The role of Pax-6 in lens regeneration

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Pax-6 is a master regulator of eye development and is expressed in the dorsal and ventral iris during newt lens regeneration. We show that expression of Pax-6 during newt lens regeneration coincides with cell proliferation. By knocking down expression of Pax-6 via treatment with morpholinos, we found that proliferation of iris pigment epithelial cells was dramatically reduced both *in vitro* and *in vivo*, and, as a result, lens regeneration was significantly retarded. However, induction of dedifferentiation in the dorsal iris was not inhibited. Pax-6 knockdown early in lens regeneration resulted in inhibition of crystallin expression and retardation of lens fiber induction. Once crystallin expression and differentiation of lens fibers has ensued, however, loss of function of Pax-6 did not affect crystallin expression and lens fiber maintenance, even though the effects on proliferation persisted. These results conclusively show that Pax-6 is associated with distinct early events during lens regeneration, namely control of cell proliferation and subsequent lens fiber differentiation.

newt | morpholino | proliferation | crystallin

The newt is one of the few adult vertebrates that can regenerate the lens after damage or removal. Newt lens regeneration is characterized by the process of transdifferentiation, whereby terminally differentiated pigment epithelial cells (PECs) of the dorsal iris dedifferentiate, proliferate, and then differentiate into lens cells (1–3). The process of transdifferentiation is rapid, with proliferation ensuing as early as 4 days after lentectomy (4, 5) and regeneration being completed in ≈30 days. Even though the ventral iris PECs initially proliferate, they do not normally contribute to the regenerative process. However, in a recent study we showed that induction of lens regeneration from the ventral iris is possible by inhibition of the bone morphogenetic protein pathway or overexpression of Six-3 with concomitant treatment with retinoic acid (6). Despite the known association of Six-3 and Pax-6 (7), it was interesting that Pax-6 was not able to elicit lens induction from the ventral iris (6).

Induction of lens regeneration from the dorsal iris differs considerably from induction of lens development where interactions between the surface ectoderm and the optic cup trigger differentiation of the lens vesicle (8, 9). However, the continued expression of genes involved in embryonic lens induction in the adult newt suggests that some genes may be involved in both processes. Indeed, lens differentiation-controlling genes such as *FGF*s, *Pax-6*, *Sox-2*, *MafB*, and *Prox-1* have been found in both dorsal and ventral irises of intact as well as regenerating newt eyes (10–14). Even though it has been established that the *Pax-6* gene is expressed during the process of lens regeneration, a direct association with events of regeneration has never been established (10, 13, 14).

Pax-6 is a known master regulator of eye development and mediator of ectopic lens formation (15–22), so a role in lens regeneration seems likely. Therefore, the present studies were undertaken to answer questions pertaining to the role of Pax-6 in lens regeneration. We used an antibody to Pax-6 to colocalize the protein with cellular events. We also established knockdown technology using morpholinos to down-regulate expression of Pax-6 to further study the events of lens regeneration associated with its expression.

Our results show that Pax-6 is a regulator of cell proliferation and lens fiber differentiation during lens regeneration. Proliferating cells in both dorsal and ventral iris are positive for Pax-6, and down-regulation of Pax-6 reduces proliferation and lens regeneration from the dorsal iris but does not abolish the inductive process of dedifferentiation. In addition, Pax-6 regulates crystallin expression and thus lens fiber differentiation during lens regeneration. These results indicate that Pax-6 plays a role in the proliferation and differentiation events of lens regeneration.

Results and Discussion

Pax-6-Expressing Cells of the Iris Proliferate During Lens Regeneration. To investigate a possible correlation between Pax-6 expression and proliferation during lens regeneration, we collected regenerating eyes at several time intervals after lentectomy. Eyes were injected with 1 μl of 10 mM BrdU 24 h before their collection to detect cells during the S phase. Beginning 5 days after lentectomy, BrdU-positive cells could be detected in both the dorsal and the ventral iris (Fig. 1 *A* and *B*). However, there were nearly 50% more proliferating cells in the dorsal iris than in the ventral iris (Fig. 1*C*). All BrdU-positive cells expressed Pax-6 (Fig. 1*B*). As regeneration continued (days 15 and 25), the number of BrdU-positive/Pax-6-expressing cells continued to increase in the dorsal iris while decreasing in the ventral iris (Fig. 1 *D–I*). Similar patterns were observed at days 7, 10, and 20 (data not shown).

Earlier studies have shown that the central portion of the dorsal iris has the most potential for regeneration (23), so we determined the spatial distribution of the dividing, Pax-6-positive cells by evaluating serial sections through the entire dorsal and ventral iris. We collected data from the central region, which was ≈60 μm thick on average, and from the peripheral regions, which consisted of 60-μm areas on either side of the central region. From 5 to 15 days after lentectomy, when the dorsal iris was proliferating, dedifferentiating, and giving rise to a new lens vesicle, Pax-6 expression and cell proliferation were mainly limited to the central portion of the iris, and only a small fraction of the proliferation occurred in the regions that flank the central portion of the iris (Fig. 1 *C* and *F*). This finding correlates perfectly with the above-mentioned study showing the ability of this part of the iris to elicit lens regeneration (23). By day 25 after lentectomy, as the regenerating lens grew in size, the

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Abbreviations: *Pax6*-Mo1, *Pax-6* morpholino1; *Pax6*-Mo2, *Pax-6* morpholino2; C-Mo, control morpholino; Mis-Mo, mismatch morpholino; PEC, pigment epithelial cell.

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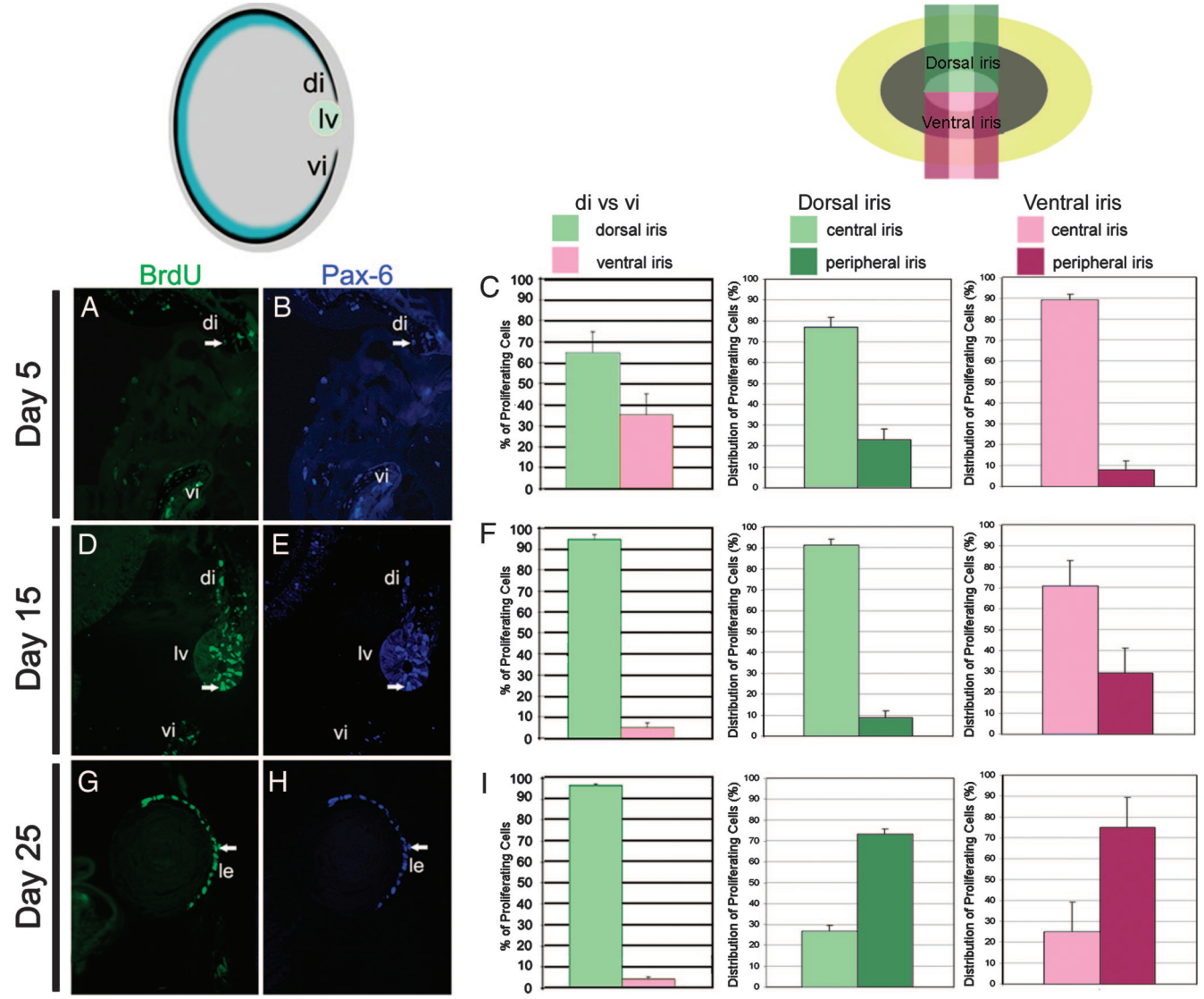

Fig. 1. Pax-6 is associated with proliferating cells in the dorsal and ventral iris during lens regeneration. Pax-6-expressing cells are shown in blue, and BrdU-positive cells are shown in green. (*A*, *D*, and *G*) BrdU-positive cells are present in both the dorsal iris (di) and the ventral iris (vi) at 5 (*A*), 15 (*D*), and 25 (*G*) days after lentectomy. (*B*, *E*, and *H*) All BrdU-positive cells in the dorsal and ventral iris also express Pax-6 (arrows). (*C*, *F*, and *I*) The percentage of proliferating cells is higher in the dorsal iris at 5 days after lentectomy (*C*), and this difference increases at 15 (*F*) and 25 (*I*) days after lentectomy. (*C* and *F*) Proliferation in the dorsal and ventral iris at 5 and 15 days after lentectomy is mostly limited to the central part of the iris and is reduced in the peripheral regions. (*G–I*) By day 25 after lentectomy the wave of proliferation (*G*) and Pax-6 expression (*H*) moves laterally along the lens epithelium (le) of the regenerating lens. lv, lens vesicle.

wave of proliferation and Pax-6 expression spread to the peripheral regions of the regenerating lens (Fig. 1 *G–I*).

A similar pattern of Pax-6 expression and proliferation localized to the central iris was also seen in the ventral iris through day 15 after lentectomy even though a much lower number of cells were proliferating (Fig. 1 *C* and *F*). This pattern of Pax-6 expression and cell proliferation tends to suggest that subsets of cells located in the central portion of both irises have the ability to respond to an injury and proliferate. There was a small number of proliferating cells in the ventral iris on day 25 after lentectomy (Fig. 1*I*).

We also studied the correlation between Pax-6 expression and apoptosis (Fig. 6, which is published as supporting information on the PNAS web site). No apoptotic cells were present through day 10 after lentectomy. There were a few apoptotic cells in lenses analyzed 15–25 days after lentectomy, but there was no correlation between apoptosis and Pax-6 expression.

Morpholinos Inhibit *Pax-6* Expression, and Loss of Pax-6 Inhibits Proliferation in Cultured PECs. Because Pax-6 is associated with proliferation of the iris PECs during lens regeneration, we proceeded with knockdown experiments to evaluate the importance of Pax-6 in lens regeneration. We tested two experimental morpholinos designed to inhibit Pax-6 expression, *Pax-6* morpholino1 (*Pax6*-Mo1) and *Pax-6* morpholino2 (*Pax6*-Mo2), in cultured iris PECs. A nonspecific control morpholino (C-Mo) and a mismatch morpholino (Mis-Mo) were used as controls. The untreated cells (Fig. 2 *A* and *F*), as well as the cells transfected with C-Mo or Mis-Mo (Fig. 2 *B*, *C*, *G*, and *H*), had high levels of Pax-6 protein. However, cells transfected with *Pax6*-Mo1 or *Pax6*-Mo2 showed a significant reduction in Pax-6 protein (Fig. 2 *D*, *E*, *I*, and *J*). Furthermore, *Pax-6* morpholinos had no effect on the protein expression of FGF receptor 1 or Six-3, two other key molecules in lens development, confirming the specific knockdown of Pax-6 by these morpholinos (Fig. 7,

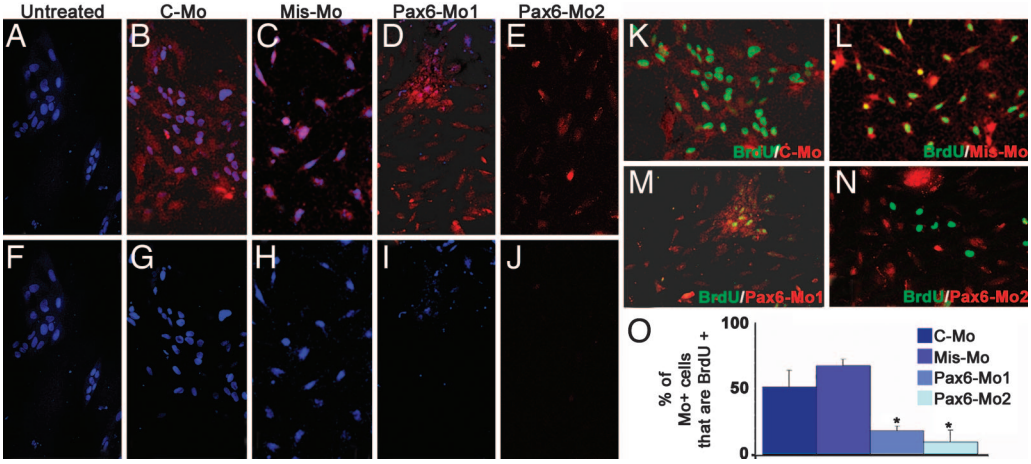

Fig. 2. *Pax-6* morpholinos decrease levels of Pax-6 and proliferation in cultured PECs. Morpholino fluorescence is red, and Pax-6 immunofluorescence is blue. Untreated cells (*A* and *F*) and cells treated with control (C-Mo) (*B*) or Mis-Mo (*C*) show robust Pax-6 immunofluorescence, whereas cells treated with morpholinos against *Pax-6* (*Pax6*-Mo1 and *Pax6*-Mo2) show reduced levels of Pax-6 (*D* and *E*). (*G–J*) Pax-6 immunofluorescence only from *B–E*. Cultured PECs transfected with Pax-6 morpholinos (*M* and *N*) show fewer BrdU-positive cells than cells transfected with the control (*K*) or the Mis-Mo (*L*). The number of proliferating cells is shown as a percentage of the number of cells transfected with the morpholinos (*O*). The reduction in proliferation is significant ($P < 0.01$). Asterisks in *O* denote statistical significance. All error bars are SEM.

which is published as supporting information on the PNAS web site).

In addition, cells transfected with *Pax-6* morpholinos showed a significant decrease in proliferation (Fig. 2 *M–O*) as assayed by BrdU incorporation compared with cells transfected with the C-Mo or Mis-Mo (Fig. 2 *K*, *L*, and *O*). These results show the *Pax-6* morpholinos are specific in down-regulating Pax-6 and indicate a functional role for Pax-6 in regulating proliferation.

***Pax-6* Morpholinos Down-Regulate Pax-6 During the Lens Regeneration Process.** Having established the specificity of the morpholinos *in vitro*, we began *in vivo* experiments to assay the role of Pax-6 during lens regeneration. In all *in vivo* experiments that follow, both *Pax-6* morpholinos had the same effect. Likewise, no differences were observed among the two control morpholinos. Therefore, to avoid redundancy, representative figures of the effects of one morpholino (experimental or control) will be presented. To determine the efficiency of the *Pax-6* morpholinos *in vivo*, we injected the morpholinos into newt eyes on day 10 after lentectomy and collected them for immunohistochemistry on day 13 after lentectomy. Animals injected with either control morpholino maintained a level of Pax-6 protein indistinguishable from the untreated animals (Fig. 3 *A*, *B*, and *D*), whereas animals injected with *Pax-6* morpholinos showed a marked reduction in Pax-6 expression in the dorsal iris (Fig. 3 *C* and *E*).

Down-Regulation of Pax-6 During Lens Regeneration Retards Lens Vesicle Formation. Having determined that the morpholinos are able to down-regulate Pax-6 *in vivo*, we proceeded with knocking down expression of Pax-6 at various times during the lens regeneration process. We first examined whether Pax-6 plays a role in lens vesicle formation. During normal lens regeneration, the lens vesicle is formed by proliferation of dorsal iris cells beginning on day 4 after lentectomy and continuing at high levels through day 15 after lentectomy (see Fig. 1). Therefore, we injected newt eyes with morpholinos 4 and 10 days after lentectomy and examined the morphology of the regenerating lenses 15 days after lentectomy. This double-injection strategy allowed us to down-regulate Pax-6 during the entire phase of lens vesicle formation.

By day 15, untreated controls had a well defined lens vesicle with elongating cells indicative of lens fiber differentiation in 18 of 20 (90%) animals (Fig. 3 *F* and *G*). Comparable lens vesicles also formed in 14 of 18 (77.8%) animals treated with C-Mo and 15 of 17 (88.2%) animals treated with Mis-Mo (Fig. 3 *F* and *H*) However, in the majority of the eyes treated with *Pax-6* morpholinos, dedifferentiation of the dorsal iris tip was obvious but formation of a lens vesicle occurred in only 4 of 16 (25%) animals treated with *Pax6*-Mo1 or 5 of 12 (41.6%) animals treated with *Pax6*-Mo2 (Fig. 3 *F* and *I*). There was no increase in cell death in the lenses of *Pax-6* morpholino-treated animals compared with animals treated with either control morpholino (Fig. 8 *A–F*, which is published as supporting information on the PNAS web site). Therefore, the retardation of regeneration in treated eyes is not due to an increase in cell death, suggesting that Pax-6 is not involved in cell survival.

Pax-6 Controls Proliferation of Cells in the Regenerating Lens. A retardation of lens regeneration and a lack of apoptotic cells as a result of *Pax-6* morpholino treatment suggest that Pax-6 down-regulation may cause a decrease in proliferation. To test this hypothesis, we injected morpholinos once at day 10 after lentectomy (a time when proliferation is at a high peak in normal regenerating lenses) and collected them 3 days later. Twenty-four hours before collecting tissues, BrdU was injected into the eyes of these animals. BrdU analysis revealed that proliferation was reduced when Pax-6 was down-regulated. On day 13 after lentectomy, 50% of cells in the lens vesicle of animals injected with either the C-Mo or Mis-Mo were proliferating. In contrast, only 28% or 35% of the cells in the lens vesicles were proliferating in animals treated with *Pax6*-Mo1 or *Pax6*-Mo2, respectively (Fig. 3 *J–O*). This reduction in proliferation is statistically significant ($P < 0.001$) and suggests that down-regulation of Pax-6 is responsible for reduced proliferation. Again, we confirmed that the decrease in the number of proliferating cells was not caused by an increase in cell death. Down-regulation of Pax-6 on days 10–13 after lentectomy did not show an increase in apoptosis because there are essentially no apoptotic cells in the lenses of animals treated with either *Pax-6* morpholino or the control morpholinos (Fig. 8 *G–L*).

Down-Regulation of Pax-6 Inhibits Differentiation in the Regenerating Lens. During lens development, Pax-6 regulates the expression of various crystallins and is required for lens fiber differentiation (16,

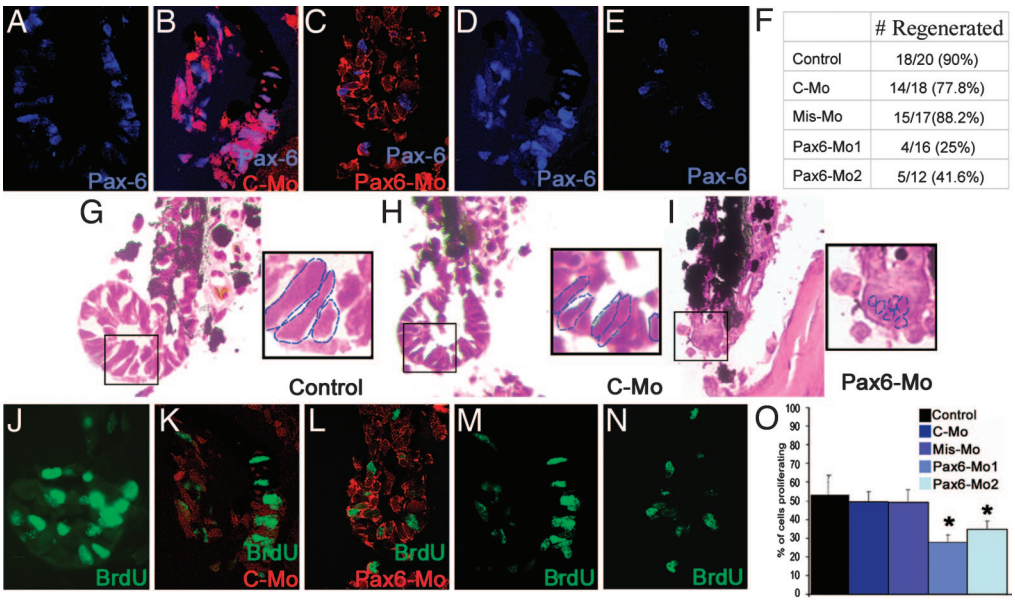

Fig. 3. Knockdown of Pax-6 decreases proliferation and inhibits lens regeneration. (*A–E*) Animals were treated with morpholinos at 10 days after lentectomy and collected at day 13 after lentectomy. Untreated control eyes (*A*) and eyes treated with C-Mo (*B*) express similar levels of Pax-6 (blue) in the regenerating lens vesicle. (*C*) Treatment with *Pax6*-Mo1 reduces expression of Pax-6. (*D* and *E*) Pax-6 staining only from *B* and *C*, respectively. (*F–I*) Animals were injected with morpholinos at day 4 and day 10 after lentectomy and collected at 15 days after lentectomy. (*F*) Animals treated with Pax-6 morpholinos showed a marked reduction in lens vesicle formation compared with untreated animals or animals injected with the C-Mo or Mis-Mo. (*G* and *H*) The lens vesicle from untreated animals and animals treated with C-Mo have elongating cells (*Insets*). The lens vesicle fails to form in animals treated with *Pax6*-Mo1; however, dedifferentiation at the dorsal tip does occur (*I*). (*J–N*) Animals were injected with morpholinos at 10 day after lentectomy and collected at 13 days after lentectomy. Lens vesicles of untreated control eyes (*J*) and of eyes treated with C-Mo (*K*) have a higher number of BrdU-positive cells compared with eyes treated with *Pax6*-Mo1 (*L*). (*M* and *N*) BrdU staining of *K* and *L*, respectively. (*O*) Morpholino knockdown of Pax-6 results in a statistically significant reduction in proliferation. The number of proliferating cells is shown as a percentage of the total number of cells in each group. All error bars are SEM. Asterisks indicate statistical significance of $P < 0.01$ when compared with animals treated with C-Mo or Mis-Mo. Sections shown in *B* and *K* and in *C* and *L* are identical and were stained for Pax-6 and BrdU, but the results are presented separately.

17, 24, 25). We therefore decided to determine whether Pax-6 plays a similar role during regeneration. Differentiation of lens fibers normally begins on day 13 after lentectomy. Thus, animals were lentectomized, injected with morpholinos on day 10 after lentectomy, collected, and assayed for α and β crystallin expression at 13 days after lentectomy. Control lens vesicles (Fig. 4 *A* and *F*) and lens vesicles from animals injected with control morpholinos (Fig. 4 *B*, *C*, *G*, and *H*) showed expression of both crystallins whereas lens vesicles from animals that were injected with the *Pax-6* morpholinos did not express either α or β crystallin (Fig. 4 *D*, *E*, *I*, and *J*). These results suggest that Pax-6 is required for the expression of crystallin genes during lens regeneration. However, it is also possible that crystallin expression is just delayed in eyes treated with *Pax-6* morpholinos because inhibition of Pax-6 expression retards lens regeneration (Fig. 3 *G–I*). To address this possibility, we injected animals with *Pax-6* morpholinos on day 10 after lentectomy, but, instead of collecting them on day 13 when crystallin expression normally begins, we allowed them to regenerate until 16 days after lentectomy before assaying for crystallin expression. These regenerating lenses did not express α or β crystallin, suggesting that Pax-6 plays a role in regulating crystallin expression (Fig. 9, which is published as supporting information on the PNAS web site).

Pax-6 Regulates Proliferation but Not Differentiation at Later Stages of Regeneration. The above results indicate that Pax-6 is needed for the early events of proliferation and initiation of lens fiber

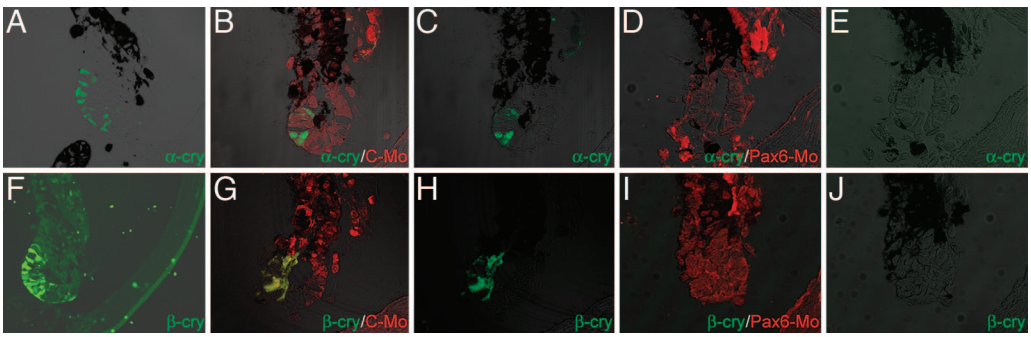

Fig. 4. *Pax-6* morpholino treatment inhibits crystallin expression. Morpholinos were injected at day 10 after lentectomy, and animals were collected for assay at day 13 after lentectomy. (*A* and *F*) Lens vesicles from untreated control eyes express α and β crystallin (α-cry or β-cry). (*B* and *G*) 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 from *G* and *I* (*H* and *J*).

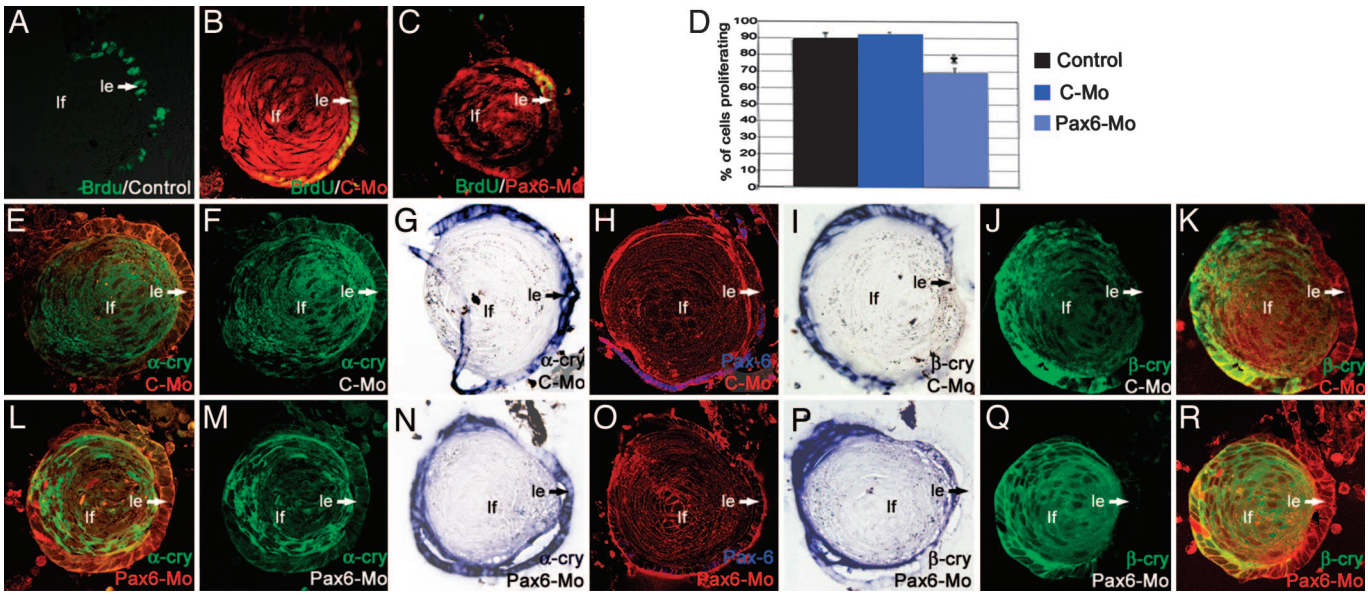

Fig. 5. *Pax-6* morpholino treatments affect proliferation of lens epithelial cells at later stages of regeneration and have no effects on the regulation of crystallin expression. Morpholinos were injected at day 20 after lentectomy and collected at day 23 after lentectomy. (*A* and *B*) Untreated eyes (*A*) and eyes treated with C-Mo (*B*) have an equivalent number of proliferating cells. (*C*) Eyes treated with *Pax6*-Mo1 show reduced proliferation. (*D*) There are ≈25% more cells proliferating in the lens epithelium of C-Mo-treated eyes than *Pax-6* morpholino-treated eyes. This difference is statistically significant ($P < 0.01$). (*E–G* and *L–N*) α crystallin expression. Eyes treated with C-Mo express α crystallin protein (*E* and *F*) and transcripts (*G*), and these expression patterns are not altered in eyes treated with *Pax6*-Mo1 (*L–N*). In *G* the ripped lens epithelium is a histological artifact. Likewise, there is no alteration in expression patterns for β crystallin protein and transcripts in eyes treated with C-Mo (*I–K*) or *Pax6*-Mo1 (*P–R*). Note that β crystallin is not expressed in the lens epithelium (le). (*H* and *O*) Adjacent sections to the sections used for *in situ* hybridization showing expression of Pax-6 in C-Mo-treated (*H*) or *Pax6*-Mo1-treated (*O*) eyes. Error bars in *D* are SEM. α-cry, α crystallin; β-cry, β crystallin; lf, lens fibers; le, lens epithelium.

differentiation. However, it is not clear whether Pax-6 is necessary for continued proliferation and maintenance of crystallin expression during the later stages of regeneration. To address this question, we injected lentectomized animals with the morpholinos on day 20 and collected the animals on day 23. By this time, a well differentiated lens with distinct epithelium and fiber expressing crystallin had formed. Animals were also injected with BrdU 24 h before collection to assay for cell proliferation. Eyes injected with *Pax-6* morpholinos showed a 23% reduction in cell proliferation in regenerating lenses when compared with eyes injected with control morpholinos (69% proliferation for *Pax-6* morpholino compared with 92% in controls) (Fig. 5 *A–D*). This decrease in proliferation did not correspond to an increase in cell death (Fig. 8 *M–R*). However, regenerating lenses from *Pax-6* morpholino-treated eyes still expressed α and β crystallin proteins (Fig. 5 *L*, *M*, *Q*, and *R*) indistinguishable from crystallin protein expression in lenses from eyes treated with control morpholinos (Fig. 5 *E*, *F*, *J*, and *K*). It is possible that we detected crystallin protein that was produced before the *Pax-6* morpholinos were introduced. To exclude this possibility, we performed *in situ* hybridization using probes for α and β *crystallin* and immunohistochemistry on consecutive sections. We show that crystallin transcripts were being produced in cells regardless of whether Pax-6 was present (C-Mo) (Fig. 5 *G–I*) or absent (*Pax-6* morpholino) (Fig. 5 *N–P*). This result confirms that Pax-6 is not needed for crystallin regulation after the initiation of lens differentiation.

Table 1. Summary of *in vivo* treatments and corresponding results

Parameter assessed	Days of morpholino injection	Day of BrdU injection	Days of tissue collection	Result
Regeneration	4 and 10	-	15	Retarded
Early proliferation	10	12	13	Reduced
Late proliferation	20	22	23	Reduced
Onset of crystallin differentiation	10	-	13 and 16	Inhibited
Maintenance of crystallin expression	20	-	23	Unaffected
Cell death	4 and 10	-	15	Unaffected
Cell death	10	-	13	Unaffected
Cell death	20	-	23	Unaffected

Conclusions

Our experiments, summarized in Table 1, clearly show that the primary role of Pax-6 during regeneration is the regulation of proliferation in both the dorsal and ventral iris. In addition to its role in proliferation, Pax-6 regulates early fiber differentiation and crystallin expression in the regenerating lens vesicle. Because dedifferentiation was not inhibited, our data suggest that Pax-6 is not directly involved in the induction of lens regeneration from the dorsal iris. Furthermore, knockdown technology in the newt opens new avenues for experimentation in this important field of regeneration.

Materials and Methods

Animals. Adult newts (*Notophthalmus viridescens*) were obtained from Mike Tolley Newt Farm (Nashville, TN) or Sullivan Newt Farm (Nashville, TN). A 0.1% 3-aminobenzoic acid (Sigma, St. Louis, MO) solution was used as an anesthetic for surgical procedures and killing.

Surgeries and BrdU Injections. Lentectomies were performed by making a slit across the cornea with a scalpel and then applying gentle pressure to the eye to remove the lens. By using a glass

micropipette, eyes were injected with 1 μl of a 10 mM BrdU solution (Roche, St. Louis, MO) 24 h before newts were killed.

Immunohistochemistry. Fixed eyes were processed for immunohistochemistry as described by Tsonis *et al.* (26) and analyzed by using an Olympus BX-51 epifluorescence microscope or an Olympus laser confocal microscope (Olympus, Melville, NY). Sources of antibodies and dilutions are listed in *Supporting Text*, which is published as supporting information on the PNAS web site.

BrdU Quantitation. The number of BrdU-positive cells, as well as the total number of cells in regenerating lenses, were counted from at least two sections from six to eight eyes per experimental group. The average number of BrdU-positive cells is shown as a percentage of the average total number of cells for each experimental group. BrdU-positive cells were then counted in the central or peripheral (nasal and temporal) iris, and their distribution is presented as a percentage of the total number of BrdU-positive cells in each dorsal or ventral iris. Statistical significance was determined by Student's *t* test. To determine the number of proliferating cells in treated cultured iris cells, the number of BrdU-positive cells, as well as the total number of cells transfected with the morpholino, was counted from 24 fields of view from six independent experiments, and Student's *t* test was performed to determine statistical significance.

Morpholinos. All special delivery morpholinos were purchased from GeneTools (Philomath, OR). All morpholinos were labeled with lissamine. The oligonucleotide sequence for each morpholino was as follows: antisense *Pax6*-1 (*Pax6*-Mo1), 5′-TGTCTCCCTTATGTAGTCCCTCATG-3′; antisense *Pax6*-2 (*Pax6*-Mo2), 5′-CCTCTCCGCTGCCGGGTCTTTAGCT-3′; Mis-Mo, 5′-CCTGTCCCCTGCCCGGTGTTTACCT-3′; C-Mo, 5′-CCTCTTACCTCAGTTACAATTTATA-3′ (underlined bases in the Mis-Mo sequence indicate differences between the Mis-Mo and *Pax6*-Mo2 sequences). For *in vitro* analysis, 14-day-old newt PECs from the iris (6) were transfected by using the special delivery method as per the manufacturer's instructions. Forty-eight hours after transfection, 150 μl of 10 mM BrdU was added, and the transfected cells were fixed 24 h later in 4% paraformaldehyde and 3% sucrose for 30 min at room temperature. Immunohistochemistry was performed to detect Pax-6, BrdU, FGFR1, and Six-3 as described in ref. 6. Results were analyzed by using an Olympus laser confocal microscope. For *in vivo* analysis, 10 μl of each morpholino was mixed with 5.6 μl of endoporter (GeneTools), and 1 μl of this solution was injected into the vitreous chamber of a lentectomized adult newt eye. For proliferation studies and analysis of Pax-6 and crystallin expression, the morpholino/endoporter injections were made 10 days or 20 days after lentectomy, and 1 μl of a 10 mM BrdU solution was injected into the eye 12 days after lentectomy or 22 days after lentectomy, respectively. These eyes were collected 24 h after BrdU was administered and processed for immunohistochemistry. For histological data, the morpholino/endoporter solution was injected twice on days 4 and 10 after lentectomy. These eyes were collected 15 days after lentectomy, fixed in Bouin's solution, and embedded in paraffin. Ten-micrometer sections were stained with hematoxylin and eosin and observed by using an Olympus BX-51 microscope. In addition to both control morpholinos, we also processed eyes with endoporter only or with no treatment at all.

***In Situ* Hybridization.** Probes for α and β crystallin were prepared by using a DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN). *In situ* hybridization was performed as described by Toresson *et al.* (27).

TUNEL. Apoptotic cells were detected with an *In Situ* Cell Death detection kit, fluorescein, or TMR red (Roche Applied Science) following the manufacturer's instructions.

The anti-Pax-6 antibody developed by Atsushi Kawakami was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by University of Iowa Department of Biological Sciences (Iowa City, IA). The crystallin antibodies were a gift from G. Eguchi (Skokei Educational Institution, Kumamoto, Japan). This work was supported by National Institutes of Health Grant EY10540 (to P.A.T.), the Miami University Dissertation Research Award (to M.M.), the Madalene and George Shetler Diabetes Research Award (to T.L.H. and K.D.R.-T.), and the Miami University Undergraduate Research Award (to C.M.M.).

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