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Lens regeneration from the cornea requires suppression of Wnt/ β -catenin signaling

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Abstract

The frog, *Xenopus laevis*, possesses a high capacity to regenerate various larval tissues, including the lens, which is capable of complete regeneration from the cornea epithelium. However, the molecular signaling mechanisms of cornea-lens regeneration are not fully understood. Previous work has implicated the involvement of the Wnt signaling pathway, but molecular studies have been very limited. Iris-derived lens regeneration in the newt (Wolffian lens regeneration) has shown a necessity for active Wnt signaling in order to regenerate a new lens. Here we provide evidence that the Wnt signaling pathway plays a different role in the context of cornea-lens regeneration in *Xenopus*. We examined the expression of frizzled receptors and wnt ligands in the frog cornea epithelium. Numerous frizzled receptors (*fzd1*, *fzd2*, *fzd3*, *fzd4*, *fzd6*, *fzd7*, *fzd8*, and *fzd10*) and wnt ligands (*wnt2b.a*, *wnt3a*, *wnt4*, *wnt5a*, *wnt5b*, *wnt6*, *wnt7b*, *wnt10a*, *wnt11*, and *wnt11b*) are expressed in the cornea epithelium, demonstrating that this tissue is transcribing many of the ligands and receptors of the Wnt signaling pathway. When compared to flank epithelium, which is lens regeneration incompetent, only *wnt11* and *wnt11b* are different (present only in the cornea epithelium), identifying them as potential regulators of cornea-lens regeneration. To detect changes in canonical Wnt/ β -catenin signaling occurring within the cornea epithelium, *axin2* expression was measured over the course of regeneration. *axin2* is a well-established reporter of active Wnt/ β -catenin signaling, and its expression shows a significant decrease at 24 hours post-lentectomy. This decrease recovers to normal endogenous levels by 48 hours. To test whether this signaling decrease was necessary for lens regeneration to occur, regenerating eyes were treated with either 6-bromoindirubin-3'-oxime (BIO) or 1-azakenpauillone – both activators of Wnt signaling – resulting in a significant reduction in the percentage of cases with successful regeneration. In contrast, inhibition of Wnt signaling using either the small molecule IWR-1, treatment with recombinant human Dickkopf-1 (rhDKK1) protein, or transgenic expression of *Xenopus* DKK1, did not significantly affect the percentage of successful regeneration. Together, these results suggest a model where Wnt/ β -catenin signaling is active in the cornea epithelium and needs to be suppressed during early lens regeneration in order for these cornea cells to give rise to

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Conflicts of Interest

The authors have no conflicts of interest.

a new lentoid. While this finding differs from what has been described in the newt, it closely resembles the role of Wnt signaling during the initial formation of the lens placode from the surface ectoderm during early embryogenesis.

Keywords

regeneration; lens; cornea epithelium; *Xenopus*; Wnt/ β -catenin signaling

1. Introduction

While examples of regeneration are widespread among the invertebrate population, few vertebrates possess the ability to regenerate complete organs lost to either damage or disease (Brookes and Kumar, 2008). In vertebrates, one organ capable of complete regeneration is the lens of the eye. However, the capacity to replace this structure is restricted to certain species of frogs, one fish, and some newts and salamanders (Henry et al., 2013; Henry and Tsonis, 2010). Newts are able to regenerate a lens via transdifferentiation of the dorsal pigmented iris epithelium, in a process referred to as Wolffian regeneration (see Henry and Tsonis, 2010). The frog *Xenopus laevis* is also capable of regenerating a lens, but instead of the iris, the lens is regenerated from the basal layer of the cornea epithelium (Freeman, 1963). This process is initiated when signals from the neural retina are able to reach the cornea epithelium, upon perforation of the cornea endothelium and removal of the lens (Freeman, 1963; Reeve and Wild, 1978). While cornea-lens regeneration has traditionally been described as transdifferentiation of the cornea, a different model has emerged suggesting that the regenerated lens may instead be derived from a population of basal stem cells or transient amplifying cells in the cornea which possess an oligopotent capacity to give rise to a new lens (Perry et al., 2013). However, the cellular signaling events needed to initiate lens regeneration in these cornea cells is not understood.

In recent years, several signaling pathways have been shown to be important for cornea-lens regeneration such as the Fibroblast Growth Factor (FGF, Bosco et al., 1997; Fukui and Henry, 2011), Retinoic acid (Thomas and Henry, 2014), and Bone Morphogenetic Protein (BMP) signaling pathways (Day and Beck, 2011). The involvement of multiple pathways suggests that this process is regulated by a complex signaling network, and it is possible that other pathways may also be involved. One pathway that has been implicated as playing a role in cornea-lens regeneration in two independent screens for genes expressed during early regeneration is the Wnt signaling pathway (Day and Beck, 2011; Henry et al., 2013; Malloch et al., 2009). In the canonical Wnt/ β -catenin signaling pathway, wnt ligands bind to corresponding frizzled receptors and associated co-receptors in order to inhibit the downstream β -catenin degradation complex (reviewed in Logan and Nusse, 2004; MacDonald et al., 2009). Inhibition of this degradation complex allows β -catenin to accumulate and translocate to the nucleus where it activates transcription through T-cell factor/lymphoid enhancer factor (TCF/LEF). In the absence of an appropriate wnt signal, the degradation complex remains in an active state and begins to degrade β -catenin. The role of Wnt signaling has been well studied during the development of the eye, and is important for the initial formation of the vertebrate lens (Fuhrmann, 2008; Graw, 2010). During vertebrate

lens development, Wnt/ β -catenin signaling needs to be suppressed in the ectoderm overlying the eye in order to initiate a lens placode (Kreslova et al., 2007; Miller et al., 2006; Smith et al., 2005). Since the cornea is derived from this surface ectoderm, it could be that the larval cornea retains some of these same signaling mechanisms deployed during development, as there appear to be many similarities between lens regeneration and initial lens development (Henry, 2003; Henry and Mittleman, 1995).

However, from observations made during Wolffian lens regeneration in the newt, active Wnt/ β -catenin signaling is necessary for lens regeneration to occur (Hayashi et al., 2006). While Wolffian lens regeneration and cornea-lens regeneration occur in different organisms and from different tissues, it appears that some signaling (e.g. FGF signaling) is somewhat conserved between these two systems (Fukui and Henry, 2011; Hayashi et al., 2004). It is possible that the role of Wnt/ β -catenin signaling in cornea-lens regeneration may also be conserved. On the other hand, recent work from our lab has shown that while Retinoic acid signaling appears to be necessary for Wolffian lens regeneration, it must be reduced during lens regeneration in *Xenopus*, which differs from observations in newt lens regeneration (Thomas and Henry, 2014; Tsonis et al., 2000; Tsonis et al., 2002). This raises the following questions: What is the role of Wnt/ β -catenin signaling in cornea-lens regeneration, and how does it compare to that of Wolffian lens regeneration?

In this study we present evidence that Wnt/ β -catenin signaling needs to be suppressed in order for cornea-lens regeneration to occur. We identified a wide assortment of wnt ligands and frizzled receptors that are expressed within the cornea epithelium and compared the expression of these genes to lens-incompetent flank epithelium. Using *axin2* expression as a readout for active Wnt/ β -catenin signaling, we demonstrated that there is a natural suppression of Wnt/ β -catenin signaling in the cornea epithelium that occurs 24 hours into the course of lens regeneration and recovers by 48 hours. Additionally, we functionally tested the necessity of this inhibition by holding the cornea in a state of active Wnt/ β -catenin signaling using 6-bromindirubin-3'-oxime (BIO) and 1-azakenpaullone, and we found that the percentage of successfully regenerating cases significantly decreased in the presence of these compounds. Finally, we inhibited Wnt/ β -catenin signaling using IWR-1, recombinant human DKK1, and transgenic expression of *Xenopus* DKK1 and found no significant effect on lens regeneration. Together these data suggest a model where Wnt/ β -catenin signaling is active in the cornea epithelium and needs to be suppressed during early lens regeneration in order for a new lens to form. This finding differs from what has been described in the newt (Hayashi et al., 2006), but resembles the role of Wnt signaling during the initial formation of the lens placode from the surface ectoderm during early embryogenesis (Kreslova et al., 2007; Miller et al., 2006; Smith et al., 2005).

2. Materials and Methods

2.1 Animals

Xenopus laevis adults were acquired from Nasco (Fort Atkinson, WI). Larvae were generated and reared as previously described (Henry and Grainger, 1987; Schaefer et al., 1999) and were developmentally staged according to Nieuwkoop and Faber (1956). All lentectomies were performed as described in Henry and Mittleman (1995) on stage 48–53

animals in a 1:2000 dilution of the anesthetic MS 222 (ethyl 3-aminobenzoate methanesulfonate, Sigma, St. Louis, MO) in 1/20x normal amphibian media (NAM; Slack, 1984). The animal care and use in this work was approved and overseen by the University of Illinois Institutional Animal Care and Use Committee and monitored by the staff of the Division of Animal Resources at the University of Illinois.

2.2 Reverse transcription polymerase chain reaction (RT-PCR)

Cornea epithelial tissue and flank epithelial tissue was collected from multiple st. 48–53 *X. laevis*. Like tissues were pooled together in microcentrifuge tubes, flash frozen in a dry ice/ethanol bath, homogenized in TRIzol (Invitrogen, Carlsbad, CA), and RNA was purified using Direct-zol RNA Miniprep (Zymo Research, Irvine, CA) according to the manufacturer's protocol. The resulting RNA was treated with DNaseI (New England Biolabs, Ipswich, MA) to ensure removal of genomic contamination. cDNA was generated from purified cornea RNA and flank RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). PCR reactions were conducted using the newly synthesized cDNA as a template, along with the primers listed in Supplemental Table 1. For this study, only *Xenopus laevis* wnt ligands and frizzled receptors from the National Center for Biotechnology Information's Reference Sequence database were examined. Negative –RT (“–”, Fig. 1) controls were generated by conducting PCR on iScript reactions that did not contain reverse transcriptase (RT). As a positive control (“+”, Fig. 1), PCR was carried out on cDNA generated from pooled embryos ranging in stages from 11–39, when these transcripts are known to be present.

2.3 Ex vivo eye culture

Eyes were lentectomized and cultured in an *ex vivo* system (formerly referred to as *in vitro* culture), as described in Fukui and Henry (2011). In the *ex vivo* culture system, eyes are lentectomized and the cornea epithelium is tucked into the eyecup to ensure close proximity to the retina. Eyes are then removed and placed into culture medium, consisting of the following: 61% L-15 powder (Invitrogen, Carlsbad, CA); 100 U/ml of penicillin and 100 ug/ml of streptomycin (Mediatech, Manassas, VA); 10% fetal bovine serum (Invitrogen, Carlsbad, CA); 2.5 ug/ml Amphotericin B (Sigma, St. Louis, IL); and 4 ug/ml Marbofloxacin (Sigma, St. Louis, IL). 1-azakenpaullone (A.G. Scientific, San Diego, CA) was diluted to a final concentration of 10 μ M from a 10 mM stock in dimethyl sulfoxide (DMSO). BIO (6-bromoindirubin-3'-oxime, Tocris Bioscience, Bristol, UK) was diluted to a final concentration of 1 μ M from a 1 mM stock in DMSO. IWR-1 (Inhibitor of Wnt Response-1; Sigma, St. Louis, IL) was diluted to a final concentration of 10 μ M from a 10 mM stock in DMSO. Recombinant human DKK1 (rhDKK1; R&D Systems, Minneapolis, MN) was diluted to a final concentration of either 200 ng/ml or 500 ng/ml from a 100 μ g/ml stock in PBS + 0.1% bovine serum albumin (BSA). An equal volume of DMSO was added to the control medium for experiments using IWR-1, 1-azakenpaullone, or BIO. For the rhDKK1 controls, an equal volume of Phosphate-buffered saline (PBS) + 0.1% BSA was added to the culture media. Culture media were changed daily. After 7 days of culture (sufficient time for a lentoid to regenerate, see Fukui and Henry, 2011; Thomas and Henry, 2014), eyes were fixed for 3 hours in 3.7% formaldehyde, embedded in Paraplast Plus (McCormick Scientific, Richmond, IL) and serially sectioned at 8 μ m (as described by

Humason, 1972). To identify regenerated lenses, immunohistochemistry was carried out on serial sections using a polyclonal anti-lens antibody (Henry and Grainger, 1987). Positive cases of regeneration were scored based on the presence of morphologically distinct lentoids that were positively stained by the anti-lens antibody.

2.4 HGEM-DKK1 Transgenesis

All F0 HGEM-DKK1 transgenic tadpoles were generated using sperm nuclear injection, following the protocol of Smith et al. (2006). The HGEM-DKK1 transgene (Fig. 5A) was kindly provided by Dr. Jonathan Slack (University of Minnesota) and has been successfully used in *Xenopus* tail regeneration studies (Lin and Slack, 2008). Only transgenic animals displaying robust GFP expression throughout the lenses of both eyes were used. Eyes were lentectomized and cultured in modified L-15 media in the *ex vivo* culture system, which allowed for individual transgenic animals to contribute one eye to the experimental group and one eye to the control group. The experimental groups received daily heat-shocks for 40 minutes at 34°C, while the control groups were maintained at room temperature (20–24°C). Eyes were then fixed, sectioned, and stained with an anti-lens antibody. Western blots were performed on HGEM-DKK1 tail tissue collected and heat-shocked in culture. Western blotting followed standard protocols (Henry et al., 2008), using the following primary antibodies: anti- β -tubulin (Sigma, T8328; expected ~50 kDa) and anti- β -catenin (Sigma, C2206; expected size 94 kDa).

2.5 Quantitative polymerase chain reaction (qPCR) Methods

For the drug validation experiments, eyes were removed from st. 48–53 tadpoles leaving the cornea epithelium attached to the underlying cornea endothelium via the central stalk. It is important to note that the lens and cornea endothelium are both undisturbed, so as not to induce regeneration. Eyes were then cultured in the appropriate drug or control medium (as described above) for 24 hours, at which point cornea epithelial tissue was collected and cDNA was generated as previously described. For the *axin2* regenerating time course, eyes were lentectomized, removed from the animal, and cultured in the culture medium (no drugs present) for either 24, 48, 72, or 96 hours. Cornea epithelial tissue was then collected, pooled, and cDNA was generated for qPCR as previously described. Control corneas for the time course experiment are simply cornea epithelial tissue that is not regenerating and has not been wounded. All qPCR experiments were performed a minimum of three times, each with triplicate technical replicates. Reactions contained SYBR Green reagent kindly provided by Dr. Jie Chen (University of Illinois at Urbana-Champaign), 500 nM of both forward and reverse primers, and 10–25 ng of input cDNA. The following primers were used: *actb* (F: 5'-CGCCCGCATAGAAAGGAGAC-3'; R: 5'-AGCATCATCCCCAGCAAAGC-3'; Thomas and Henry, 2014), *axin2* (F: 5'-TGCAGCCAGTATCAACGACAG-3'; R: 5'-CAAAGACACTTGTCCATTGGC-3'; Myers et al., 2014), and *odc* (F: 5'-GCCATTGTGAAGACTCTCTCCATTC-3'; R: 5'-TTCGGGTGATTCTTGCCAC-3'; Heasman et al., 2000). Changes in relative expression were determined using the comparative CT method (Schmittgen and Livak, 2008), and normalized to a reference gene, either *ornithine decarboxylase* or *beta-actin*.

2.6 Statistical Tests

Statistical significance of the percentage of cases that successfully regenerated was determined using Fisher's exact test (Fisher, 1922), under the two-tailed condition. Statistical significance of qPCR experiments was determined using an unpaired *t* test. Statistical differences were considered significant if the *p*-values were less than 0.05. All error bars represent standard error.

3. Results

3.1 Expression of frizzled receptors and wnt ligands in lens competent and incompetent tissue

To identify frizzled receptors and wnt ligands that are expressed in the cornea, RT-PCR was performed on cornea epithelial tissue (Fig. 1). Of the nine frizzled receptors investigated, eight appear to be transcriptionally expressed at some level: *fzd1*, *fzd2*, *fzd3*, *fzd4*, *fzd6*, *fzd7*, *fzd8*, and *fzd10*. Only *fzd5* was found to be absent in the larval cornea epithelium. Numerous wnts are also expressed in cornea epithelium: *wnt2b.a*, *wnt3a*, *wnt4*, *wnt5a*, *wnt5b*, *wnt6*, *wnt7b*, *wnt10a*, *wnt11*, and *wnt11b*. Of those examined, only *wnt1*, *wnt8a*, and *wnt8b* do not appear to be expressed in this larval tissue.

In order to better understand any differences in the expression of these genes in an epithelial tissue that is not competent to regenerate a lens, we reanalyzed the same genes in st. 48–53 flank epithelium, which is lens regeneration incompetent after stages 30/31 (Arresta et al., 2005). Flank expressed many of the same transcripts including: *fzd1*, *fzd2*, *fzd3*, *fzd4*, *fzd6*, *fzd7*, *fzd8*, *fzd10*, *wnt2b.a*, *wnt3a*, *wnt4*, *wnt5a*, *wnt5b*, *wnt6*, *wnt7b*, and *wnt10a* (Fig. 1). Others, including *fzd5*, *wnt1*, *wnt8a*, *wnt8b*, *wnt11*, and *wnt11b* were not expressed in flank. Only two of these genes were found to be different between flank and cornea epithelium: *wnt11* and *wnt11b*, which are present only in the cornea epithelium.

3.2 Time Course of *axin2* expression during lens regeneration

As the cornea epithelium expresses many of the appropriate signals and receptors to be participating in Wnt signaling, we wanted to quantitatively assess the activity of canonical Wnt/ β -catenin signaling in the cornea over the first four days of regeneration. To do this, *axin2* expression was examined using quantitative polymerase chain reaction (qPCR), as its expression is regulated by Wnt/ β -catenin signaling and is commonly used as a readout for active canonical signaling (Jho et al., 2002; Myers et al., 2014). Larvae were lentectomized and allowed to regenerate for either 24, 48, 72, or 96 hours, at which point cornea epithelial tissue was isolated for qPCR analysis. Expression was normalized to *beta-actin* (*actb*) and regenerating expression levels were compared to the expression level in control cornea epithelium that had not been wounded and was not regenerating. At 24 hours post-lentectomy there is a 46.6% reduction in *axin2* expression, representing a statistically significant decrease ($p=0.0006$, Fig. 2). This is a particularly strong effect, considering that not all cells of the cornea epithelium are thought to respond to the retinal signals to initiate a new lens (Freeman, 1963). By 48 hours post-lentectomy, the levels of *axin2* return to that of the control and are maintained at that level through 96 hours post-lentectomy (Fig. 2).

3.3 Activation of Wnt signaling significantly reduces successful lens regeneration

To test whether the suppression of Wnt/ β -catenin signaling observed by qPCR was functionally significant, lentectomized eyes were treated with activators of Wnt signaling to see if holding the eyes in an active state of Wnt/ β -catenin signaling would result in a failure to regenerate a lens. The Wnt signaling activators used were 1-azakenpauellone and BIO (6-bromoindirubin-3'-oxime), which both activate Wnt/ β -catenin signaling by inhibiting Glycogen synthase kinase 3 β (Gsk3 β ; Kunick et al., 2004; Meijer et al., 2003). Gsk3 β is an important member of the β -catenin degradation complex, responsible for the phosphorylation and subsequent degradation of β -catenin via the proteasome (see MacDonald et al., 2009). Thus, inhibition of Gsk3 β prevents the degradation of β -catenin, permitting the activated Wnt/ β -catenin signaling pathway. Eyes were lentectomized and cultured *ex vivo*, in the presence of either 1-azakenpauellone or BIO for seven days. In the *ex vivo* system, lentectomized eyes are removed and the cornea epithelium is tucked inside of the eyecup to ensure close proximity to the neural retina (Fukui and Henry, 2011; Thomas and Henry, 2014). Positive cases of regeneration are histologically scored based on the presence of morphologically distinct lentoids that are positively stained by an anti-lens antibody. A 10 μ M treatment of 1-azakenpauellone reduced regenerative success from 94.4% (34/36 eyes; control) to 26.5% (9/34 eyes; treated). This difference represents a statistically significant decrease in lens regeneration ($p < 0.0001$; Fig. 3A, D-G). Although not as dramatic as the effect seen with 1-azakenpauellone, a 1 μ M treatment of BIO also resulted in a statistically significant difference ($p = 0.0031$), decreasing regenerative success from 95.5% (21/22 DMSO treated eyes) to 55.0% (11/20 BIO treated eyes; Fig. 3B, H-K). To confirm that these compounds were having the desired effect on Wnt/ β -catenin signaling, the relative expression levels of *axin2* were measured using qPCR (Fig. 3C). Corneas treated for 24 hours in either 10 μ M of 1-azakenpauellone or 1 μ M of BIO showed a statistically significant increase in the relative expression levels of *axin2*, as expected ($p = 0.0226$ and $p < 0.0001$, respectively).

3.4 Inhibition of Wnt Signaling does not prevent lens regeneration

It has been shown that Wnt/ β -catenin signaling is necessary for lens regeneration to occur in Wolffian lens regeneration (Hayashi et al., 2006), and it could be that perturbing Wnt signaling in either direction could affect lens regeneration, so we also assessed lens regeneration under Wnt signaling inhibition using three different approaches. First, we challenged regeneration in the presence of the small molecule Inhibitor of Wnt Response-1 (IWR-1). This molecule has been shown to be a potent inhibitor of Wnt/ β -catenin signaling by stabilizing the β -catenin degradation complex (Lu et al., 2009). It has also been used to inhibit Wnt signaling in several *Xenopus* specific applications (Borday et al., 2012; Myers et al., 2014). Using the *ex vivo* eye culture system, lentectomized eyes were treated in 10 μ M IWR-1 continuously for seven days and were then assessed for the presence of lenses. This concentration is sufficient to inhibit tail fin regeneration in zebrafish (Chen et al., 2009). Eyes cultured in the control medium successfully regenerated 77.8% (14/18 eyes) of the time, while the IWR-1 treated eyes regenerated at a very similar percentage of 76.9% (10/13 eyes; Fig. 4A, D-G). While these experiments showed no effect on the percentage of regenerative success ($p = 1.0000$), this concentration of IWR-1 did successfully suppress Wnt

signaling, as measured by *axin2* levels using qPCR on cornea tissue treated with 10 μ M of IWR-1 ($p < 0.0001$; Fig. 4C).

Next, we treated eyes in the same *ex vivo* lens regeneration system in the presence of recombinant human Dickkopf1 (rhDKK1; Glinka et al., 1998) protein. DKK1 inhibits Wnt signaling by binding to the low density lipoprotein receptor-related proteins (Lrp), Lrp5/6, which serve as critical co-receptors in canonical Wnt signaling (Niehrs, 2006). At concentrations of 200 ng/ml, treated eyes (14/14; 100%) were still able to regenerate as well as the control eyes (13/14; 92.9%, Fig. 4B, H-K). Increasing the concentration of rhDKK1 to 500 ng/ml gave the same result, with treated eyes (19/19; 100%) regenerating at percentages near the control eyes (17/19; 89.5%, Fig. 4B, L-O). Neither of these differences was statistically significant ($p=1.0000$ and $p=0.4865$, respectively), although both treated groups showed an increase in the percent of successful regenerates. Suppression of Wnt signaling using rhDKK1 was also confirmed using qPCR of *axin2* expression ($p=0.0081$, Fig. 4C).

Finally, we transgenically expressed *Xenopus* DKK1 under the control of a heat shock inducible *hsp70* promoter (Lin and Slack, 2008; Fig. 5A). This “Heat-shock Green-Eyed Monster” (HGEM) construct also contains a lens-specific γ -crystallin promoter driving the expression of GFP, providing an easy way to screen for transgenic tadpoles (Fig. 5B, C). HGEM-DKK1 F0 transgenic tadpoles had both lentectomized eyes removed and cultured *ex vivo* in modified L-15 medium, either in the control group (no heat-shock) or the experimental group (daily heat-shocks). Eyes were then fixed, sectioned, and stained with a polyclonal anti-lens antibody to identify regenerated lenses (Fig. 5D, G). Inhibition of Wnt/ β -catenin signaling in the cornea did not significantly affect the percentage of successful regeneration ($p=0.3706$) between heat-shocked eyes (22/32; 68.8%, Fig. 5H) and control eyes (23/28; 82.1%, Fig. 5H). In order to confirm that DKK1 had the desired effect on Wnt/ β -catenin signaling, Western blots of β -catenin (CTNNB1) were carried out on either heat-shocked or control tissue collected from the tails of HGEM-DKK1 tadpoles. As expected, levels of β -catenin were greatly diminished in the transgenic tail tissue expressing DKK1 after heat-shock (Fig. 5I).

4. DISCUSSION

Wnt signaling is known to be an important regulator in the development of the vertebrate lens (Fuhrmann, 2008). Within the developing lens itself, active Wnt signaling is needed for proper differentiation of lens fiber cells and lens epithelial cells (Chen et al., 2006; Stump et al., 2003), as well as for the proper growth and orientation of lens fiber cells (Chen et al., 2008; Sugiyama et al., 2011). However, earlier in development, active Wnt signaling in the presumptive lens ectoderm prevents this tissue from giving rise to a lens (Kreslova et al., 2007; Smith et al., 2005). This comes from observations that holding the surface ectoderm in a state of active Wnt signaling results in the loss of lens formation (Miller et al., 2006; Smith et al., 2005). Additionally, β -catenin loss-of-function has no effect on the ability of a lentoid to form and is actually sufficient to induce lentoid formation in murine nasal and periocular ectoderm (Kreslova et al., 2007; Smith et al., 2005).

Far less is known about the involvement of Wnt signaling during lens regeneration. The Tsonis lab challenged lens regeneration in the newt by treating iris explants with either an activator or inhibitor of Wnt signaling and then implanting the treated iris into lentectomized newt eyes, but no effect on lens regeneration was reported (unpublished data discussed in Grogg et al., 2006). However, Hayashi and colleagues (2006) were able to demonstrate that the addition of media conditioned with either *Xenopus* DKK1 or human sFRP1 (both inhibitors of Wnt signaling) to cultures of dorsal iridies results in the inability to regenerate a lens in the newt. Interestingly, the addition of WNT3A, in conjunction with FGF2, was able to induce lentoid formation in the ventral iris, which does not usually give rise to a lens (Hayashi et al., 2006). These experiments demonstrated a necessity for active Wnt signaling during Wolffian lens regeneration, but it was unclear if this finding was broadly applicable to all lens regeneration or if it was specific to the Wolffian system.

In cornea-lens regeneration in *Xenopus*, even less is known about the role of Wnt signaling. Wnt signaling has been implicated in two independent screens for genes expressed during early lens regeneration. From a cDNA library screen for genes upregulated during the process of lens regeneration, three genes specific to the Wnt signaling pathway were identified, including two inhibitors and one ligand: *sfrp3*, *sfrp5*, and *wnt7b* (Malloch et al., 2009). Another study of global transcriptional expression during this process also revealed many components of the Wnt signaling cascade, including, *fzd7*, *fzd8*, *wnt2*, *wnt3*, *wnt5b*, *wnt6*, *wnt7a*, and other components further down the signaling cascade (Day and Beck, 2011). Interestingly, Day and Beck (2011) also found the inhibitor *sfrp2* to be upregulated during lens regeneration. The RT-PCR data from the present study shows that the cornea epithelium is normally transcribing a wide assortment of frizzled receptors and wnt ligands, demonstrating that this tissue expresses the appropriate signals and receptors involved in active Wnt signaling (Fig. 1). It is important to note that the cornea epithelium is not made up of a homogenous population of cells, so it is possible that not every cell in this tissue expresses all of these genes.

In order to identify any differentially expressed genes between cornea and other skin that is not competent to regenerate a lens, we looked at the expression of the same genes in flank epithelium (Arresta et al., 2005). Flank epithelium expresses most of the same wnts and frizzleds, with the notable difference being *wnt11* and *wnt11b*, which were both expressed in the cornea epithelium but not in the flank (Fig. 1). While nothing is known about these genes in the context of lens regeneration, *wnt11* has been shown to be specifically expressed in the limbal region of human corneas, where the population of stem cells that replenish the mature cornea reside (Nakatsu et al., 2011). This is interesting since there is now evidence that the basal layer of the larval cornea epithelium, which serves as the source of regenerated lenses in *Xenopus* (Freeman, 1963), also appears to contain a population of oligopotent stem cells and their transient amplifying cells (Perry et al., 2013). It could be that *wnt11* and/or *wnt11b* help to maintain the oligopotency of these cells, either through a non-canonical or canonical mechanisms. *wnt11* has traditionally been defined as a non-canonical wnt (Rao and Kühl, 2010) but there has also been a report in *Xenopus* of *wnt11* working in a canonical fashion (Tao et al., 2005), so its specific involvement in Wnt signaling cascades has some dependency on the biological context.

To better understand how the levels of active Wnt/ β -catenin signaling in the cornea epithelium might be changing during the early events of lens regeneration, we analyzed the expression levels of *axin2* by qPCR. *axin2* is a commonly used readout for the level of active canonical Wnt/ β -catenin signaling, as its expression is regulated through TCF/LEF (Jho et al., 2002). qPCR analysis of *axin2* during cornea-lens regeneration revealed a significant reduction in *axin2* expression at 24 hours post-lentectomy, representing a reduction in Wnt/ β -catenin signaling during that time (Fig. 2). This timing coincides with Freeman lens regeneration Stages 1 and 2, when the cells of the inner layer of the cornea epithelium transition from squamous to cuboidal to form a thickened placode (Freeman, 1963). Additionally, the recovery of *axin2* expression levels at 48 hours suggests that the observed decrease in Wnt/ β -catenin signaling is occurring within the cornea and not lens tissue, as 48 hours is not sufficient time for the formation of the lens vesicle or fiber cells (Freeman, 1963). While this result is specific to the canonical Wnt/ β -catenin signaling pathway, it does not rule out a possible role for non-canonical Wnt signaling, such as Wnt/Planar Cell Polarity (PCP) during this regenerative process. In fact, as the new lens continues to form it seems likely that Wnt/PCP signaling could be necessary for the proper orientation of differentiated lens fiber cells (Sugiyama et al., 2011).

To functionally test the observed inhibition from the qPCR experiment, we impeded the ability of the cornea epithelium to decrease Wnt signaling by culturing regenerating eyes *ex vivo* in the presence of an activator of Wnt/ β -catenin signaling. Both 1-azakenpaullone and BIO treatments resulted in a statistically significant decrease in the percentage of cases that successfully regenerated (Fig. 3). These data suggest that suppression of Wnt/ β -catenin signaling is necessary in order for the cornea epithelium to transition towards the lens fate to regenerate a new lens. This is not the only regenerative system where Wnt/ β -catenin signaling needs to be inhibited for regeneration to occur, as both retina regeneration in the chick (Zhu et al., 2014) and head regeneration in planaria (Liu et al., 2013; Sikes and Newmark, 2013; Umesono et al., 2013) display a similar phenomenon. It is important to note that because retinal tissue is a necessary component of the *ex vivo* culture system, it is impossible to rule out the possibility that treatment of the neural retina may be contributing to the observed results. Regardless of whether or not the required suppression is specific to the cornea epithelium, these data demonstrate that the role of Wnt signaling differs from what has been described during Wolffian lens regeneration in the newt, where active Wnt signaling is necessary for lens regeneration (Hayashi et al., 2006).

In fact, to confirm this difference, we inhibited Wnt signaling using IWR-1 treatment, rhDKK1 treatment, and transgenic expression of *Xenopus* DKK1, and none of these resulted in a statistically significant change in regeneration (Fig. 4 and 5). As these two forms of lens regeneration occur in completely different organisms, and the lens is regenerating from different tissues (cornea epithelium vs. dorsal iris) within these systems, this result may not be surprising. A recent study concluded that Retinoic acid (RA) signaling differs between the newt and cornea-lens regeneration systems (Thomas and Henry, 2014). In the newt, RA signaling has been shown to be necessary for lens regeneration (Tsonis et al., 2000; Tsonis et al., 2002), while Thomas and Henry (2014) demonstrated that in *Xenopus* RA signaling must be suppressed. These differences help illustrate the importance of studying regenerative

mechanisms in a variety of organisms, as there appear to be distinct molecular pathways to regenerate a lens.

While different from Wolffian lens regeneration, our findings do appear to resemble what is known about the role of Wnt signaling during the initial development of the vertebrate lens. Holding Wnt signaling in an active state prevents the ability of the surface ectoderm to form a lens (Miller et al., 2006; Smith et al., 2005), which is similar to what was observed when cornea epithelium (which is derived from the surface ectoderm) is cultured in the presence of Wnt signaling activators (Fig. 3). In contrast, disruption of β -catenin through loss-of-function experiments during lens development, has no effect on the ability of the surface ectoderm to form a lentoid (Kreslova et al., 2007; Smith et al., 2005). Again, this matches our results from inhibiting Wnt signaling in regenerating eyes (Fig. 4 and 5). In fact, β -catenin loss-of-function in murine periocular and nasal ectoderm is sufficient to induce lentoid formation in these tissues during development (Kreslova et al., 2007; Smith et al., 2005). We find it particularly interesting that in the rhDKK1 experiments, both the 200 ng/ml and 500 ng/ml treatments resulted in 100% of the cases regenerating lenses, which was not observed in any other experiment (Fig. 4B). While this did not represent a statistically significant increase from the controls, which regenerated well themselves, it is a tantalizing thought that this inhibition may have been so effective that it helped promote lens formation as has been observed in periocular and nasal ectoderm (Kreslova et al., 2007; Smith et al., 2005). Taken together, these data suggest that the involvement of Wnt signaling during lens regeneration may parallel that of early embryonic lens development.

In light of the findings from this study, it is also interesting that both of the screens for genes involved in cornea-lens regeneration found secreted frizzled-related proteins (*sfrp2*, *sfrp3*, and *sfrp5*), known inhibitors of wnt signaling, to be upregulated in the cornea early during regeneration (Day and Beck, 2011; Malloch et al., 2009). Perhaps these are key inhibitory factors responsible for diminishing canonical Wnt signaling in the cornea during this process. As not every cell of the cornea epithelium responds to the retinal signals to regenerate a lens (Freeman, 1963), it is unlikely that there is a global upregulation of these inhibitors. Instead, it seems more likely that *sfrp* upregulation would occur in a localized subset of cells which ultimately give rise to the lens.

One possible explanation as to why Wnt/ β -catenin signaling would need to be suppressed in the cornea epithelium is that it could be helping to maintain the oligopotency of stem cells and/or their transient amplifying progeny that exist in the basal layer of the larval cornea epithelium (Perry et al., 2013). Studies of Wnt/ β -catenin signaling in human cornea cells have indicated that the pathway may help to maintain cornea epithelial stem cells (Lu et al., 2011; Lu et al., 2012; Nakatsu et al., 2011). Active Wnt/ β -catenin signaling has been observed in the human cornea in a subset of basal epithelial cells that reside in the limbus (Nakatsu et al., 2011) and helps to regulate proliferation of these human limbal stem cells. Additionally, studies have shown that active Wnt/ β -catenin signaling helps to maintain human corneal epithelial cells in a less differentiated state (Lu et al., 2011; Lu et al., 2012). It is possible that the larval cornea epithelium in *Xenopus* also possesses active Wnt/ β -catenin signaling that helps to maintain cornea stem cells (or their transient amplifying progeny) in the basal layer that are capable of giving rise to a new lens. However, in order

for the cornea tissue to ultimately respond to the lens-forming cues released from the retina, these cells, or at least a subset of cells, must first reduce the level of Wnt signaling.

5. CONCLUSIONS

Our work demonstrates that suppression of Wnt/ β -catenin signaling is necessary in order for lens regeneration from the cornea epithelium to occur in the frog. This result is different from iris-derived lens regeneration in the newt (where Wnt signaling has been shown to be necessary), and helps to highlight the importance of studying regenerative phenomenon in different systems. Our results also provide insight into our broader understanding of how some tissues and animals are capable of regenerating while others are not. Finally, the required suppression of Wnt/ β -catenin signaling appears to be similar to the role that Wnt/ β -catenin signaling plays in the surface ectoderm during early phases of embryonic lens development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BIO	6-bromoindirubin-3'-oxime
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
DKK1	Dickkopf-1
DMSO	Dimethyl sulfoxide
FGF	Fibroblast Growth Factor
GFP	Green Fluorescent Protein
GSK3β	Glycogen Synthase Kinase 3 Beta
HGEM	Heat-shock Green-eyed Monster
IWR-1	Inhibitor of Wnt Response 1
LEF	Lymphoid Enhancer Factor
LRP	Low-density lipoprotein Receptor-related Protein
NAM	Normal Amphibian Media
PBS	Phosphate-Buffered Saline

PCP	Planar Cell Polarity
qPCR	Quantitative Polymerase Chain Reaction
RA	Retinoic Acid
rhDKK1	Recombinant Human Dickkopf-1
RT	Reverse Transcriptase
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SFRP	Secreted Frizzled-Related Protein
TCF	T-Cell Factor

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Highlights

- Numerous wnt ligands and frizzled receptors are expressed in the cornea epithelium.
- Active Wnt/ β -catenin signaling, as measured by *axin2* levels, decreases in the cornea during early lens regeneration.
- Activation of Wnt/ β -catenin signaling significantly reduces the ability of the cornea to regenerate a lens.
- Inhibition of Wnt/ β -catenin signaling does not reduce the ability of the cornea to regenerate a lens.
- The role of Wnt/ β -catenin signaling during cornea-lens regeneration resembles that of early lens development and differs from Wolffian lens regeneration.

Figure 1. Expression of wnts and frizzled receptors in larval cornea and flank epithelium

RT-PCR was conducted on total RNA isolated from larval cornea epithelium ("C") or flank epithelium ("F"). "+" denotes a positive control from pooled embryonic total RNA. "C-" and "F-" are reverse transcriptase negative controls. Primers and corresponding product sizes are listed in Supplemental Table 1.

Figure 2. *axin2* expression decreases early during lens regeneration

axin2 transcriptional levels in cornea epithelium regenerating for either 24, 48, 72, or 96 hours. Control expression is from cornea epithelium that is not wounded and is not undergoing regeneration. *axin2* expression was normalized to *beta-actin* (*actb*). Asterisk denotes a statistically significant decrease (p value = 0.0006). Error bars indicate standard error.

Figure 3. Activation of Wnt signaling reduces successful lens regeneration

Lens regeneration percentages for treatment with Wnt signaling activators, including 10 μ M 1-azakenpauillone (A) or 1 μ M BIO (B), compared to DMSO treated controls. “Percent Regenerative Success” refers to the percentage of cases that successfully regenerated based on the presence of morphologically distinct lentoids that were positively stained by an anti-lens antibody. (C) Relative expression levels of *axin2* in corneas treated with 10 μ M 1-azakenpauillone or 1 μ M BIO. Asterisks indicate statistical significance ($p < 0.05$) and error bars indicate standard error. *axin2* expression was normalized to *ornithine decarboxylase* (*odc*). (D-K) Representative examples of positive cases of lens regeneration for BIO or 1-azakenpauillone treated eyes. D, F, H, and J show histological eye sections labeled using an

anti-lens antibody (red fluorescence). Arrowheads indicate regenerated lenses. Sometimes higher background signal is observed in retinal tissue (**H** and **J**), but lenses are easily identified due to their enhanced signaling and distinct morphology. **E**, **G**, **I**, and **K** show an overlay of anti-lens staining with the corresponding DIC images. Dark tissue is retinal pigment epithelium. All images are at the same scale, scale bar in **K** is 200 μm .

Figure 4. Wnt inhibition has no effect on lens regeneration

Lens regeneration percentages for *ex vivo* eyes treated with either 10 μ M IWR-1 (A) or rhDKK1 protein at either 200 ng/ml or 500 ng/ml (B). “Percent Regenerative Success” refers to the percentage of cases that successfully regenerated based on the presence of morphologically distinct lentoids that were positively stained by an anti-lens antibody. Neither of these experiments showed statistically significant differences. (C) Both IWR-1 and rhDKK1 treatment significantly decreased *axin2* transcriptional levels in treated cornea tissue, verifying the Wnt inhibitors had the desired effect on Wnt signaling. Asterisks

indicate statistical significance ($p < 0.05$), and error bars indicate standard error. *axin2* expression was normalized to *ornithine decarboxylase (odc)*. **(D-O)** Representative examples of positive cases of lens regeneration for IWR-1 or rhDKK1 treated eyes. **D, F, H, J, L,** and **N** show fluorescent (red) labeling of histological eye sections using an anti-lens antibody. Arrowheads indicate regenerated lenses. Sometimes higher background signal is observed in retinal tissue (**H** and **J**), but lenses are easily identified due to their enhanced signal and distinct morphology. **E, G, I, K, M,** and **O** show anti-lens staining overlaid with the corresponding DIC images. Dark tissue is retinal pigment epithelium. All images are at the same scale, scale bar in **O** is 200 μm .

Figure 5. Transgenic expression of DKK1 does not inhibit lens regeneration

(A) A diagram of the HGEM-DKK1 bi-cistronic transgene. The heat-shock inducible *hsp70* promoter drives expression of *Xenopus* DKK1, and a gamma-crystallin promoter drives GFP expression specific to the lens. (B-C) HGEM-DKK1 transgenic eye showing constitutive expression of GFP in the lens (C). (D-G) Anti-lens antibody staining of regenerated lenses in a sectioned control eye kept at room temperature (“Cont.”, D and E) and a heat-shocked eye (“HS”, F and G). Arrowheads indicate regenerated lenses. Dark tissue in DIC images is retinal pigment epithelium (D and F). All images are at the same scale, scale bar in K is 200

µm. **(H)** Regeneration percentages of HGEM-DKK1 eyes are not significantly different ($p=0.3706$). “Percent Regenerative Success” refers to the percentage of cases that successfully regenerated based on the presence of morphologically distinct lentoids that were positively stained by an anti-lens antibody. Error bars represent standard error. **(I)** Depletion of β -catenin (CTNNB1; 94 kDa) in western blots of HGEM-DKK1 transgenic tissue. β -tubulin (TUBB; 50 kDa) is the loading control.