et al., 1980; Cioni et al., 1982). Furthermore, experiments reveal that the lens and inner cornea do not produce inhibitors of lens regeneration. For instance, regeneration of a new lens will occur in the presence of the original lens provided an opening is created that allows for retinal factors to reach the corneal epithelium (Reeve and Wild, 1978; Filoni et al., 1978, 1980; Bosco et al., 1979, 1980). The process of lens regeneration can be recapitulated in culture (Bosco et al., 1993, 1994, 1997a,b). For example, transdifferentiation will occur in co-cultures of cornea epithelial and neural retina tissues (Figure 2D). In fact, the efficiency is nearly as good, or even better than that which occurs in vivo. This represents a very convenient system in which to study this process under a more controlled set of conditions. Direct contact between the cornea epithelium and the neural retina does not appear to be required for lens regeneration to take place, as lens cell differentiation will occur in cultures of corneal epithelium grown in retina-conditioned media (Bosco et al., 1997a). Together, these findings indicate that the retinal tissue provides diffusible factors sufficient to support lens regeneration.

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Through a variety of tissue transplantation experiments, it has been shown that only the larval cornea and the pericorneal epidermis, which includes a combined area twice the diameter of the eyecup, has the potential to form lens cells in *X. laevis* (Freeman 1963). The competence of this larval tissue to respond to these signals is established by developmental history, which includes the initial series of early and late embryonic lens inductive interactions and the presence of the eyecup (Bosco and Filoni 1992; Cannata et al., 2003; Arresta et al., 2005). Once competence is established, the continued presence of the eyecup does not appear to be required to maintain this condition (Cannata et al., 2003), since the eye can be removed late during embryogenesis or from the older larvae and the overlying tissues will still retain the ability to respond if transplanted into the vitreous chamber.

The success and extent of lens regeneration gradually decreases as the larvae approach metamorphosis (Freeman, 1963, Filoni et al., 1997). This appears to be due to the increasing rapidity with which the inner corneal endothelium heals back to cover the pupillary opening, and may also be related to the extent of differentiation of the cornea epithelium. Experimentally, one can demonstrate that the capacity to differentiate lens cells is still present in the corneal epithelium throughout this time interval and apparently even after metamorphosis, if one exposes this tissue to the key retinal factors via transplantation into the vitreous chamber (Freeman and Overton, 1962; Filoni et al., 1997). Likewise, the factors provided by the neural retina are still present in adult eyes (Bosco et al., 1992). Researchers have demonstrated that corneal epithelium of postmetamorphic frogs can undergo transdifferentiation, if implanted into the vitreous chamber of either pre- or post-metamorphic frogs (Freeman and Overton, 1961; Bosco and Willems, 1992; Bosco et al., 1992; Filoni et al., 1997). More recently, Yoshii et al. (2007) observed examples of lens regeneration in adult *Xenopus* and suggested that these may arise from small numbers of lens epithelial cells that remain in the eye following incomplete removal of the original lens (though no attempt to trace these cells was made in that study to prove this claim).

X. tropicalis is also capable of regenerating lenses though the success rate is much lower than that found in *X. laevis* (Figure 2A–C; Henry and Elkins, 2001). In the former species the inner corneal endothelium heals more rapidly compared to that in *X. laevis*, and this tends to cut off the critical signaling factors required to support lens regeneration. Another species, *X. borealis* does not normally regenerate a lens following lentectomy, due to the rapidity with which the inner cornea heals back to cover the pupillary opening (Filoni et al., 2006). On the other hand, the corneal epithelium of *X. borealis* is capable of forming a lens if implanted directly into the vitreous chamber where it can be continuously exposed to the key inducing factors. In contrast, the corneal epithelium of other frog species, (e.g., members of the genera *Rana, Bufo, Hyla* and *Disscoglossus*) do not appear to have the competence to respond to these inductive signals; however, it appears that the eyes of all frog species examined do produce the factors required to support regeneration, as established in xenoplastic (cross-species) transplantation experiments (reviewed by Henry and Elkins, 2001; Henry, 2003 and Filoni et al., 2006).

As mentioned above, the new lens normally arises from the tissue that lies directly over the pupillary opening. This suggests that the signals that trigger lens regeneration are either short ranged and/or the response is concentration dependant. The initial observations made by Freemen (1963) indicate that lens-forming transformations initially occur at multiple foci within this region (each reaching stages 1–2, refer to Figure 1 for description of these stages), however, only one of these foci eventually forms a lens. The other foci appear to regress. Rarely does one see the formation of multiple lenses. This suggests that some mechanism may operate to ensure that only one lens is ultimately formed, possibly involving some form of lateral inhibition (see further discussion below).

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2.2. Molecular Aspects of Lens Regeneration in Xenopus

Patterns of crystallin expression have been characterized during lens regeneration in *X. laevis* and some interesting differences have been found compared to the situation encountered during embryonic lens formation. The use of antibodies reveals that α- and β-crystallin proteins are detected in both fiber cells and lens epithelial cells beginning at Freeman stage 4 of lens regeneration, while γ-crystallin proteins are apparently restricted only to lens fiber cells (Campbell, 1965, Brahma and McDevitt, 1974; Campbell and Truman, 1977, Reeve and Wild, 1978; Brahma, 1980; Henry and Mittleman, 1995). During embryonic lens development translation of various crystallins, however, is restricted to lens fiber cells, beginning around stage 29/30 of development (stages of Nieuwkoop and Faber, 1956). The transcription of certain crystallin genes has also been examined during lens regeneration (Schaefer et al., 1999; Mizuno et al., 1999a, Malloch et al., 2009). Again, some interesting differences have been observed compared to the patterns of expression detected during embryonic stages. For instance, αA-, and βB1-crystallin transcripts are first detected in presumptive lens fiber cells of the regenerated lens vesicle (middle to late Freeman stage 3), and subsequently only in differentiated lens fiber cells at later stages. γ-crystallin transcripts are not detected until early Freeman stage 4 of regeneration and only in lens fiber cells. However, during embryogenesis, αA-βB1- and γ-crystallin transcripts are detected simultaneously in the lens placode, and only in differentiated lens fiber cells at later stages of development. These finding indicate that there are some differences in the regulation of crystallin expression during regeneration vs. development of the lens. In contrast, Mizuno et al. (2005) demonstrated that the expression of βB1-crystallin during lens regeneration requires the same promotor elements as those required during embryonic lens development, suggesting that elements of a shared regulatory network appear to be operating in both of these lens-forming processes.

A large number of transcription factors play key roles in eye and lens development (Beebe, 1994; Wride, 1996; Cvekl an Piatigorsky, 1996; Graw, 1996; 2003; Oliver and Gruss, 1997; Tomarev, 1997; Fini et a., 1997; Jean et al., 1998; Kondoh, 1999; Ogino and Yasuda, 2000; Wawersik and Mass, 2000; Hanson, 2001; Chow and Lang, 2001; Zhang et al., 2002; Graw and Loster, 2003; Zuber et al., 2003; Zaghloul et al., 2005; Lupo et al., 2006; Adlar and Canto-Soler, 2007; Cvekl and Duncan, 2007), including *Otx2, Pax6, Sox3 Pitx3*, and *Prox1* as well as others. For instance, *Otx2* encodes an orthodenticle-related transcription factor that contains a bicoid class DNA-binding homeodomain. Otx2 acts as a “head-field selector” and is required for the development of the forebrain and midbrain (Chow and Lang, 2001, Ogino et al., 2008). *Otx2* is expressed early in embryonic head ectoderm, including the presumptive lens ectoderm (Pannese et al., 1995; Kablar et al., 1996; Zygar et al., 1998), and is required for the formation of the lens and retinal pigmented epithelium in mice (Martinez-Morales et al., 2001). Otx2 has recently been shown to play an important role in regulating Notch induced *FoxE3* expression (a forkhead transcription factor related to *Xenopus lens1*), which is essential for lens formation (Kenyon et al., 1999; Ogino et al., 2008). Pax6 represents a central transcriptional regulator of eye development, containing two separate DNA binding domains that include a homeodomain and a paired box (Ashery-Pedan and Gruss, 2001). *Pax6* is expressed early during development in a relatively broad area of anterior embryonic ectoderm and plays specific roles in eye development and lens cell differentiation, including the regulation of crystallin gene expression (Pannese et al., 1995; Kablar et al., 1996; Cvekl and Piatigorsky, 1996; Zyger et al., 1998; Schaefer et al., 1999; Cui et al., 2004). As is the case in other organisms, ectopic expression of *Pax6* leads to the formation of ectopic eyes and lenses in *Xenopus* (Altmann et al., 1997; Chow et a., 1999). Sox3 is a member of the SOX family of SRY testis determining factor family of HMG box transcription factors known to play important roles in eye development (Penzel et al., 1997; Furuta and Hogan, 1998; Zyger et al., 1998; Ashery-Pedan et al., 2000; Koster et al., 2000).

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Sox proteins play roles in regulating crystallin expression (Uwanogho et al., 1995; Kamachi et al., 1995, 1998, 2001; Nishiguchi et al., 1998; Chow an Lang, 2001; Ishibashi and Yasuda, 2001). Pitx3 is a paired-like (bicoid) homeodomain containing transcription factor expressed early in presumptive and placodal lens ectoderm (Chang et al., 2001; Pommereit et al., 2001). *Pitx3* and a related gene *Pitx1* are required for normal lens and retinal development (Semina et al., 2000; Khosrowshahian et al., 2005; Shi et al., 2005; Medina-Martinez et al., 2009). Another gene, *Prox1* encodes a prospero-like transcription factor, which is initially expressed in the lens placode (Oliver et al., 1993, Tomarev et al., 1996, 1998; Schaefer et al., 1999; Chow and Lang, 2001; Reza et al., 2002). Prox1 is important for lens fiber cell differentiation (Wigle et al., 1999) and serves to activate crystallin expression (Cvekl and Piatigorsky, 1996; Ring et al., 2000).

Significantly, *Otx2, Pax6, Sox3, and prox1* are all expressed during lens regeneration in *Xenopus* (Schaeffer et al., 1999; Mizuno et al., 1999b; Cannata et al., 2003). Furthermore, Henry et al. (2002) showed that *Pax6* is expressed in larval corneal epithelium prior to removal of the lens. Following lens removal, *pax6* expression appears to be up-regulated in the cornea and regenerating lens (Schaefer et al., 1999; Mizuno et al., 1999b; Cannata et al., 2003). Gargioli et al. (2008) examined the expression of *otx2, Pax6, Sox3, pitx3, prox1* and *βB1-cry* prior to lens removal in *Xenopus*. These investigators noted that only *Pax6* is expressed in the larval ectoderm, and further, its expression is restricted to the lentogenic area that includes both the corneal and pericorneal ectoderm. They further demonstrated that injections of exogenous *Pax6* in early embryonic cells (at the 2-cell stage) trigger ectopic, endogenous *Pax6* expression. Presumably this occurred through auto-regulation of *Pax6* expression, though they did not actually demonstrate that the injected, exogenous message was translated. This treatment, however, was apparently sufficient to impart lentogenic capacity in flank epidermis, as assayed by transplanting this tissue inside the vitreous chamber. These findings suggest that *pax6* expression is linked with the acquisition of lens regeneration competence in larval ectoderm. Other researchers have described patterns of *Pax6* expression as being linked with embryonic lens-forming competence in other vertebrate systems (e.g., chicken and rat, Li et al., 1994; Fujiwara et al., 1994).

In order to further characterize the molecular level events that underlie the process of lens regeneration in *Xenopus laevis*, a subtracted cDNA library enriched for genes expressed during the first four days of this process was prepared (Freeman stages 1–3; Henry et al., 2002; Malloch et al., 2009). Representative clones were sequenced and the information was submitted for BLAST analysis to characterize gene expression during the early stages of lens regeneration. A searchable database of information pertaining to each of these sequences is available on-line at www.life.illinois.edu/henry/EST_databases.html. A total of 734 unique genes were identified. The assemblage of genes is typical of those associated with metazoan organismal development and includes a high concentration of genes involved in regulating transcription and signal transduction. Groups with more prevalent representation include transcription factors, proteins involved in RNA synthesis and processing, integral membrane and transmembrane proteins, components of a number of conserved signaling pathways, proteins involved in protein processing, transport and degradation, proteins involved in matrix remodeling including a number of matrix metalloproteases (MMPs), regulators of the cell cycle, metabolism, apoptosis, inflammation and immune responses, proteins involved in chromatin remodeling in addition to known lens proteins (e.g., crystallins).

Of the transcription factors identified in this set, a number have not been previously implicated in the process of lens formation; though some, including eyes-absent homolog 2 and Prox1, are clearly important for eye/lens formation (Malloch et al., 2009). On the other hand, elements of several signal transduction cascades are also present, which have

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previously been shown to play important roles in lens development, regeneration and differentiation, including the Bone Morphogenetic Protein (BMP), Delta-Notch, Wnt, FGF, Hedgehog, TGF-beta, Rho-Ras and retinoic acid signaling pathways (Manns and Fritzsch, 1991; Hyuga et al., 1993; Kastner et al., 1994; Bosco et al., 1994, 1997b; Kodama and Eguchi, 1995; Graw 1996; Cvekl and Piatogorsky, 1996; Li et al., 1997; McDevitt et al., 1997; Del Rio Tsonis et al., 1997; 1998; Gopal-Srivastava et al., 1998; Enwright and Grainger, 2000; Wagner et al., 2000; Tsonis et al., 2000, 2002; 2004; de Inogh et al., 2001; Onuma et al., 2002; Kawamorita et al., 2002; Stump et al., 2003; Beebe et al., 2004; Ang, et al., 2004; Lang, 2004; Lupo et al., 2005; Lovicu and McAvoy, 2005; Grogg et al., 2005, 2006 ; Chen et al., 2008).

Expression patterns for most (703) of the genes identified by Malloch et al. (2009) were examined during embryonic development. 634 of these exhibited some embryonic expression, Significantly, 57.7% of the latter are expressed in developing eye tissues and nearly half (46.8%) included the developing lens. These findings indicate that there are significant similarities, as well as some differences, between the processes of lens development and lens regeneration. Some of the differences may be associated with the processes of wound healing, as well as cellular de-differentiation that may be associated with lens regeneration (Carinato et al., 2000; Henry et al., 2002; Malloch et al., 2009). It will be important to decipher the roles of those genes that only appear to be expressed in regenerating lenses.

Comparison of the genes expressed during lens regeneration in *Xenopus* with those expressed in other regenerating tissues/organisms, did not reveal a substantial conserved core of genes that appear to underlie different regenerative phenomena (Malloch et al., 2009). There are, however, some commonalities. For instance, MMPs appear to be expressed in a number of different regenerating tissues, including regenerating lenses (Yang an Bryant, 1994, Miyazaki et al., 1996; Yang et al., 1999; Kerif et al., 1999; Carinato, et al., 2000; Kato et al., 2003; Vinarsky et al., 2005; Odelberg, 2005; Malloch et al., 2009). In addition, elements of the stress, inflammation and immune response pathways also appear to be expressed in many regenerating systems, including *Xenopus* lens regeneration (Lu and Richardson, 1991; Meyer-Franke et al., 1998; Leon et al., 2000; Patruno et al., 2001; Imokawa and Brockes, 2003; Harty et al., 2003; Imokawa et al., 2004; Mescher and Neff, 2005; Makino et al., 2005; Kanao and Miyachi, 2006; Brockes and Kumar, 2006; Filbin 2006; Godwin and Brockes, 2006; Mescher et al., 2007; Pearl et al., 2008; Malloch, et al., 2009). These researchers propose conflicting hypotheses as to whether stress, inflammation and immune response pathways stimulate or inhibit regeneration.

2.3. Signaling Factors Involved in Xenopus Lens Regeneration

The inductive signals that trigger lens regeneration have not yet been identified. There is some evidence, however, that they may be similar to those that are responsible for embryonic lens induction (Henry and Mittleman, 1995; Cannata et al., 2003, 2008; Arresta et al., 2005). Studies indicate that members of the FGF family and specific BMPs (BMP4, and BMP7) play key roles in eye and lens development (Furuta and Hogen, 1998; Belecky-Adams et al., 2002; Chow and Lang, 2001; Yang, 2004; Esteve and Bovolenta, 2006; Cvekel and Duncan, 2006; Wordinger and Clark, 2007; Adler and Canto-Soler, 2007).

Using cultures of isolated larval corneal epithelium, Bosco et al., 1994, 1997b; demonstrated that addition of FGF1 appears to be sufficient to trigger lens cell differentiation. Furthermore, Arresta et al. (2005) found that a commercial polyclonal antibody that recognizes the FGFR2 receptor (*bek* variant) labels stage 53 larval cornea epithelium but not dorsal head or flank epidermis (stages of Nieuwkoop and Faber, 1956). In addition, this antibody was found to label dorsal head epidermis if this was previously subjected to eyes

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implanted at stage 46 of larval development. These observations link FGFR2 expression with the acquisition of lentogenic capacity, and provide further evidence that FGF signaling may be involved in lens regeneration in *Xenopus*. The Henry lab (University of Illinois, Urbana-Champaign) also has several lines of evidence that FGF signaling is functionally required *in vivo* for lens regeneration in *Xenopus laevis* (Fukui and Henry, unpublished data).

In another set of studies, Dr. Caroline Beck's lab (University of Otago, New Zealand) has shown that over-expression of Noggin in transgenic *Xenopus* larvae inhibits lens regeneration, suggesting that elements of the TGF-β signaling pathways, specifically those involving the Bone Morphogenetic Proteins (BMPs) play an important role in this process (Caroline W. Beck, personal communication).

As mentioned above, the early observations of Freeman (1963) suggest that multiple, small populations of cells scattered over the pupillary opening begin to undergo transdifferentiation (reaching Freeman stages 1–2) but only one of these foci appears to form the news lens. An element of the Delta-Notch signaling pathway known to play roles in lateral inhibition and boundaries-inductive mechanisms (Artavanis-Tsakonas, et al., 1999; Schweisguth, 2004; Gazave et al., 2009) is expressed during lens regeneration (including *Mind bomb homolog 1*, which encodes a E3 ubiquitin protein ligase known to regulate Delta-mediated Notch signaling, Malloch et al., 2009). Perhaps Delta-Notch signaling plays a role in regulating the development of these foci. As argued above, some mechanism may operate to ensure that only a single lens is ultimately regenerated. A similar mechanism could also function in the context of embryonic lens development given that a larger field of competent tissue is established early during development than actually generates the developing lens. As mentioned above, direct contact between the cornea epithelium and the retina is not required for lens regeneration, and this might preclude the involvement of delta-notch signaling in that particular interaction.

3. Studies of Lens Regeneration and Development in *Xenopus*

As a model vertebrate system, *Xenopus* offers significant advantages for the study of regeneration, which have been extolled in a number of recent reviews (Slack et al., 2004, 2008; Callery, 2006; Tseng and Levin, 2008; Henry et al., 2008; Beck et al., 2009; Filoni, 2009). Tremendous molecular and genomic resources exist for this system (Klein et al., 2002, 2006; Carruthers and Stemple, 2006). Likewise, *Xenopus* has been used widely in the study of eye development (Henry et al., 2008). Maintenance of a colony of adult frogs is rather simple and inexpensive, and the adults provide an abundant supply of embryonic material, which is available year-round. Furthermore, the large eggs and embryos develop externally and rapidly. In addition, a vast array of molecular tools are available to facilitate studies of gene function in these animals. As previously mentioned, these include the availability of a sequenced genome (http://genome.jgipsf.org/Xentr4/Xentra4.home.html). The *X. tropicalis* genome has a high number of regions with long stretches of synteny with mammalian genomes (each typically a hundred or more genes). Hence, it is evolutionarily well positioned to identify conserved elements in vertebrate genomes (e.g. Ogino et al., 2008).

It is a relatively simple matter to carry out both gain- and loss-of-function analyses to examine the necessity and sufficiency of specific genes. For example morpholinos may be used to knockdown translation of specific messages (Ekker, 2000; Corey and Abrams, 2001; Nutt et al., 2001; Heasman, 2002; Gene Tools, LLC, Philomath, OR; www.gene-tools.com). Affymetrix produces micro-arrays to study gene expression in both *X. laevis* and X. *tropicalis* (www.affymetrix.com). A reverse genetics/TILLING resource (Stemple, 2004) at

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the Sanger Centre (Cambridge, UK) has recently led to the identification of the first null mutation in the *Rx* gene (a key homeobox regulator of eye formation, S. Carruthers, D. Stemple and R. Grainger. University of Virgina, Unpublished, see also Mathers et al., 1997) and other mutations are in the initial stages of characterization.

The relative ease with which one can prepare transgenic frogs is a tremendous benefit and facilitates molecular functional studies at more advanced stages of larval development, which is essential for studying regenerative phenomenon (Kroll and Amaya, 1996; Amaya and Kroll, 1999; Amaya et al. 1998; Huang et al., 1999; Marsh-Armstrong et al. 1999; Offield et al., 2000; Sparrow et al., 2000; Hirsch et al., 2002; Ogino et al., 2006; Pan et al., 2006; Slack et al., 2008: Beck et al., 2009). For instance, transgenic frogs carrying various genes under the control of the heat-shock (*hsp70*) promoter (see Wheeler et al., 2000) have been used to study tail and limb regeneration (Beck and Slack, 2001; Beck et al., 2003; 2006; Slack et al., 2004; Lin and Slack, 2008). Beck et al. (2003, 2006, see also Slack et al., 2004) investigated the activity of the BMP and Notch signaling pathways during spinal cord and muscle regeneration in the tail and limb, akin to the unpublished study by Beck's lab that examined lens regeneration (as described above). Knocking down either BMP or Notch inhibited regeneration while activation of either promoted regeneration. These experiments showed further that BMP acts upstream of Notch in spinal cord regeneration, but these two pathways appeared to act independently in the case of muscle regeneration. Lin and Slack (2008) also used transgenic frogs to show that FGF and Wnt signaling are important in *Xenopus* tail regeneration by expressing the Wnt inhibitor dkk1 and a dominant negative (truncated) form of an FGF receptor under the control of the *hsp70* heat-shock promoter.

Tissues isolated from transgenic frogs carrying a GFP reporter driven by the cytomegalovirus (CMV) promoter have been used in transplantation experiments to follow the fates of cells that contribute to regenerated tissues in the tail (Gargioli and Slack, 2004). This study demonstrated that notochord and spinal cord both regenerated from prexisting notochord and spinal cord stump tissues, respectively. However, muscles arose from a population of satellite "stem" cells, rather than from pre-existing myofibers. Chen et al. (2006) showed further that this population of satellite cells specifically express *Pax7*. Using a similar approach, Lin et al, (2007) showed that melanophores do not arise from a pre-existing population of unpigmented, melanophores precursors during tail regeneration. The cre/lox system has also been employed to generate transgenic frogs that express yellow fluorescent protein as a muscle specific lineage marker (under control of the *cardiac actin* promoter) to show that differentiated muscle cells do not undergo transdifferentiation to form other cell types during tail regeneration (Ryffel et al., 2003). Transgenic *Xenopus tropicalis* lines carrying a **γ**1-crystallin promoter driving lens GFP expression have proven to be very useful for the study of lens development and regeneration (Offield et al. 2000; Henry and Elkins, 2001, see Figure 2A–C). Labs, including our own, are now applying these transgenic approaches to study lens regeneration.

Finally, tremendous NIH-funded community web resources are available that provide valuable information on *X. laevis* and X. *tropicalis* biology and genomics (e.g, "Xenbase," www.xenbase.org and see also "Xine," http://blumberg.bio.uci.edu/xine/index.htm). A long-standing course taught at Cold Spring Harbor covers topics related to the cell and developmental biology of *Xenopus*, and the Embryology and Physiology Courses taught at the Marine Biological Laboratory also cover *Xenopus* biology, training future generations of scientists in the use of this system. These resources continue to position *Xenopus* as a key system for the study of regeneration and development for many years to come.

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4. LENS REGENERATION IN NEWTS

4.1. Morphological events

In the case of the newt, lens regeneration involves transdifferentiation of the pigmented epithelial cells of the iris (PECs). Following removal of the lens, the PECs re-enter the cell cycle, dedifferentiate, and lose their characteristic pigmentation. *In vivo*, regeneration occurs from the dorsal iris population of PECs. Despite the apparent similarity between the dorsal and ventral PECs, the ability to regenerate through transdifferentiation under normal conditions, belongs exclusively to the dorsal PECs. Interestingly, however, culturing of both dorsal and ventral PECs results in lentoid body formation (Del Rio-Tsonis and Tsonis, 2003; Tsonis and Del Rio-Tsonis, 2004). This means that the ventral PECs have the potential for transdifferentiation, but this is not permited *in vivo*.

Early events of dedifferentiation occur through day 8 of the regeneration process. Cell proliferation is initiated by day 4 post-lentectomy. Visible dedifferentiation and loss of pigment from the PECs begins by day 8. By day 10 of the process, a lens vesicle has formed, which represents the precursor to what will become a fully differentiated lens. After the lens vesicle has formed, the posterior cells start to elongate, express crystallins and differentiate to lens fibers. The anterior cells become lens epithelium (Figure 3) (Eguchi, 1963; Eguchi, 1964).

Once lens differentiation has started the process is remarkably similar to lens development (as far as the sequential appearance of the different crystallins is concerned). Indeed, studies using antibodies to crystallins or cDNA probes have conclusively shown that there is a parallel of their synthesis during developing and regenerating lens. For instance, αA, βB1 and γ-crystallins appeared all at the same time at the ventral-posterior portion of the lens vesicle (Sato stage IV–V; nearly 10–12 days postlentectomy (Yamada, 1977), with γ-crystallin (in contrast with the other two) being specific for the lens fibers and not the lens epithelial cells (Mizuno et al., 2002).

4.2. Gene Regulation

Focusing on the possible mechanisms of genetic regulation, it would only make sense to hypothesize that different genes are at play in the dorsal and ventral iris creating two very different populations of cells, one that regenerates and one that does not, respectively. There are of course, many genes and proteins to consider in the regeneration process. As previously mentioned, a master regulator gene in eye development, *Pax-6* is found in both the dorsal and ventral iris during Wolffian lens regeneration (Del Rio-Tsonis et al., 1995; Madhavan et al., 2006). The same is true for *Six-3*, a partner of *Pax-6* and by itself sufficient to induce lens when ectopically expressed in frogs and fish (Oliver et al, 1996; Altmann et al., 1997). *Prox-1* is a transcriptional regulator of crystallin synthesis and is also expressed just before cells of the vesicle elongate and express crystallins (Del Rio-Tsonis et al., 1999). Interestingly these genes are also expressed in the ventral iris tissues. This finding was unexpected and it means that the ventral iris does initially undergo similar events as the dorsal iris In fact there may be a general inhibitory effect that prevents further regeneration from taking place.

To gain more insights about gene expression patterns, a microarray analysis with newt cDNA was utilized and it revealed that gene signatures in the dorsal and ventral iris are in fact very similar (Makarev et al., 2007). Even genes responsible for tissue remodeling, such as collagenase and cathepsin are present and up-regulated in both dorsal and ventral irises during regeneration. Surprisingly expression levels for some of these genes are even higher in the ventral iris. It is not unreasonable to suggest, given these unexpected results on gene expression, that we might encounter novel regulatory events during newt regenerative

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processes (Makarev et al., 2007). EST analysis from irises 8 days after lentectomy, when dedifferentiation of PECs is ongoing, has also revealed very important information. An initial set of 10449 cDNA sequence reads were obtained. The total length of these sequences was 9.85 Mbp that was reduced to 4.82 Mbp by assembling overlapping reads using CAP3 software (Huang and Madan, 1999). This assembly was composed of 4725 unique sequences (1368 contigs and 3357 singlets). Of 1219 contigs and 3357 singlets, 780 contigs and 1666 singlets were annotated by Blast2GO (Conesa et al., 2005). The 15 most frequently counted biological process terms, cellular component terms, and molecular functions were reported (Maki et al, 2010). It is interesting to note here that when compared with a same analysis performed during Xenopus lens regeneration the most frequent GO annotations were very similar (Table 1). GO (Gene Ontology) is used to describe the function of a gene as it pertains to processes and place in the cell.. There are 3 independent sets of ontologies, the molecular function (or molecular component, i.e. DNA binding), the biological process (i.e. transcription) and the cellular component (or compartment) where the gene product can be found (i.e. nucleus).

4.3. Signaling pathways in newt lens regeneration

A number of cell signaling pathways have been implicated in the process of lens regeneration in the newt. Members of the hedgehog signaling pathway are also expressed during the regeneration process; Sonic hedgehog (*Shh*) and Indian hedgehog (*Ihh*) only being expressed in the regenerating and developing lens, and no longer expressed once the lens is fully reformed. When one interferes with this signaling pathway, the overall regeneration process is inhibited. Also, cell proliferation rates are decreased and differentiation in the regenerating lens suffers (Tsonis et al., 2004). Retinoic acid receptors are also expressed in the lens during regeneration and their inhibition might account for aberrant lens formation (Tsonis et al., 2000; Tsonis et al., 2002).

Exogenous FGFs have been shown to elicit regeneration of a second lens from the dorsal iris (Del Rio-Tsonis et al., 1997; Hayashi et al, 2004). It has been suggested that this action of FGFs is mediated via an induction of cell proliferation. The FGF pathway seems to collaborate with the Wnt pathway. When explants of dorsal iris were treated with FGF2 in the presence of Wnt inhibitors, the action of FGF2 was inhibited. Combined addition of FGF2 and Wnt3a was in fact able to induce lens transdifferentiation of ventral explants as well (Hayashi et al., 2006).

Interestingly, research on another signaling pathway, the Bone Morphogenetic Protein pathway (BMP), revealed that its inhibition allows the ventral iris to transdifferentiate into lens. Importantly, such induction was also observed when ventral PECs were transfected with *Six-3* and treated with retinoic acid (Grogg et al., 2005). These manipulations of signaling pathways and regulatory genes clearly indicates that induction of the ventral iris is possible and such observations might open new avenues in experimenting with higher animals that are unable to regenerate eye tissues.

4.4. MicroRNAs

MicroRNAs, or miRNAs are short RNAs, about 22 nucleotides long, which can bind to complementary sequences of RNA and subsequently prevent mRNA translation. miRNAs may have multiple binding sequences due to their short size and can block translation on a wide scale. As such they might be involved in the transition from one cell type to another as we see during regeneration. Cloning of newt miRNA from the eye has pinpointed differentiatial regulation in both dorsal and ventral iris. Some of the targets of the cloned miRNAs were predicted to be *FGFR2*, and *SOX9* (for miR-124a), *PAX3*, *chordin*, and *TGFβR1* for *let7b* (Makarev et al., 2006). Based on this, further study of the role of miRNA

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regulation in regeneration showed that members of the *let7* family were found to be down-regulated in the regenerating dorsal iris. Examination of the ventral iris revealed that miR-148 is up-regulated in both intact and regenerating ventral iris when compared with dorsal counterparts (Tsonis et al., 2007). Thus, miRNAs might be very useful regulators of regenerative processes.

Our previous study on the expression of miRNAs during lens and hair cell regeneration in newts was the first to indicate a possible involvement and regulation. The present study shows that, indeed, miRNAs can have a role in regeneration. Other recent reports support this as well. During zebrafish fin regeneration it has been shown that miR-203 regulates the Wnt signaling pathway transcription factor Lef1. Down-regulation of Lef1 by miR-203 blocks regeneration, while loss of miR-203 results in up-regulation of Lef1 and fin overgrowth (Thatcher et al., 2008). In another study it was found that depletion of miR-133 promotes fin regeneration and that this is FGF-dependent (Yin et al., 2008). In a different study it was shown that miR-196 is involved in axolotl tail regeneration. Inhibition of miR-196 blocks regeneration by acting up-stream *BMP4* and *Pax-7* (Sehm et al., 2008).

4.5. Knock-down technology and gene expression in newts

Transgenesis and knockout technologies are not as developed in the newt when compared to Xenopus or even the axolotl (another salamander that can regenerate body parts such as tail and limb, but is unable to regenerate the lens). Nevertheless knock-down technology has been developed in the newt and it promises to shed more light to the mechanisms of regeneration. Application of such technology in the adult newt has been quite informative on the role of *pax-6* in this process. When *pax-6* expression is decreased in the eye, lens regeneration suffers dramatically due to the decreases in proliferation of the pigmented epithelial cells. When *Pax-6* is knocked down during later regeneration events, crystallins are not made and lens fibers production is decreased (Figure 4) (Madhavan et al., 2006).

4.6. Transdifferentiation in newts: A model for stem cell differentiation?

One could argue that during the process of lens regeneration the dedifferentiated cells become stem cell-like, going from a terminally differentiated state to an undifferentiated state and can then further differentiate into the cells needed to create a new lens. Such a hypothesis was first proposed in 2000, using the example of mesenchymal stem cell similarity to limb blastema cells (Tsonis, 2000; Tsonis, 2004). Similar processes may also occur during lens regeneration in the frog (Henry et al., 2003). Research in this area is being pursued, and some initial studies in the newt support this hypothesis. For example, the stem cell nuclear protein nucleostemin (also a cancer cell marker) is found highly expressed in the nucleus of undifferentiated cells, like pluripotent embryonic stem cells, as well as multipotent stem cells of the nervous system and primitive cells of the bone marrow (Maki et al., 2007). As the onset of differentiation of these cells occurs, accumulation of nucleostemin within the nucleus is shown to decrease. When dedifferentiated PECs were studied during regeneration, as compared to non-regenerating PECs, this stem cell nuclear protein was found to be highly expressed in both dorsal and ventral regenerating PECs. The amount of protein present decreased faster in the ventral PECs, and after lens differentiation the amount of nucleostemin also decreased in dorsal PECs (Maki et al., 2007).

Further studies were undertaken to characterize gene expression during lens regeneration in the newt. A list of the lens-regenerating iris ESTs, possible candidate genes for participating in nuclear regulation during newt dedifferentiation have been identified (Maki et al, 2010). This is similar to the types of genes expressed during lens regenerationin the frog (Malloch et al., 2009). Epigenetic regulation, a range of heritable chromatin modifications including histone modifications, DNA methylation and chromatin remodeling, play a pivotal role in

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the control of differentiation and maintenance of cellular identity. So it is expected that epigenetic regulation plays an important role during newt PEC dedifferentiation. Indeed, many factors involved in reprogramming were identified in the ESTs and interestingly were also found in newt ovarian tissues as well.

The expression patterns of these genes may be significant in the field of cellular reprogramming, stem cells and regeneration in general. While the newt does not reprogram its adult differentiated cells to form pluripotent progenitors, it does reprogram them to specific progenitor stem cell state according to their origin and to their contribution, as evidenced by their ability to regenerate with precision only those parts lost to injury (Kragl et al., 2009). Indeed, aggregates from dorsal iris PECs will transdifferentiate only to lens even if transplanted to regenerating limb (Ito et al, 1999; Tsonis, 2000). These data might suggest an underlying mechanism for this property in the newt.

Recently, it has been shown that differentiated mammalian cells, including human, can also be reprogrammed to become induced pluripotent stem cells (iPSCs) that can subsequently differentiate to different tissues. This reprogramming is mediated by inducing the expression of four factors, *Oct4, Sox-2, c-myc* and *Klf4*. Another factor, nanog, seems to be also important (Takahashi et al., 2006, 2007; Okita et al., 2007; Wernig et al., 2007). All these are transcriptional factors expressed in embryonic stem cells. The critical question that arises from these studies is: Does reprogramming that mediates the formation iPSCs share any similarities to reprogramming during regeneration in newts? A recent study examined expression of these stem cell pluripotency-maintaining factors during newt lens regeneration (Maki et al., 2009). Interestingly, there was significant regulation of three of the factors that we examined, *Sox-2, Klf4* and *c-myc*. *Oct4* and nanog were not detected in these tissues beyond the levels of negative control (-RT), however, they were expressed in control ovarian tissues (meaning that the newt does have these genes).

During lens regeneration, *Sox2* and *Klf4* were upregulated during the very early stages of regeneration (day 2), while c-myc showed a peak of expression at day 8. Day 2 marks an early response to lens removal and is expected to be characterized by events that may prepare pre-existing tissues for reprogramming and cell cycle re-entry. In fact, cell proliferation is not detected until day 4. Those rapid responses to lens removal prior to cell cycle re-entry are similar to those observed for nucleostemin (Maki et al., 2007). *c-myc* showed quite opposite patterns to *Sox2* and *Klf4*. It was highly expressed at day 8, which correlated with the establishment of the lens vesicle, but without major differences between dorsal and ventral iris. It should be noted here that the regeneration-incompetent ventral iris does show activity such as cell cycle re-entry and gene expression, which must be suppressed later (Grogg et al., 2005; Madhavan et al., 2006). Therefore, expression differences between dorsal and ventral iris might be correlated with these events.

The specific stage-related regulation of *Sox2, Klf4* and *c-myc*, and the absence of *Oct4* and *nanog* expression might indicate why the newt cells do not become pluripotent. Manipulating these factors in the newt will open new avenues in the field and should be of enormous importance to compare or contrast the biology of regeneration in classical models and in stem cells.

5. Xenopus and Newt Lens Regeneration: Some Additional Questions

While there have been many advances in our understanding of the cellular and molecular mechanisms that control lens regeneration in *Xenopus*, and newts, a number of key questions still need to be addressed in these systems, in addition to those already raised above. The process of “transdifferentiation” is described as one that takes a population of differentiated cells though an initial process of cellular de-differentiation, followed by re-

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differentiation along an altered trajectory (e.g., Eguchi and Kodama, 1993; Burke and Tosh, 2005). While it is clear this does take place during Wolffian lens regeneration in the newt, investigators have questioned whether the process of lens regeneration in *Xenopus* actually involves de-differentiation. In fact, the cornea is not fully differentiated until after the process of metamorphosis is completed (Yamada, 1982, McDevitt and Brahma, 1982, Bosco, 1988, Henry 2003). The larval cornea and lens are originally derived from the same embryonic tissue, and the phenomenon of lens regeneration in *Xenopus* may simply represent a condition in which there is persistent competence of embryonic and larval ectoderm to respond to key lens inducing signals. Likewise, the precise relationships between embryonic lens development and lens regeneration, though apparently similar processes, is not entirely clear (see Schaefer et al., 1999; Mizuno et al., 1999a, 2005; Henry et al., 2002; Henry, 2003; Cannata et al., 2003; Malloch et al., 2009; Filoni, 2009; Mizuno et al., 2002). Answers to these questions will require careful molecular characterization and comparison of the genes expressed in these tissues and the changes that occur during the processes of lens regeneration and development.

It is unclear whether the larval cornea or the adult iris contains populations of specialized somatic stem cells. For instance, it is known in other vertebrates that there exists a population of limbal stem cells that supports repair of the cornea epithelium (Buck 1985; Cotseralis et al., 1989; Pellegrini et al., 1999; Lavker, et al., 1998; Auran et al., 1995; Huang and Tseng, 1995; Tseng and Sun 1999). We have looked for a population of slowly dividing stem cells (BrdU-retaining-cells) in *Xenopus* and have not found any evidence that these exist in the limbal region. Rather, these preliminary investigations revealed that dividing cells are seen scattered throughout the cornea epithelium (see also Freeman, 1963). Hence, it is not clear if there is a specialized subpopulation of stem cells within the cornea that support regeneration. Likewise it is not believed at the present time that the newt iris contains a stem cell population. Rather, all the evidence testifies for a clear case of transdifferentiation. However, more studies on cell populations using advanced techniques will eventually clear this issue.

Though the regenerated lenses appear to be normal on the basis of morphological and histological criteria, no attempt has been made to examine lens function or visual acuity following regeneration in either *Xenopus* or newts. In addition, it is unclear whether other associated structures are also fully regenerated, such as the muscles that reposition the lens, etc.

Finally, we do not understand what factors may prevent lens regeneration from occurring in other vertebrates, including humans. Do all vertebrates possess a similar conserved suite of genes that could be active in these processes? Some evidence suggests that there might be species specific differences that underlie some regenerative phenomena (e.g., limb regeneration in newts, Kumar et al., 2007; Brockes and Kumar, 2008; Garza-Garcia et al., 2010). In the case of lens regeneration, barriers may be present to ensure that abnormal, supernumerary lenses do not form within the eye that would prevent regeneration from occurring. This could involve the production of specific inhibitors of lens regeneration, or it is possible that key inductive signals are either not present or the receptors or downstream elements of the signal cascade are missing in eye tissues. Once we understand the factors required to support lens regeneration, and the reasons why some vertebrates are unable to regenerate these structures, it may be possible to “restore” this capacity, even in humans, as a therapeutic approach to treat injured or diseased lenses.

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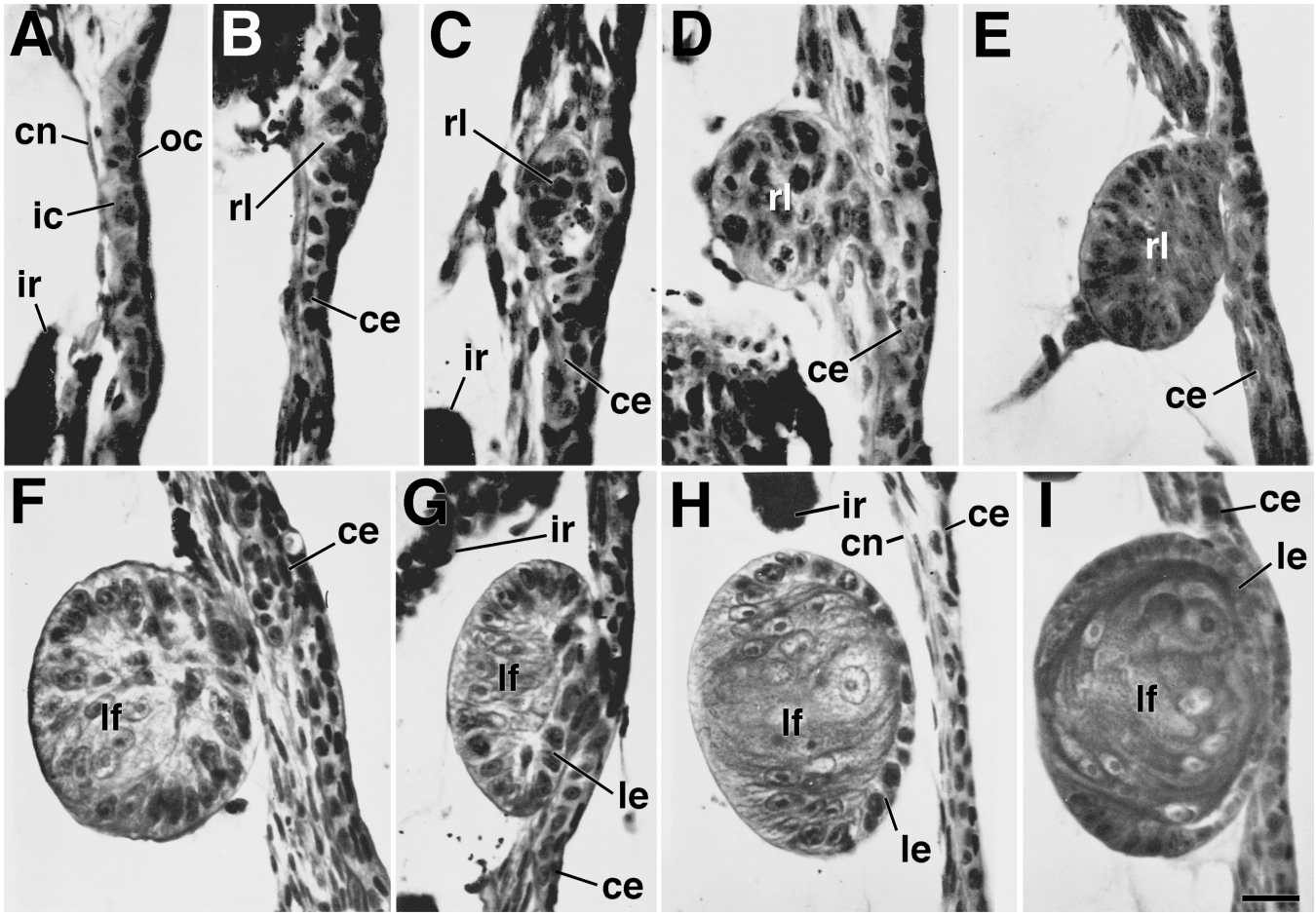

Figure 1.

Cornea-lens transdifferentiation in *X. laevis*. Sections show different stages of lens regeneration following removal of the lens (stages follow the convention of Freeman,1963). A. Stage 1. During this stage cells of the inner corneal epithelium assume a cuboidal shape within 24 hours following lens removal. B. Stage 2. During this stage, cells begin to assume a thickened placodal arrangement. Nuclei of these cells are found to contain one nucleolus (characteristic of lens epithelial cells), rather than two (characteristic of cornea epithelial cells). C. Early stage 3. D. Middle stage 3. E. Late stage 3. During stage 3, a loosely organized aggregate of cells begins to separate from the corneal epithelium. These cells begin to organize with distinct apical-basal polarity to form a lens vesicle. F. Early stage 4. G. Middle stage 4. H. Late stage 4. During stage 4 a definitive lens vesicle is present. Elongated primary lens fiber cells have formed on the side closest to the vitreous chamber. Typically the lens vesicle is separated from the overlying corneal epithelium at this stage. I. Early stage 5. During this stage, secondary lens fiber cells are being added from the lens epithelium at the equatorial zone. Nuclei of the primary fiber cells begin to disappear. This stage is followed by further addition of secondary fiber cells and growth of the lens. ce, corneal epithelium; cn, corneal endothelium; ic, inner layer of the corneal epithelium; ir, iris; oc, outer layer of the corneal epithelium; le, lens epithelium; lf, lens fibers; rl, regenerating lens vesicle. Refer to text for further details. Figure from Henry (2003, after Freeman, 1963). Scale bar equals 25μm.

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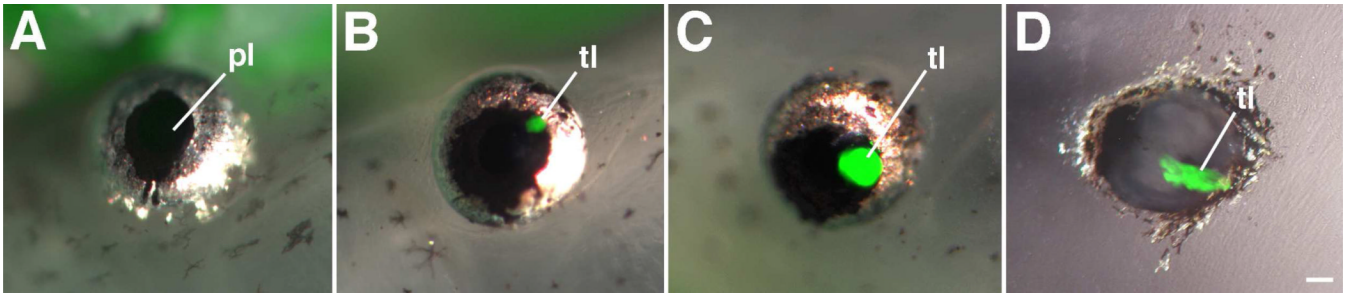

Figure 2.

In vivo and *in vitro* lens regeneration in living transgenic *X. tropicalis* and *X. laevis* larval tissues carrying a transgene encoding the jellyfish green fluorescent protein (GFP) coupled to a γ-crystallin enhancer/promoter. Combined light and epifluorescence micrographs show expression of GFP (green) in the regenerating lenses of these examples. A. Eye of transgenic *X. tropicalis* following removal of the lens. Note absence of differentiated lens cells and GFP in the eye at one week following lens removal. B. Eye at 10 days following lens removal. Note the presence of a small regenerating lens containing GFP expressing cells. C. Eye at 21 days following lens removal. Note the growth of a larger regenerating lens containing GFP expressing cells. D. *In vitro* transdifferentiation in co-cultures of transgenic *X, laevis* corneal epithelium and non-transgenic larval eye cup. Note GFP expressing lens cells derived from the transgenic corneal epithelium. pl, pupil; tl, transdifferentiating lens cells. A–C from Henry et al. (2008, after Henry and Elkins, 2001). D, from L. Fukui and J. Henry (unpublished data, University of Illinois, Champaign-Urbana). Scale bar equals 200μm.

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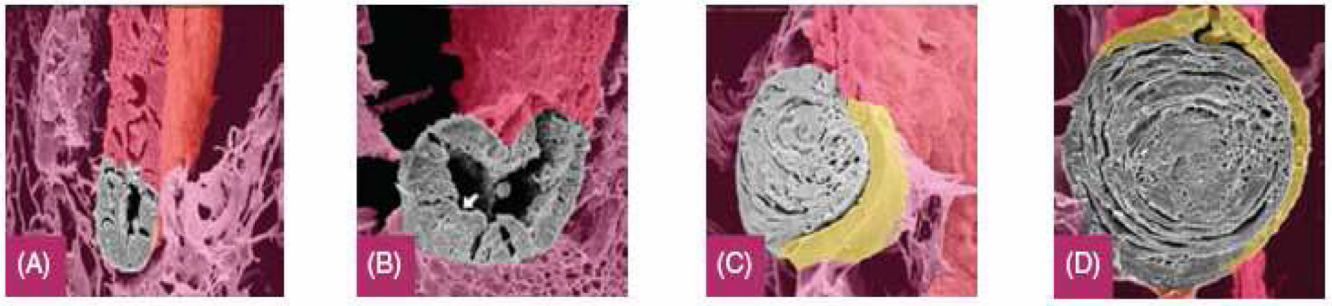

Figure 3.

The process of lens regeneration in newts. Lens formation from the dorsal iris during regeneration shown through scanning electron microscopy. (A) Day 10 after lentectomy, a lens vesicle forms at the dorsal margin of the iris. (B) Day 14, elongation and further lens development as differentiation of lens fibers occur at the posterior part (arrow) of the vesicle. (C) Day 20 of lens reformation. The anterior portion has become the lens epithelium (colored yellow). In contrast, lens fiber cells are forming in the posterior region. (D) Day 30, regeneration has concluded.

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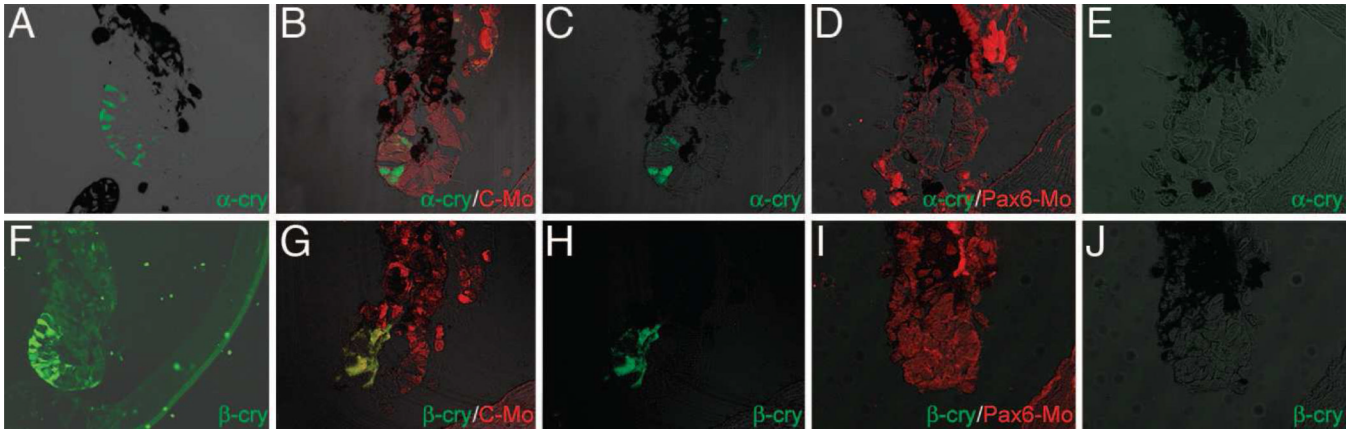

Figure 4.

Pax-6 morpholino treatment inhibits crystallin expression. Morpholinos were injected at day 10 after lentectomy, and animals were collected for assay at day 13 after lentectomy. (*A* and *F*) Lens vesicles from untreated control eyes express α- and β- crystallin (α-cry or β-cry). (*B* and *G*) Eyes treated with C-Mo also express α and β crystallin. (*D* and *I*) Lens vesicles from eyes treated with *Pax6*-Mo1 do not show any α- or β- crystallin expression. (*C*, *E*, *H*, and *J*) α- or β- crystallin staining only from *B* and *D* (*C* and *E*) and β- crystallin staining only from *G* and *I* (*H* and *J*).

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Table 1

The 5 most frequent GO identified in *Xenopus* and Newt. In red are the common ones.

	Xenopus	**Newt**
GO	Regulation of transcription, DNA dependent	Regulation of transcription, DNA dependent
	Transcription	Signal transduction
Biological Process	Transport	Ribosome biogenesis
	Signal transduction	Translation
	Multicellular organismal development	Electron transport
	Nucleus	Cytoplasm
	Membrane	Nucleus
Cellular Compartment	Cytoplasm	Integral to membrane
	Integral to membrane	Transcription factor complex
	Intracellular	Extracellular space
	Protein binding	Protein binding
	Metal ion binding	ATP binding
Molecular Component	DNA binding	Zinc ion binding
	Nucleotide binding	RNA binding
	Zinc ion binding	DNA binding