Unique Expression Patterns of the Retinoblastoma (*Rb*) Gene in Intact and Lens Regeneration-Undergoing Newt Eyes

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ABSTRACT

Based on the role of retinoblastoma (*Rb*) in lens development and in the cell cycle reentry of muscle cells during limb regeneration, we have analyzed expression or *Rb* patterns in intact and lens regeneration-undergoing newt eyes. We find that in intact newt eye *Rb* is expressed in the retina as a gradient with higher levels in the photoreceptor layer and virtually no expression in the ganglion layer. In addition, a second gradient was detected within the photoreceptor layer with expression diminishing at the dorsal and ventral regions. In the intact lens, *Rb* is expressed in the lens epithelium and in the differentiating lens fibers at the bow region. During lens regeneration, *Rb* is expressed very strongly in the differentiating lens fibers, but not in the lens epithelium. Using an antibody specific to the hyperphosphorylated form of Rb, we detected the inactive protein only in the pigment epithelial cells of the iris. These distinct patterns might be related to the regenerative potential of the lens in the newt. Anat Rec Part A 271A:185–188, 2003.

Key words: newt; lens regeneration; retina; retinoblastoma

Lens regeneration in the adult vertebrates is only possible in some newts and begins with dedifferentiation and proliferation of dorsal iris pigment epithelial cells (PECs). By dedifferentiation we mean the loss of characteristics that define the pigment epithelial cells, such as pigmentation. Dedifferentiation initiates molecular events, such as reentering 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 (Yamada, 1977). At about 10 days postlentectomy, a lens vesicle is formed from the depigmented dorsal PECs. Around 12 to 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 (Yamada, 1977). This marks the beginning of primary lens fiber differentiation. During days 15 to 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 to 20 days the PECs have stopped proliferating, and the lens fibers continue to accumulate crystallins. Lens regeneration is considered complete by day 20 to 25 (Tsonis, 2000, 2001). Lens regeneration, therefore, is possible by transdifferentiation, which is the transformation of one cell type to another (in this case, pigment epithelial cells 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

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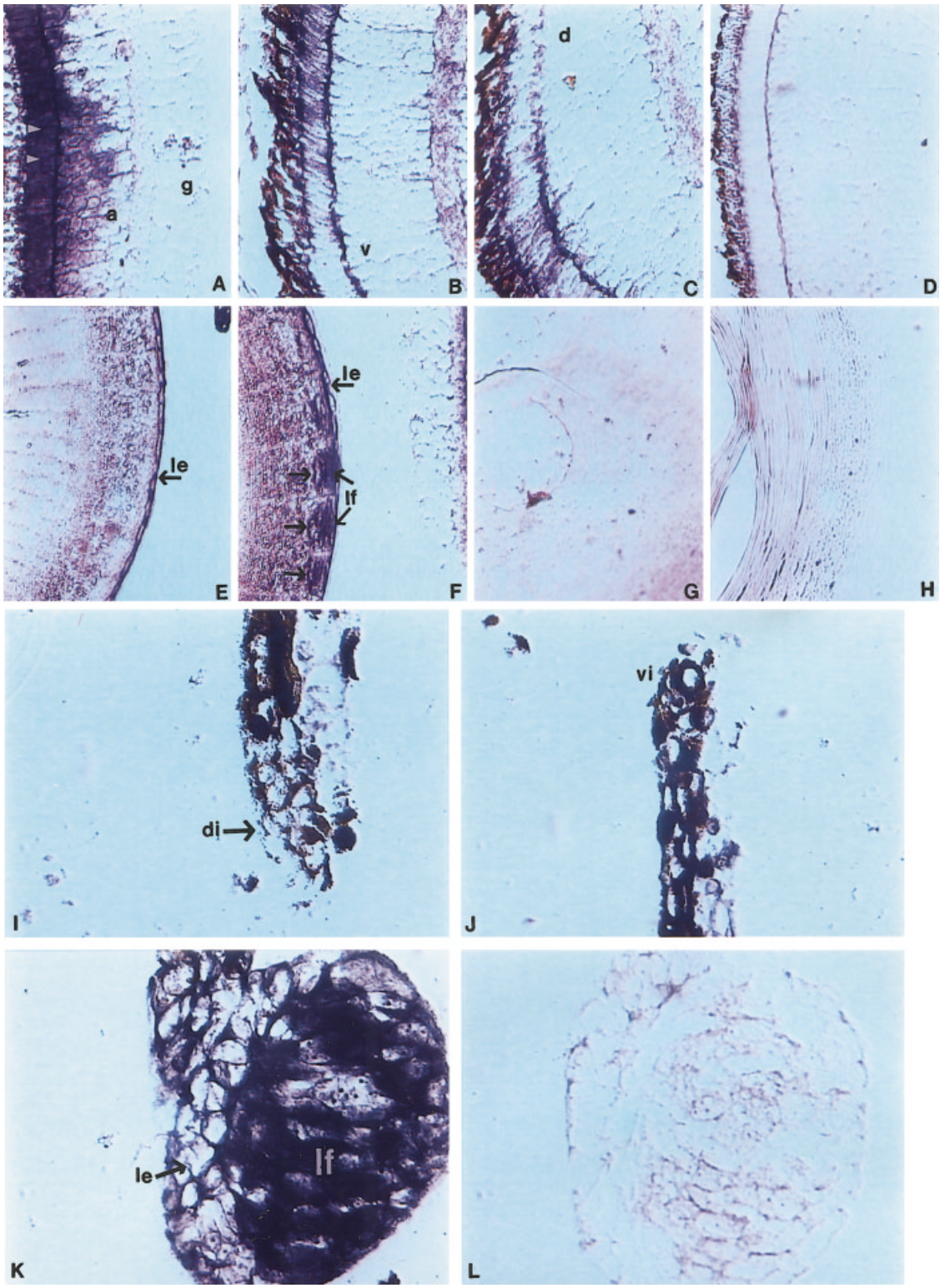

Fig. 1. Expression of *Rb* in intact adult newt eye. **A:** Expression as a gradient in the neural retina. Note strong expression in the photoreceptor (arrowheads) layer and part of the amacrine layer (a). No expression is seen in the ganglion layer (g). **B:** Expression in the photoreceptor layer is higher in the center and lower in the ventral part (v) of the retina. **C:** Expression is lower in the dorsal part (d) of the retina. **D:** Negative control; hybridization with the sense probe. **E:** Expression in the anterior lens epithelial cells (le). **F:** Expression in the lens epithelial cells at the bow region (le) and the differentiating fibers (lf, arrows). **G:** Lack of expression in the lens fibers at the center of the lens. **H:** Negative control; a portion of the anterior lens hybridized with the sense probe. **I-L:** Expression of *Rb* during lens regeneration. I: Lack of expression in the dedifferentiated tip (arrow) of the dorsal iris (di) 10 days postlentectomy. J: Lack of expression in the tip of the ventral iris (vi). The dark color is due to the heavy pigmentation of the pigment epithelial cells. K: Strong expression in the lens fibers (lf) of a regenerating lens 20 days postlentectomy. Note that the lens epithelial cells (le) are rather negative. L: Negative control using a similar section as in C and the sense probe.

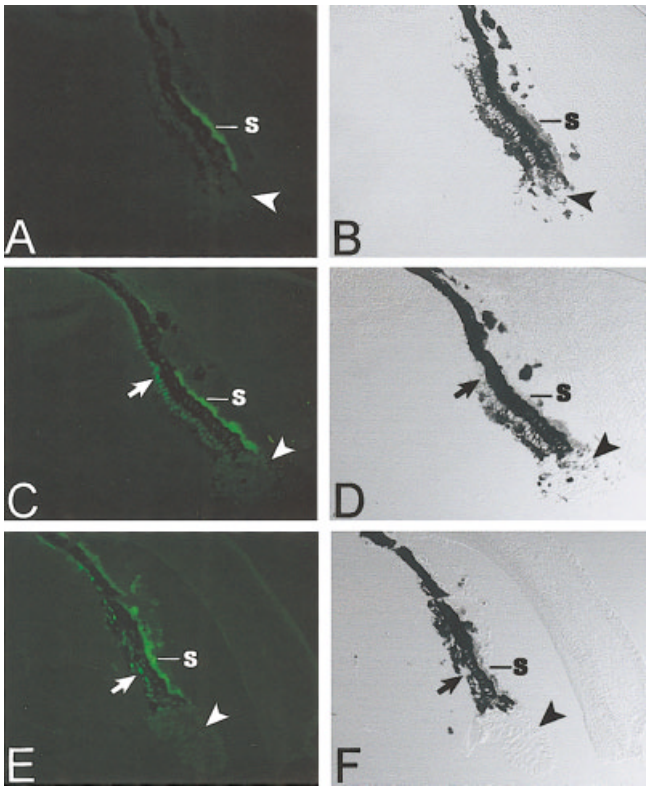

Fig. 2. Rb protein detection by immunofluerescence in the pigment epithelial cells (PECs) of the newt iris. **A:** Negative control; incubation with the primary antibody was omitted. This is a section through a regenerating eye 10 days postlentectomy. **C:** A section through a regenerating eye ten days postlentectomy. Presence of Rb in the PECs of the dorsal iris (arrow). The lens vesicle (arrowhead) is negative. **E:** A section through a regenerating eye 15 days postlentectomy. Note presence of Rb in the PECs (arrow) and absence in the lens vesicle (arrowhead). Stroma (S) always shows unspecific autofluorescence. **B, D, F:** Corresponding differential inference contrast (DIC) images of A, C, and E, respectively.

transdifferentiation can be observed (Kodama and Eguchi, 1995; Tsonis, 2001). As the PECs proliferate, they become depigmented and then transdifferentiate to lens cells.

The presence of retinoblastoma (Rb) and the regulation of its phosphorylation is an important event for reentry to the cell cycle. Such an event is especially important during lens development and controls proliferation and apoptosis that lead to lens fiber differentiation (Morgenbesser et al., 1994). In addition, Rb phosphorylation is correlated with the ability of newt myotubes to reenter the cell cycle, an important step in the dedifferentiation process that leads to limb regeneration (Tanaka et al., 1997). Because the newt lens can be regenerated by the transdifferentiation, it is paramount to examine mechanisms of cell cycle reentering. Given the important role of Rb in cell cycle regulation we decided to examine its expression patterns in the intact and lens regeneration-undergoing eye of the adult newt.

Animals

Adult newts, *Notophthalmus viridescens*, were lentectomized under anesthesia, 0.1% MS222 (Sigma, St. Louis, MO), and their eyes were collected in time intervals up to 20 days postlentectomy. The eyes were processed for expression studies using in situ hybridization and immunohistochemistry.

In Situ Hybridization

This was carried out as described previously (Del Rio-Tsonis et al., 1997, 1999). The newt *Rb* probe was a gift from Dr. J.P. Brockes (Tanaka et al., 1997).

Immunofluorescence

The mouse monoclonal antibody 51beta7 directed against the hyperphosphorylated form of Rb (Rubin et al., 2001) was used in immunohistochemistry with eye sections. Frozen sections were collected from different stages during lens regeneration. The sections were air-dried for 30 min at room temperature (RT) and fixed in 4% paraformaldehyde for 10 min. The sections were then permeabilized in 100 mM glycine pH 7.0, 0.2% Triton X-100, incubated with 10% sodium dodecyl sulfate (SDS) in phosphate-buffered saline (PBS) for 5 min, and washed in 1× PBS/0.2% bovine serum albumin (BSA) 3× for 5 min each. Sections were then blocked with 0.2% BSA, 0.1% Tween-20, 10 mM NaF, 10 mM B-glycerolphosphate for 10 min. Following blocking, sections were incubated with primary antibody at 37°C for 1 hr, or at 4°C overnight in 0.2% BSA, 0.1% Tween-20, 10 mM NaF, 10 mM B-glycerolphosphate, and the Rb antibody at 20 ug/ml. Subsequently, sections were washed in PBS/0.2% BSA 3×, 5 min each, and then were incubated with secondary antibody (0.2% BSA/PBS, 1:1,000 dilution of Fluorescein) 2×, 30 min, at RT. Finally, sections were washed in PBS/0.2% BSA 3×, 5 min each, and mounted with gel mount.

RESULTS AND DISCUSSION

In the adult intact eye, *Rb* was expressed in both the neural retina and the lens. In the neural retina, *Rb* was expressed as a gradient with higher expression levels in the photoreceptor layer and no expression in the ganglion cell layer (Fig. 1A). In addition, this expression was most pronounced in the central part of the photoreceptor layer and not in the dorsal or ventral part (Fig. 1B,C). These expression patterns are quite different from the ones observed during development of mouse eye, where expression was exactly opposite, with the signal mostly seen in the ganglion cell layer (Jiang et al., 1997) or from adult human retina where *Rb* was found in all cell layers (Nork et al., 1994). *Rb* was expressed in the intact lens as well, both in the lens epithelium and in the differentiating lens fibers at the bow region (Fig. 1E,F) but not in the fibers at the center of the lens (Fig. 1G).

After lentectomy, PECs from the dorsal iris dedifferentiate and reenter the cell cycle. They lose their pigments and produce a vesicle that is destined to become the lens epithelium, which subsequently differentiates to lens fibers (Tsonis, 2000, 2001). The lens fibers are postmitotic and Rb inactivation (as shown during mouse development) is sufficient to cause proliferation of lens fibers. In this case, Rb is needed for withdrawal of fiber cells from cell cycle before their end-stage differentiation (Morgenbesser et al., 1994). During the process of regeneration, we found that *Rb* transcripts were absent during the early events of vesicle formation (Fig. 1I,J). During the formation of the regenerating lens, *Rb* was expressed very

strongly in the lens fibers but it was virtually absent in the lens epithelium (Fig. 1K). This is an interesting difference between the intact and regenerating lens, because the lens epithelium of the intact lens was rather positive for *Rb*. This indicates a fundamental difference between the genetic activity of lens epithelium during regeneration, which might reflect the fact that other factors, not belonging to the Rb family, might be involved in lens epithelium cell proliferation (Nguyen et al., 2002). Expression in the retina of the eyes undergoing lens regeneration was virtually the same as in the retina of the intact eyes. Due to heavy pigmentation in the iris, we were unable to detect expression of Rb using in situ hybridization in the PECs. Among the several Rb antibodies that we tried, we were able to detect a signal with a specific Rb antibody directed against the hyperphosphorylated form of mouse Rb (Rubin et al., 2001). This form is inactive and leads to dissociation from E2F and entering of the cell cycle. We found that this form was only expressed in the pigment epithelial cells of the iris, especially of the dorsal iris (Fig. 2). Expression was not detected when the sections were treated with phosphatase (not shown). This pattern is novel and consistent with a role of Rb in allowing these cells to reenter the cell cycle, proliferate, and contribute to the dedifferentiation process. The absence of signal in any other tissue, especially the intact and regenerating lens, suggests that the hypophosphorylated form of Rb might be the predominant one. This is consistent with its association with cell cycle withdrawal (Morgenbesser et al., 1994; Rampalli et al., 1998).

Overall, expression of *Rb* seems to be unique in the newt eye and such patterns might be correlated with their regenerative properties. Further studies with specific inhibitors of Rb phosphorylation might shed light to its role in lens transdifferentiation.

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