

Selective Activation of Thrombin Is a Critical Determinant for Vertebrate Lens Regeneration

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Summary

The regeneration of structures in adult animals depends on a mechanism for coupling the acute response to tissue injury or removal with the local activation of plasticity in residual differentiated cells or stem cells [1]. Many potentially relevant signals are generated after injury, and the nature of this mechanism has not been elucidated for any instance of regeneration. Lens regeneration in adult vertebrates always occurs at the pupillary margin of the dorsal iris, where pigmented epithelial cells (PEC) reenter the cell cycle and transdifferentiate into the lens [2–5], but the basis of this striking preference for the dorsal margin over the ventral is unknown. In this study, we report that a critical early event after lentectomy in the newt is the transient and selective activation of thrombin at the dorsal margin. The thrombin activity was blocked with two different irreversible inhibitors and was shown to be strictly required for cell cycle reentry at this location. The axolotl, a related urodele species, can regenerate its limb, but not its lens, and thrombin is activated in the former context, but not the latter. Our results indicate that selective activation of thrombin is the pivotal signal linking tissue injury to the initiation of vertebrate regeneration.

Results

Regeneration of the lens in adult vertebrates is confined to various species of newt within the urodele amphibians [6]. It was first described in the 1890s by Colucci and by Wolff, who noted a fundamental asymmetry in the process following lentectomy [7]. The new lens is always derived from the pupillary margin of the dorsal iris, where pigmented epithelial cells (PEC), the sole precursor cells for lens regeneration, reenter the cell cycle, lose pigmentation, and transdifferentiate into the lens [2–5]. The basis of this asymmetry within the centrosymmetric context of the eye has been a mystery, particularly as it is clear that both dorsal and ventral PEC from amphibian, avian, and mammalian species can be stimulated to transdifferentiate into lens cells in dissociated culture [3, 8–10]. The importance of thrombin activation in urodele regeneration has been suggested by studies of cell cycle reentry in multinucleate myotubes during newt limb regeneration [11–13]. Thrombin-mediated proteolysis can generate a distinctive activity in serum to which newt,

but not mammalian, myotubes respond in cell culture by entering and traversing S phase [12, 14]. It has recently been shown that both dorsal and ventral PEC from the newt iris are equally responsive to the thrombin-derived activity in dissociated culture [15]. Furthermore, thrombin is locally activated in the newt limb blastema at a stage at which cell cycle reentry and reversal of differentiation occurs in endogenous and implanted muscle cells [12, 16]. Reentry is an early event after lentectomy, and lens regeneration offers a favorable context for a critical test of the functional role of thrombin in regeneration.

Thrombin Is Transiently Activated at the Dorsal Margin after Lentectomy

The newt iris was frozen and sectioned at 3 days after lentectomy, and sections were overlaid with a translucent membrane impregnated with a fluorogenic thrombin substrate [12]. Thrombin activity was readily detected in the dorsal, but not the ventral, iris and was particularly strong at the pupillary margin (Figure 1A). Adjacent sections were overlaid in the presence of the irreversible thrombin inhibitor PPACK, which alkylates the histidine residue of the catalytic center [17], and showed no significant signal (Figure 1B). The activity at the dorsal margin was not detectable at 1 min after lentectomy (Figures 1C and 1G), but it was present at 20 and 30 min (Figures 1D and 1H) and was comparable up to 4 days (Figures 1A, 1E, 1I, 1F, and 1J). It was diminished at 5 days (Figures 1K and 1M), was at the limit of detection by 7 days, and, at 15 days, when the new lens was clearly visible (Figures 1L and 1N), there was no detectable activity. Thrombin thus appears in a location, and with a time course, that is consistent with a role in the initiation of lens regeneration.

The activation of thrombin after injury depends on the release of prothrombin from the vasculature [18]. The distribution of vessels in the newt iris was analyzed after perfusion with fluorescein dextran. Although the distribution was asymmetric, there was not a marked dorsoventral difference in the density of vessels, and, interestingly, no vessels were detected in the zone around the pupillary margin (Figure 1O). This latter observation suggests that prothrombin may be released from a non-iris source after lentectomy and then selectively activated on the dorsal margin.

Inactivation of Thrombin Blocks S Phase Reentry by Dorsal PECs

After injection of tritiated thymidine or bromodeoxyuridine (BrdU) at 5 days after lentectomy, there is selective labeling of PEC at the dorsal pupillary margin, and thymidine-labeled cells at this location eventually give rise to the new lens [2]. In order to evaluate the functional significance of selective thrombin activation for S phase reentry, we injected PPACK or vehicle control into the anterior chamber at several different times after lentectomy, followed by intraperitoneal injection of BrdU at

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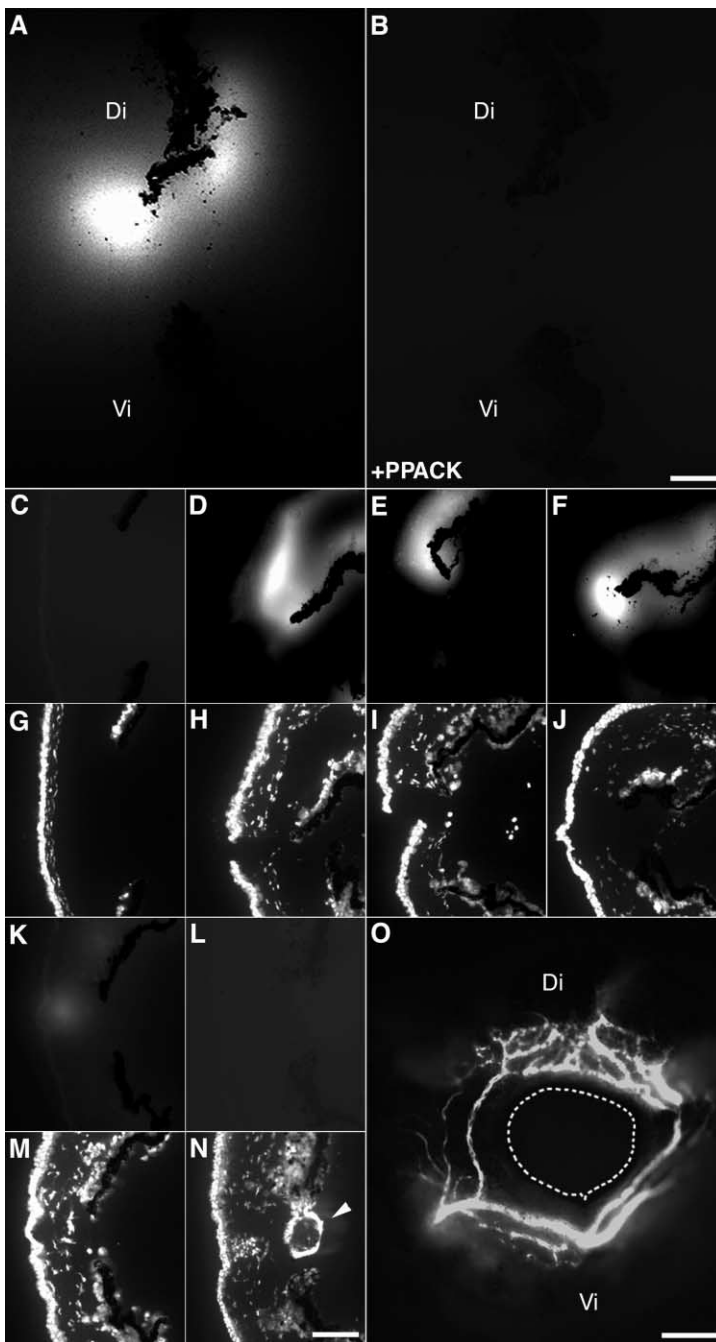


Figure 1. Expression of Thrombin Activity in the Dorsal Iris after Lentectomy

(A) Enzyme overlay assay on a section of the anterior orbit showing fluorescent reaction product associated with the dorsal, but not ventral, iris.

(B) A parallel section reacted in the presence of PPACK.

(C–O) (C) Assay at 1 min postlentectomy. (G) A fluorescent image showing nuclei in the section. (D and H) The same as (C) and (G) at 30 min, (E and I) 8 hr, (F and J) 22 hr, (K and M) 5 days, and (L and N) 15 days. Note the new lens in (N) (arrowhead) growing from the dorsal margin. (O) Distribution of blood vessels in a whole mount of the newt iris after perfusion with FITC-dextran. The pupillary window is enclosed with a dotted line. Note the absence of vessels around the pupillary margin. Di, dorsal iris; Vi, ventral iris.

The scale bars in (A) and (B) represent 100 μm , the scale bars in (C)–(N) represent 200 μm , and the scale bar in (O) represents 300 μm .

day 5. The PPACK injections led to an absence of thrombin activity on the dorsal margin, as determined by the overlay assay. The iris was removed at day 6, was analyzed as a whole mount, and was stained with antibody to BrdU to detect nuclei that had entered S phase. After injection of vehicle, the whole mounts showed the expected labeling of the dorsal cohort of PEC (Figures 2A and 2B); however, after PPACK injection, there was little or no cell labeling in this location (Figures 2C and 2D). The total number of labeled cells on the dorsal and ventral margins is indicated for the control and PPACK-injected groups in Figure 3A, showing that this treatment led to a >90% inhibition of reentry. This in turn led to

extensive inhibition of the extent and time course of lens regeneration observed after 1 month (data not shown).

In order to inhibit thrombin activity by a different mechanism, we have injected the protein Antithrombin III (ATIII) after lentectomy. ATIII is a physiological inhibitor of thrombin that acts by forming a covalent complex with the critical serine residue at the active center [19]. The injection of ATIII also led to effective inhibition of S phase reentry relative to vehicle- or serum albumin-injected controls (Figure 3B). We conclude from the activity of the two inhibitors that thrombin activation on the dorsal iris is essential for S phase reentry in this location after lentectomy.

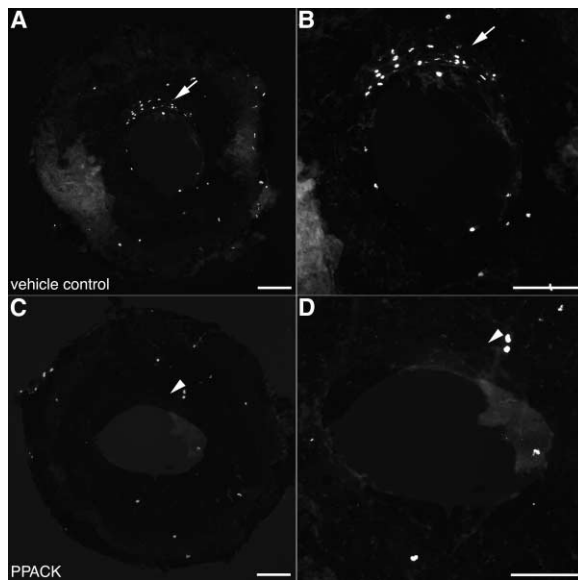


Figure 2. S Phase Labeling of Cells in the Iris at 6 Days Postlentectomy

(A) Whole mount of an iris after injections of vehicle control showing BrdU-labeled nuclei at the dorsal pupillary margin (arrowed) and elsewhere.

(B) Higher-magnification image of the pupillary margin from (A).

(C) Whole mount of iris after injections of PPACK showing no labeled nuclei at the dorsal margin (arrowhead) but some labeling elsewhere. (D) Higher-magnification of the pupillary margin from (C). The scale bar represents 300 μm .

The Axolotl Activates Thrombin after Limb Amputation, but Not after Lentectomy

While the urodele amphibians in general have the most extensive regenerative ability among vertebrates, adult lens regeneration is confined to the various species of newt [6, 7]. The axolotl, a neotenus species of salamander, can regenerate its appendages and other structures, but not its lens [6]. The axolotl iris was analyzed at various times after lentectomy and was negative for thrombin activity (Figures 4A and 4B), while control experiments on the axolotl limb blastema showed clear activity in the mesenchymal tissue; this finding confirmed earlier results in the newt (Figures 4C and 4D). We hypothesize that, although it is closely related to the newts, the axolotl cannot regenerate its lens because it has lost the ability to activate thrombin on the dorsal margin. It may be possible in the future to engineer the selective appearance of thrombin at this location and hence to determine if this triggers regeneration.

Discussion

Our experiments indicate that selective activation of thrombin is a critical signal that couples lentectomy to lens regeneration. After limb amputation, prothrombin is released from severed vessels and is activated throughout the mesenchymal tissue at the end of the stump so as to trigger hemostasis and other events of wound healing. After lentectomy, we observe that thrombin is selectively activated on the dorsal margin

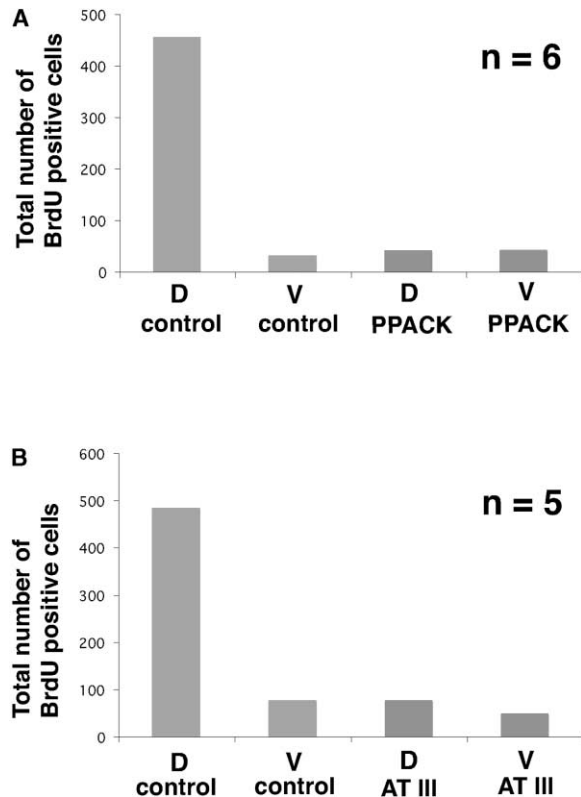


Figure 3. S Phase Labeling of Cells at the Dorsal and Ventral Margins after Inhibition of Thrombin Activity

(A) The aggregate number of S phase-labeled cells on the dorsal (D) and ventral (V) margins is shown at 6 days postlentectomy. The control group was injected with 10 mM HCl (vehicle), and the experimental group was injected with PPACK/10 mM HCl; six newts were in each group.

(B) The same as (A), except that the control group was injected with buffer and the experimental group was injected with ATIII; 5 newts were in each group. Control groups injected with bovine serum albumin solution gave comparable results to buffer controls. Note the marked inhibition of S phase labeling on the dorsal margin in both experimental groups.

of the iris at 20 min and is detected at this location for about 5–7 days. In view of the apparent absence of vessels at this location, the precursor prothrombin could be released from a non-iris source before its activation on the dorsal iris. The lens is attached to the ciliary body through elastic fibers [20], and it is plausible that ruptured vessels in this location, or conceivably the ciliary muscle itself, may be the source; although, further work is required to test these possibilities. The activation of prothrombin after its release is known to depend on the expression of Tissue Factor (clotting factor III), which is the sole integral membrane protein among the clotting factors and nucleates the formation of complexes containing Factors Xa and Va, which activate prothrombin [18] (Figure 5 and legend). We therefore suggest that the difference between dorsal and ventral iris cells in situ, and between the newt and axolotl iris, is in the local expression of Tissue Factor, which leads to the selective activation of prothrombin after lentectomy (Figure 5); this possibility can be addressed in future studies.

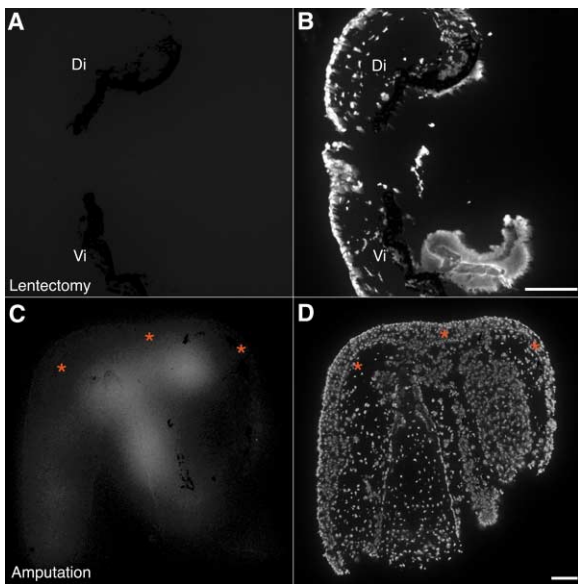


Figure 4. Thrombin Is Activated after Amputation of the Axolotl Limb, but Not after Lentectomy

(A) Enzyme overlay assay on section of axolotl iris at 1 day postlentectomy. (B) Fluorescent image of nuclei in the section. No thrombin activity is detectable, and comparable results were obtained at 1–4 days postlentectomy. (C and D) (C) Overlay assay on section of axolotl limb at 1 day postamputation. (D) A fluorescent image of nuclei in the section. Note the thrombin activity in the blastemal mesenchyme. Comparable results were obtained at 1–3 days postamputation, and a diminished signal was obtained at 4 days. Di, dorsal iris; Vi, ventral iris. The scale bar represents 200 μm .

In earlier work in cell culture, it has been shown that the action of thrombin on serum is able to generate a protein that activates S phase reentry of both newt myotubes and iris PECs, and that both dorsal and ventral PECs are equally sensitive to this activity [12, 15]. This is consistent with the key role for localized thrombin activation: determining the site of regeneration by promoting reentry at the dorsal margin (Figure 5). It is possible that PECs in higher vertebrates might have lost responsiveness to the activator, as is known to be the case for mouse versus newt myotubes [11, 12, 14], as well as that dorsal PECs may have lost the ability to activate thrombin; it is now important to evaluate these possibilities. It is interesting that the recent transcriptional profiling of embryonic and adult stem cells has identified responsiveness to thrombin as a core stem cell property; although, in this case, it is direct responsiveness as mediated by one of the thrombin receptors [21]. The pivotal role of thrombin activation in urodele regeneration provides a new focus for attempts to extend regenerative ability in other vertebrates.

Experimental Procedures

Membrane Overlay Assay for Thrombin in Sections

Newt (*Notophthalmus viridescens*) irides at various times postlentectomy were frozen on dry ice in Tissuetek (Sakura Finetek) and were sectioned at 10 μm . Sections were collected on superfrost plus microscope slides (BDH), air dried at room temperature for 20

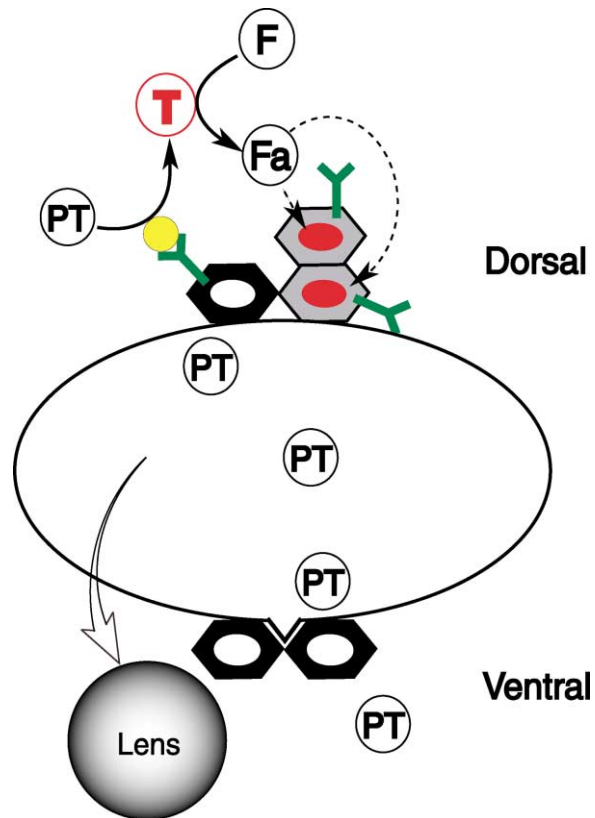


Figure 5. Schematic Diagram of Hypothesis for Events that Couple Lentectomy to Cell Cycle Reentry by PECs

After lens removal, prothrombin (PT) and clotting factors (yellow circle) are released into the anterior chamber, possibly from ruptured vessels in the ciliary body. The site of PT activation is determined by the local expression of Tissue Factor (green “Y”), which nucleates conversion of PT to thrombin (red “T”); thrombin thus appears on PECs at the dorsal margin. Thrombin converts the plasma protein F to its active form, Fa, which triggers cell cycle reentry of PECs (shown as a red nucleus) and depigmentation (gray cytoplasm), as shown earlier for cultured PECs [15]. The plasma protein F may be released along with PT upon lentectomy or may be present in the aqueous humor. Note the pivotal role of T in coupling the acute events of tissue removal with the longer-term activation of plasticity in PECs.

min, fixed in methanol, rehydrated in PBS, stained in 1.5 $\mu\text{g/ml}$ propidium iodide, and placed in PBS + 0.1 mg/ml bovine serum albumin (BSA). Membranes impregnated with thrombin substrate (7-amino-4-trifluoromethylcoumarin-D-Phe-Pro-Arg; AFC-64, Enzyme System Products), were hydrated briefly in water, then PBS, and were placed immediately onto the sections [12, 22]. After removal of excess liquid, a coverslip was applied and sealed with nail varnish. Images were collected within 1 hr at room temperature on a Zeiss Axiophot with a cooled CCD camera by using Image Pro Plus software (Photonic Science). Parallel control sections were incubated with 1 μM PPACK (Calbiochem) before exposure to the membrane. The propidium-stained nuclei were visualized under fluorescence optics with appropriate filters.

Inhibition of Thrombin after Lentectomy

Lentectomy was performed in a bath of PBS supplemented with thrombin inhibitors where appropriate. At 4 hr postlentectomy, 100 nl of 10 mM PPACK or vehicle control was injected through the cornea and was subsequently injected at days 1, 2, and 4. For ATIII, 400 nl of 2.5 mg/ml ATIII (Enzyme Research Laboratories) was

injected at the same time points. The vehicle control was either 2.5 mg/ml BSA or ATIII buffer.

BrdU Labeling of Newt Iris

5-deoxy-2'-bromouridine (Roche) was injected intraperitoneally (0.25 mg/g body weight) at 5 days postlentectomy [16], and the iris was harvested after 24 hr, fixed in 1% paraformaldehyde for 30 min as a whole mount, and washed in PBS. After fixation in methanol at -20°C for 15 min, the iris was floated in 2 N HCl for 1 hr at 37°C , neutralized in 0.1 M borate buffer (pH 8.4), rinsed in PBS, and blocked in PBS with 10% goat serum and 0.2% Triton X-100 (PTG buffer). After incubation in monoclonal antibody to BrdU in PTG at 4°C for 3 days, the iris was washed in PTG and was incubated in fluorescein-conjugated rabbit anti-mouse antibody for 48 hr. The whole mount of the iris was washed in PTG, placed on a slide, and observed under a coverslip.

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