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Effects of a CDK inhibitor on lens regeneration

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Lens regeneration in adult newts is always initiated from the dorsal iris by transdifferentiation of the pigment epithelial cells. One of the most important early events should be the ability of pigment epithelial cells to dedifferentiate and re-enter the cell cycle. As a first step in an attempt to study this event, we have decided to examine the effects of a cyclin-dependent kinase-2 inhibitor on lens regeneration. At the appropriate concentration, this inhibitor completely abolished the ability of pigment epithelial cells to form a new lens, but it did not stop them from dedifferentiating and forming a small lens vesicle. The effects of this inhibitor seem to be mediated by its opposite effects on cell proliferation and apoptosis. The inhibitor significantly reduced cell proliferation and enhanced apoptosis of pigment epithelial cells both in vitro and in vivo and of the regenerating lens in vivo. (WOUND REP REG 2004;12:24-29)

Lens regeneration, as occurring in adult salamanders, is controlled by events that are intimately linked to the cell cycle. Upon removal of the lens, the pigment epithelial cells (PECs) from the dorsal iris undergo a dedifferentiation process. These terminally differentiated cells are capable of re-entering the cell cycle, proliferating, and finally transdifferentiating into lens epithelial cells, which consequently will give rise to lens fibers and to the complete regenerated lens.^{1,2} The differentiation of the lens epithelial cells to fibers is also dominated by the loss of organelles and by apoptosis.^{3,4} However important these events of proliferation and apoptosis are for the process of lens regeneration, little is known about the mechanisms and the molecules involved. We have begun to address these issues and as a first step we have decided to

BrdU	5-bromodeoxyuridine
CDK	Cyclin-dependent kinase
CKI	CDK inhibitor
DMSO	Dimethylsulfoxide
PBS	Phosphate buffered saline solution
PEC	Pigment epithelial cells
Rb	Retinoblastoma protein
TBS	Tris buffered saline solution
TUNEL	Terminal deoxynucleotidyl transferase nick end labeling

examine the role of a specific inhibitor of cyclin-dependent kinases (CDKs), which are largely known as important regulators of proliferation, apoptosis, and differentiation in lens development.⁵

CDKs control the progression through the G1 phase of the cell cycle and the initiation of DNA synthesis (S phase). The activities of CDKs are in turn regulated by specific inhibitors called CDK inhibitors (CKIs). There are two classes of CKIs. The first includes the INK4 proteins, named as such because they inhibit CDK4 and also CDK6. These proteins are the p16^{INK4a}, p15^{INK4b}, p18^{INK4c} and p19^{INK4c}. The other class contains the inhibitors of the Cip/Kip family, which affect the activities of cyclin D-, E-, and A-dependent kinases. This class includes the proteins p21^{Cip1}, p27^{Kip1} and p57^{Kip2}.⁵⁻⁷ One of the targets of these inhibitors is (via inhibition of CDKs) phosphorylation of the retinoblastoma protein (Rb), which has been shown to be important in lens differentiation as well.^{8,9} When Rb is hypophosphorylated it is active and

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this results in lens fiber differentiation and fiber denudation. This pathway of events does not require p53. However, when Rb is hyperphosphorylated (inactive), this leads to proliferation and via the activation of p53 to apoptosis in the lens.³ Obviously, the control of Rb phosphorylation, which is mediated by CDK inhibitors p27 and p57, is paramount in establishing the stage for the interplay between proliferation and differentiation of lens cells.⁹

Given the importance of the mechanisms involved in cell proliferation and apoptosis, we have decided to probe the effects of a specific CDK inhibitor on the process of lens regeneration. This inhibitor is 1.8-fold and 9-fold more selective for cdk2 than cdk4 and cdk1, respectively.¹⁰

MATERIALS AND METHODS

A novel cdk2-selective inhibitor, SU9516 from SUGEN (South San Francisco, CA) was used. SU9516, 3-[1-(3*H*-imidazol-4-yl)-meth-(*Z*)-ylidene]-5-methoxy-1,3-dihydroindol-2-one, is a novel 3-substituted indolinole compound.¹⁰ For use in our experiments SU9516 was dissolved in dimethylsulfoxide (DMSO).

Animals and operations

The newt, *Notophthalmus viridescens*, was used in our studies. After lentectomies, the animals were placed in inhibitor solutions. Several concentrations, ranging from 1 μ M-100 μ M, were examined in this study. Control animals were treated with appropriate DMSO solutions. The newts were kept for 20 days, at which time the eyes were collected and examined histologically. All operations were performed under anesthesia according to animal care protocols approved by the University of Dayton IACUC. MS222 (Sigma Chemical, St. Louis, MO) was used as anesthesia.

BrdU incorporation in cultured cells

To examine the effects of SU9516 on cell proliferation, we treated cells with the compound (10 μ M for 24 hours and pulsed them with 5-bromodeoxyuridine [BrdU] for 60 minutes). For these studies, we used a rat cell line (LacZ rat gliosarcoma, CRL-2200) and primary cultures of PECs isolated from the dorsal iris. For this procedure, we used the In Situ Cell Proliferation Kit, FLUOS, Cat. no. 1810740 from Roche Molecular Biochemicals (Indianapolis, IN). To the culture medium, 1/10 volume of BrdU labeling solution was added, and incubated in a humidified chamber at 25 °C for 60 minutes. After that, cells were washed three times with phosphate buffered saline solution (PBS) and fixed with 3 volumes 50 μ M glycine solution pH 2.0 with 7 volumes 100 percent ethanol at 4 °C for 30 minutes. Following fixation, the cells were washed with PBS and HCl-

denaturation solution was added at room temperature for 20 minutes. Cells were then rinsed three times with PBS and were incubated with incubation buffer at room temperature for 10 minutes. Anti-BrdU-FLUOS antibody working solution was added to cells and incubated in a humidified chamber at 37 °C for 45 minutes. Cells were then washed twice with PBS, covered with GEL/MOUNT (Biomedica Corp., Foster City, CA), and allowed to dry before analysis. Several areas in different slides were selected and positive vs. negative cells were counted.

Apoptosis analysis in vitro

The same cells used for the proliferation assays were also examined for the effects of the inhibitor on apoptosis using the terminal deoxynucleotidyl transferase nick end labeling (TUNEL) procedure. The TdT-FragEL™, DNA Fragmentation Detection Kit, Cat# QIA33 from Oncogene Research Products (Boston, MA) was used. After treatment (10 μ M, 24 hours) cells were fixed with 4 percent formaldehyde (in PBS) at room temperature for 10 minutes. Cells were then pelleted by centrifugation for 5 minutes and resuspended in 80 percent ethanol, fixed (100 μ l) onto poly L-lysine coated glass slides and stored at 4 °C. Slides were rehydrated by immersion in tris-buffered saline solution (TBS) for 15 minutes at room temperature. Sections were incubated with 20 μ g/ml proteinase K at room temperature for 5 minutes and then the slides were rinsed two to three times with TBS. To inactivate endogenous peroxidase the slides were treated with 3 percent H₂O₂, incubated at room temperature for 5 minutes, and rinsed two to three times with TBS. For labeling, sections were covered with 1X TdT Equilibration Buffer and incubated at room temperature for 30 minutes followed by TdT Labeling Reaction Mixture, which was placed onto sections. Each specimen was covered with a piece of parafilm and the slides were incubated in a humidified chamber at 37 °C for 1.5 hours. After that time, parafilm was removed from the slides, and slides were rinsed two to three times in TBS. To terminate the reaction, sections were incubated with Stop Solution at room temperature for 5 minutes and then rinsed two to three times with TBS. For detection, sections were covered with Blocking Buffer at room temperature for 10 minutes. The blocking buffer was blotted from the sections and immediately sections were covered with diluted 1X conjugate and incubated in a humidified chamber at room temperature for 30 minutes. Sections were then incubated with diaminobenzidine solution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and incubated for 15 minutes at room temperature, rinsed with deionized H₂O and covered with Crystal/Mount (Biomedica Corp.) and allowed to dry before observation. Several areas in different slides were selected and

positive vs. negative cells were counted. The student's *t*-test was used for statistical analysis.

In vivo proliferation and apoptosis determination

Animals were treated with the CDK inhibitor (25 μ M) for 4 days between day 12 and 15 postlentectomy. After the treatment (the 16th day), eyes were injected with BrdU and were collected 1 day later. For analysis of apoptosis, animals were treated the same way and eyes were collected at the end of the treatment. We selected this treatment and not a continuous one because continuous treatment does not result in any regenerating lens (see Results), and thus we would not be able to observe proliferation and cell death patterns in the regenerating lens. The protocols for BrdU and cell death detection were similar to the ones used for cultured cells, but from different kits. The In Situ Cell Death detection kit TMR-red (Roche) was used for apoptosis and the BrdU labeling and detection kit I (Roche) was used for proliferation. After collection, the eyes were fixed in 4 percent formaldehyde for 10 minutes and then were treated with 30 percent sucrose for cryoprevention. The whole eyes were sectioned and proliferation and apoptosis profiles were observed in selected sections, which represented the same area unit in all samples. The student's *t*-test was employed for statistical analysis.

RESULTS AND DISCUSSION

Lens regeneration in the adult newt begins with dedifferentiation and proliferation of dorsal iris PECs. By

dedifferentiation we mean the loss of characteristics that define the PECs, such as pigmentation. Dedifferentiation initiates molecular events, such as re-entering the cell cycle, which is necessary for cell proliferation and the subsequent regeneration of the lens. The first peak of cell proliferation in the dorsal iris is observed between 4 and 6 days postlentectomy.¹¹ At about 10 days postlentectomy, a lens vesicle is formed from the depigmented dorsal PECs. Around 12–16 days postlentectomy, the internal layer of the lens vesicle thickens and synthesis of crystallins begins. From 12 to 15 days postlentectomy, a second peak of cell proliferation is observed in the dorsal iris.¹¹ This marks the beginning of primary lens fiber differentiation (Figure 1a). During days 15–19, proliferation and depigmentation of PECs slow down. In the internal layer of the regenerating vesicle, the lens fiber complex is formed and in the margin of the external layers nondividing secondary lens fibers appear. By 18–20 days the PECs have stopped proliferating and the lens fibers continue to accumulate crystallins (Figure 1b). Lens regeneration is considered complete by day 20–25.^{1,2}

Therefore, lens regeneration is possible by transdifferentiation, which is the transformation of one cell type to another (in this case PECs to lens cells). The process of transdifferentiation has been proven beyond any doubt in this system. When single PEC cells are placed in culture the process of transdifferentiation can be observed.^{1,12} As the PECs proliferate, they become depigmented and then transdifferentiate to lens cells.

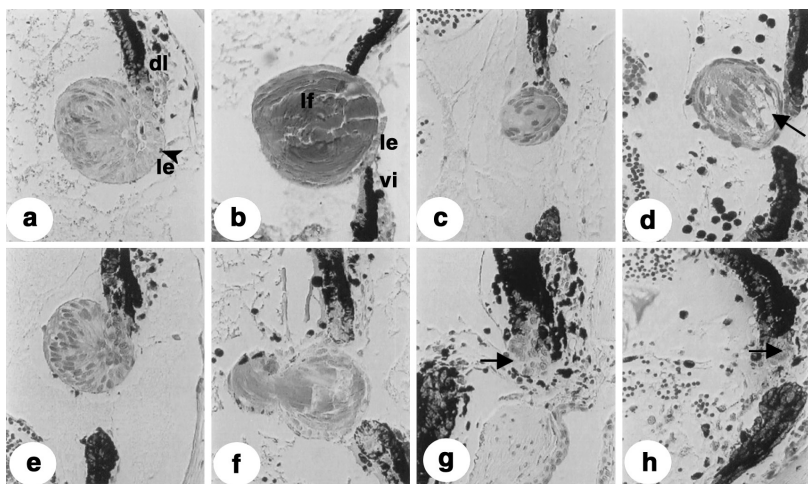


FIGURE 1. Histologic appearance of lens tissue following *in vivo* treatment of newts with a CKI. (a) Normal lens regeneration 15 days postlentectomy. Note the lens fiber differentiation from the posterior part of the lens (arrow); le: lens epithelium, di: dorsal iris. (b) Normal lens regeneration 20 days postlentectomy. Note that the lens has been regenerated and is packed by lens fibers (lf); le: lens epithelium, vi: ventral iris. (c) A regenerated lens 15-days postlentectomy after treatment with the CKI during days 4–6 postlentectomy. Note that the lens is smaller. (d) Same treatment as in (c), but the lens was examined 20 days postlentectomy. Note that the lens is vacuolated (arrow). (e, f) Regenerated lens 20 days after lentectomy following treatment with the CKI during days 12–15 postlentectomy. In (e) we can see a retarded lens and in (f), regeneration of two lenses. (g, h) Two cases of inhibited lens regeneration, but with a small dedifferentiated vesicle (arrows) in eyes treated continuously with 25 μ M of the CDK inhibitor. The eyes were examined at day 20 postlentectomy. (Original magnification for all sections \times 40)

Therefore, while in many other regenerative tissues stem cells may play a role, such a possibility is very unlikely for lens regeneration.

Several treatments with the CKI were performed (see Table 1 for a summary of the treatments and effects). Continuous treatment at 1 μM concentration did not affect lens regeneration at all. Also, treatment at 100 μM was lethal for the animals. The most dramatic effects that were not lethal were observed when the animals were treated at 25 μM concentration. When animals treated during the first peak of proliferation (4–6 days postlentectomy) were examined, only two lenses out of 14 were affected. These lenses were of a good size (even though smaller than the untreated control), but they were vacuolated (Figure 1c, d). When animals treated during the second peak of proliferation (12–15 days postlentectomy) were examined, three out of 16 lenses were affected. Of these three lenses, two were retarded and the other case was a double lens (Figure 1e, f). When animals were treated continuously (from day 1 postlentectomy), examination of 18 eyes showed that in eight eyes lens regeneration was completely inhibited and in the remaining ten lenses regeneration was severely impaired with only a small vesicle differentiated from the dorsal iris (Figure 1g, h). The major point of these results is that a continuous treatment is necessary for the CKI to exert its effect on the morphogenesis of the regenerated lens. Short treatments can affect regeneration somewhat, but obviously the process is able to recover. The vacuolated lenses are reminiscent of cataracts observed in lenses. The only case of double lens is, of course, interesting. Such a result is very rare and we have never seen it during normal regeneration. The only other cases of such double lenses we have seen were obtained by the use of exogenous fibroblast growth factor.¹³ We believe that cells at the equator change polarity and differentiate to fiber cells on the posterior part of the eye as well as creating two lenses. Abnormal proliferation patterns could be the reason for such an effect. The fact that the majority of the cases treated continuously at the 25 μM concentration were totally inhibited or only showed early vesicle differentiation is very interesting. Obviously, the inhibitor does not completely inhibit the dedifferentiation process, but it does inhibit the fiber differentiation process in 100 percent

Table 1. Number of affected regenerated lenses due to the CKI treatment

Duration of treatment	CKI concentration (μM)		
	1	25	100
4–6 days postlentectomy	No effect	2/14	lethal
12–15 days postlentectomy	No effect	3/16	lethal
Continuous	No effect	18/18*	lethal

*In 8/18 complete inhibition was observed and in 10/18 only a small lens vesicle was formed.

of the cases. This might be explained by the effects of this inhibitor on cell proliferation and apoptosis (see below).

Having established the effects of this CKI on morphogenesis of the regenerating lens, we decided to examine its effects on cell proliferation and apoptosis. For this, we analyzed profiles of treated cells in vitro and during lens regeneration in vivo. For the in vitro studies, we used an established rat cell line as well as primary cultures of PECs isolated from the dorsal iris of the adult newt. The rat cell line was used only as a reference point. When cells were treated with the inhibitor, incorporation of BrdU was much lower than in cells treated with only DMSO for both cell types (Figure 2A). The effects on apoptosis were opposite, with the inhibitor treatment resulting in a significant increase of apoptosis (Figure 2B). The figures for apoptosis were higher for the rat cell line than the PECs, but this is probably due to the

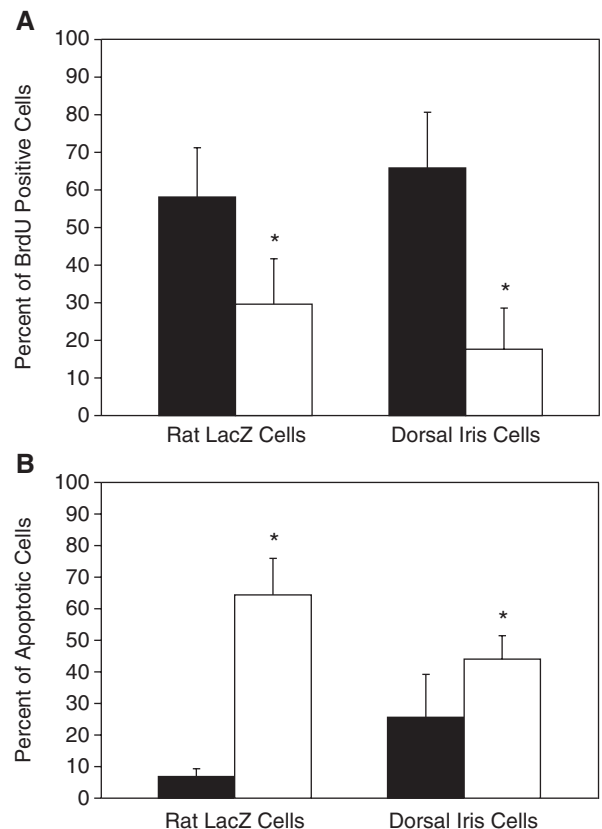


FIGURE 2. Effect of in vitro CKI inhibitor treatment on cell proliferation and apoptosis. (A) Effects of the cdk-2 inhibitor on cell proliferation. Cells were treated in vitro and analysis was performed 24 hours later. Treatment of cells significantly inhibited proliferation as indicated by the incorporation of BrdU in the cell line Rat LacZ and in primary dorsal iris cells from the newt. (B) Effects of the cdk-2 inhibitor on apoptosis of the same cells as in (A). Treatment resulted in an increased rate of cell apoptosis. The differences between control (black bars) and treated cells (white bars) are statistically significant (asterisks indicate $p < 0.001$; t -test, standard deviation). Five different areas were examined and from the total cell number, the percentage of the positive cells was calculated.

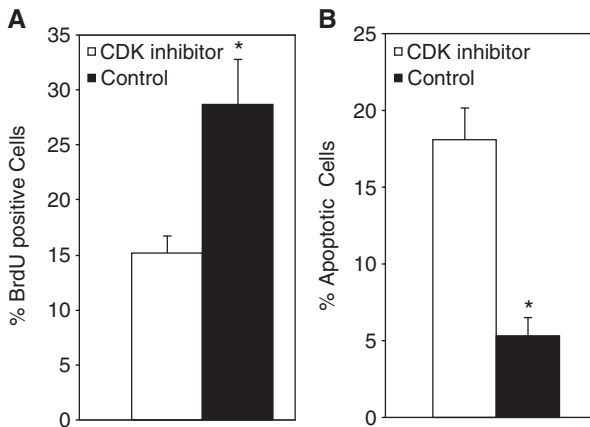


FIGURE 3. Effect of in vivo CKI treatment on cell proliferation and apoptosis. (A) Effects of the CKI on cell proliferation as measured by BrdU incorporation. The percentage of positive cells was taken by counting lens cells from seven eyes treated with the inhibitor and five control eyes. (B) Effects of the CKI on apoptosis in the regenerating lens. The same number of eyes was examined as in the proliferation assay. From all lens sections, we selected the ones from the center as the area unit. The same area unit was considered in all eyes. Asterisk indicates $p < 0.001$ (two tailed, two sample equal variance t -test between corresponding data sets).

fact that the PECs are primary cells. Similar effects were obtained in previous studies using different cancer cell lines.¹⁰ When proliferation and apoptosis profiles were examined during lens regeneration in vivo it was found that the CKI reduced cell proliferation and increased apoptosis in the regenerating lens.

As explained in the Methods section, we selected short treatments between 12 and 15 days postlentectomy because continuous treatments do not result in a visible differentiated lens and because at the selected period of treatment (12–15 days) there is active differentiation of the lens fibers and proliferation. We examined seven eyes from treated animals and five eyes from controls. The positive cells (either BrdU-labeled, or TUNEL-positive) were counted and the percentage was calculated with respect to the total cell number in selected area units (the area unit was the two most center sections from a lens). As in the case with the cultured cells, we found that the CKI decreased proliferation and increased apoptosis in the regenerating lens. These data are shown in Figure 3. Representative sections showing the effects of the inhibitor on proliferation and apoptosis are shown in Figure 4. Green fluorescence shows incorporation of BrdU in control

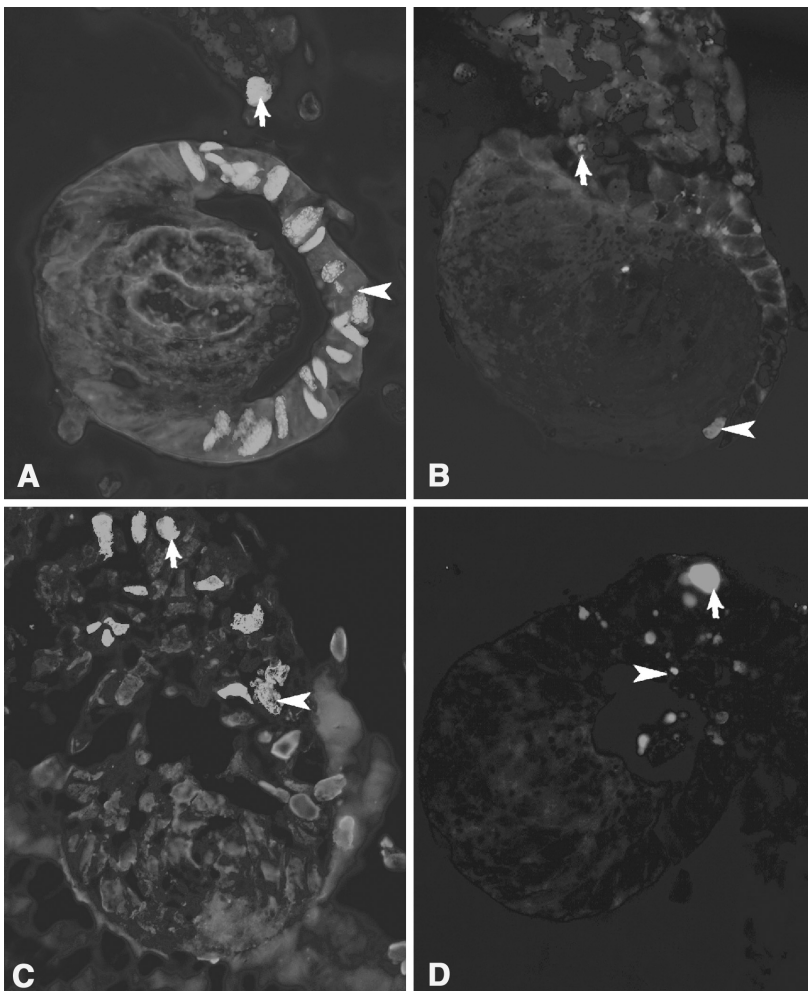


FIGURE 4. Effects of the CKI on cell proliferation and apoptosis during lens regeneration. (A) BrdU incorporation in the dorsal iris PECs (arrow) and in the regenerating lens (arrowhead) in a control eye 16 days postlentectomy. (C) BrdU incorporation in the dorsal iris PECs (arrow) and in the regenerating lens (arrowhead) in a CKI-treated eye 16 days postlentectomy. Note the dramatic decrease of positive cells in the lens epithelium of the treated eyes. (B) Apoptosis in a control regenerating lens 16 days postlentectomy. Note that there is one apoptotic cell in the dorsal iris (arrow) and one in the regenerating lens (arrowhead). (D) Apoptosis in a CKI-treated regenerating lens 16 days postlentectomy. Note a dramatic increase in cells with fragmented DNA.

DMSO-treated (Figure 4A) and CKI-treated (Figure 4C) eyes undergoing lens regeneration. Red fluorescence shows fragmented DNA in apoptotic cells in control (Figure 4B) and CKI-treated (Figure 4D) samples. Note the marked decrease of BrdU-positive cells and increase of apoptotic cells due to the influence of the CKI.

The present results strongly suggest that lens regeneration could be regulated by specific interplay of mechanisms involved in cell proliferation and cell death. The downstream targets of this inhibitor (and of course of cdk2, and cdk4) in lens regeneration are not at the present known, but eventually such CKIs as the one tested in the present work should prove valuable in the future. One possible candidate might be Rb, because it is expressed during lens regeneration¹⁴ and the inhibitor has been shown to reduce Rb phosphorylation in human colon carcinoma cell lines.¹⁰ It is interesting to note here that the same effects seen by the use of SU9516 can be seen in cell lines with the use of E2F-derived oligopeptides.¹⁵ These peptides block the phosphorylation site of cyclin A/cdk2 and cyclin E/cdk2 complexes. E2F is a protein that binds Rb but dissociates upon Rb phosphorylation. It is believed that the apoptotic effects of SU9516 may relate to its effects on the cyclin A/cdk2 complex rather than cyclin E/cdk2. This is because cyclin A/cdk2 can negatively regulate E2F and thus inhibition of cdk2 allows an increase in free E2F, which can induce both cell proliferation and apoptosis.^{16,17}

Proliferation also occurs in the ventral iris after lenticectomy, but is short lived. This may mean that regulation of proliferation and apoptosis might be vital to ensure that the ventral iris does not transdifferentiate to lens as its dorsal counterpart. Therefore, detailed studies on control of proliferation and apoptosis using the factors discussed in this paper might shed light on this important regulative process and on the molecular mechanisms of lens regeneration.

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