A critical role for thrombin in vertebrate lens regeneration

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Lens regeneration in urodele amphibians such as the newt proceeds from the dorsal margin of the iris where pigment epithelial cells (PEC) re-enter the cell cycle and transdifferentiate into lens. A general problem in regeneration research is to understand how the events of tissue injury or removal are coupled to the activation of plasticity in residual differentiated cells or stem cells. Thrombin, a pivotal regulator of the injury response, has been implicated as a regulator of cell cycle re-entry in newt myotubes, and also in newt iris PEC. After removal of the lens, thrombin was activated on the dorsal margin for 5–7 days. Inactivation of thrombin by either of two different inhibitors essentially blocked S-phase re-entry by PEC at this location. The axolotl, a related species which can regenerate its limb but not its lens, can activate thrombin after amputation but not after lens removal. These data support the hypothesis that thrombin is a critical signal linking injury to regeneration, and offer a new perspective on the evolutionary and phylogenetic questions about regeneration.

Keywords: transdifferentiation; tissue factor; urodele; newt

1. INTRODUCTION

The only adult vertebrates that are able to regenerate the lens are various species of newts, a term used to refer to salamanders with a predominantly aquatic habitat, and some species of cobitid fish (Sato 1961; Mitashov 1966). Newts are urodele amphibians, an order that possesses the most extensive regenerative ability among vertebrates. Lens regeneration in urodeles was described in the 1890s by Colucci and by Wolf (Reyer 1954), and it remains a subject of great interest for students of regeneration (Del Rio-Tsonis & Tsonis 2003). After removal of the lens (lentectomy), the new lens regenerates as a vesicular structure from the pupillary margin of the dorsal iris. In comparison with other examples of urodele regeneration it is noteworthy that the site of regeneration, the iris margin, is not contiguous with the lens, the structure that is removed. The lens is suspended in the eye chamber by ligaments or zonules that are connected to the ciliary body which regulates its curvature (Bron *et al.* 1997).

Although it had been surmised that the PEC of the dorsal iris were the precursor cells for the new lens, this was established definitively by the experiments of Eguchi, Okada and their collaborators (Eguchi & Okada 1973; Eguchi *et al.* 1974; Eguchi 1998). These investigators established culture conditions that induced PEC to lose pigmentation and convert to lens bodies (lentoids) expressing definitive markers of lens differentiation (figure

1; Agata *et al.* 1993). These conditions included the use of phenylthiourea to inhibit melanogenesis, as well as fibroblast growth factor. The term transdifferentiation was introduced to refer to this direct conversion from one differentiated cell type into another (Okada 1991). This is associated with S-phase re-entry by the PEC both in clonal cell culture, and in the animal after lentectomy (Eguchi & Shingai 1971; Okada 1991). It is clearly necessary to generate more cells to allow formation of the new lens as well as reconstruction of the iris, but the precise relationship between transdifferentiation and re-entry is still unclear and is discussed later in this article. It is noteworthy that PEC from both the dorsal and the ventral iris are able to undergo transdifferentiation in culture, and furthermore that PEC from all vertebrates including man can respond in culture in this way (Eguchi *et al.* 1974; Yasuda *et al.* 1978). This widespread potentiality contrasts with the restriction of lens regeneration from the PEC of the adult iris to certain newt and fish species (Reyer 1954; Sato 1961; Mitashov 1966; Stone 1967).

It is likely that the occurrence of regeneration is controlled by the early events that link tissue injury or removal to the activation of plasticity in residual differentiated cells or stem cells (Brockes 1997; Brockes *et al.* 2001). Regeneration in urodeles is particularly associated with the plasticity of differentiated cells (Brockes 1997). This involves re-entry to the cell cycle, and in most cases the loss of differentiated properties. It is important to point out that lens regeneration is an example which is encompassed within the general framework of urodele plasticity (figure 1; Brockes & Kumar 2002). One striking experimental demonstration of this point is the implantation of pieces of iris into various locations in an adult newt (Reyer *et al.* 1973). Although the iris cells retain their pigmented identity in most places, the implants are effectively converted into lenses within the limb blastema, the mesenchymal growth zone

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Figure 1. Plasticity of differentiated cells during regeneration of the newt heart and lens (Brookes & Kumar 2002). (a) After removal of the apex of the ventricle the heart is sealed by a clot and the adult cardiomyocytes return to the cell cycle in a zone (green) around the clot, proliferate and regenerate the ventricle. If cardiomyocytes are dissociated into a culture dish, they can divide and form beating groups of cells. (b) After removal of the lens, the PEC of the dorsal margin of the iris give rise to the new lens. See also figure 9. If the iris is dissociated, PEC in cell culture will re-enter the cell cycle, de-pigment and transdifferentiate into lentoid bodies which express lens markers.

Figure 2. Thrombin activity counteracts the post-mitotic arrest in newt myotubes (Tanaka *et al.* 1999; Brookes & Kumar 2002). Thrombin acts indirectly by cleaving a substrate (F) that is present in vertebrate serum and plasma to induce cell cycle re-entry. The resulting activity, referred to as Fa, has no action on mononucleate cells, whereas growth factors such as PDGF have the reverse specificity. Mouse myotubes are completely refractory to the Fa activity.

that gives rise to a new limb after amputation. The blastema apparently provides an environment that destabilizes the differentiated state and is able to act both on the PEC of the iris as well as on the mesenchymal cells of the limb. This article is concerned with the issue of how regeneration is initiated in relation to the events of tissue injury or removal, rather than with the processes of transdifferentiation and lens morphogenesis. There has been considerable recent progress in these last areas, for example in relation to the role of FGF and the expression of the master

regulatory gene *pax-6* (Del Rio-Tsonis *et al.* 1995, 1997). The centre-piece of the present account is the activation of the zymogen PT to the protease thrombin, a process that has been extensively studied because of its crucial importance for haemostasis and other aspects of the response to injury. The hypothesis that thrombin may also have a critical function in initiating regeneration has come from studies on differentiated newt cells in culture, but it is the context of lens regeneration that has allowed a direct experimental test in the animal (Imokawa & Brookes 2003).

Figure 3. Thrombin activation may link the acute events of tissue injury with cell cycle re-entry and regeneration. After rupture of a vessel (top), blood gains access to cells expressing TF in the plasma membrane. This leads to sequential activation of clotting factors as diagrammed to form a prothrombinase complex resulting in localized thrombin activation. Thrombin has many activities in relation to injury including formation of the fibrin clot. It is possible that the F/Fa connection (see figure 2) may link activation to cell cycle re-entry and regeneration as shown.

2. REGULATION OF S-PHASE RE-ENTRY IN CULTURED NEWT MYOTUBES

A key step in vertebrate skeletal myogenesis is the fusion of mononucleate myoblasts to give a multinucleate myotube. Further growth and differentiation of the myotube leads in turn to the formation of a striated myofibre. The myotube enters a state of post-mitotic arrest after fusion so that it is completely refractory to the growth factors acting on its mononucleate precursors. This is true for myotubes derived from primary myoblasts, as well as for muscle cell lines. Newt myotubes derived from the

adult limb mononucleate A1 cell line provide a striking exception to this generalization. After exposure to high concentrations (10–20%) of serum from various sources, the myotubes re-enter and traverse S phase and arrest in G2/M (Tanaka *et al.* 1997). A necessary aspect of this response is the inactivation of the Rb by phosphorylation, thereby allowing transit through the G1/S restriction point. In addition to its familiar role in relation to the cell cycle, Rb is also implicated in the regulation of expression of muscle genes from late promoters (Novitsch *et al.* 1999). It is noteworthy that cultured mouse myotubes missing both copies of the Rb gene can also be induced to re-enter

S phase (Schneider *et al.* 1994). An important parallel between newt and mouse myotubes is that both are refractory to the familiar serum growth factors that act on mammalian myoblasts, and thus the post-mitotic arrest operates in both contexts (Tanaka *et al.* 1997, 1999). The mouse cells have apparently lost the ability to respond to some other ligand present in serum.

Serum is the soluble fraction of clotted blood and results from the activation of thrombin and formation of the fibrin clot. Thrombin activity appears to be critical for generation of the activity on newt myotubes in culture (Tanaka *et al.* 1999). It is possible to take subthreshold concentrations of serum and incubate with thrombin, thereby generating significant levels of re-entry activity (figure 2). Thrombin does not act directly on the muscle cells because it can be inactivated before the products of the digestion reaction are added to the cells (Tanaka *et al.* 1999). Responsiveness to the thrombin-derived activity is a property of newt myotubes but not of mouse myotubes. Although the mammalian myotubes are unresponsive (figure 2), it is possible to fuse newt and mouse mononucleate cells to form cross-species hybrid myotubes (Velloso *et al.* 2001). These can be grown at an intermediate temperature, and they express markers of muscle differentiation, and show a post-mitotic arrest relative to mononucleate newt and mouse cells. Nonetheless the mouse nuclei, as well as the newt, can be stimulated to enter S phase after activating the newt pathway with the thrombin-based manipulations (Velloso *et al.* 2001); this indicates that mammalian nuclei can respond to the intracellular consequences of activation, presumably in part the phosphorylation of Rb. The further delineation of this pathway, in particular the identification of the ligand and its receptor, is an important goal, but the main concern here is to consider the significance of thrombin as a potential signal linking tissue injury to regeneration.

The pathway for activation of thrombin from its zymogen PT has been studied in considerable detail (figure 3) (Hutton *et al.* 1999). In brief, the rupture of blood vessels leads to the interaction of blood proteins with cells expressing the membrane protein called TF. This leads to the sequential activation of clotting factors to form an activation complex for PT on the cell surface. This in turn leads to the formation of active thrombin which cleaves fibrinogen to form the fibrin clot. The activation and subsequent inactivation of thrombin is exquisitely regulated in space and time by a network of interacting proteins (figure 3; Hutton *et al.* 1999). It is critical, for example, that the formation of a clot occurs very rapidly, but equally critical that it does not propagate once it is formed. The lens is a particularly interesting context because of the displacement between the iris margin, the location of the regenerative response, and the ciliary muscle, the presumed location of an injury response. One important prior issue is to determine if the pigment cells of the iris are responsive to the thrombin-derived activity in culture.

3. NEWT IRIS PIGMENT EPITHELIAL CELLS ARE RESPONSIVE IN CULTURE

Newt PEC were dissociated from the adult newt iris with dispase and plated into dishes coated with collagen type IV (figure 4). The dissociation and plating were

Table 1. Stimulation of cultured newt iris PEC to re-enter the cell cycle. Cultured PEC were exposed to various treatments as outlined previously (Simon & Brockes 2002), and then labelled with BrdU to determine S-phase re-entry. TAF, thrombin activated 0.25% FCS.

treatment of PEC	fold stimulation S-phase entry
0.25% FCS	1
10% FCS	15.6
crude thrombin	7.8
TAF	5.6
TAF: dorsal PEC	5.4
TAF: ventral PEC	6.5

performed in serum-free medium so as to obtain cells which were appropriately quiescent in relation to S-phase entry (Simon & Brockes 2002). Unlike newt myotubes, the newt PEC were responsive to the protein growth factors PDGF-C, FGF 2 and epidermal growth factor. The thrombin-based manipulations were assayed in the presence of 0.25% FCS because the background level at this concentration was relatively low. Crude preparations of bovine thrombin contain significant activity on newt myotubes even after removal of thrombin enzymatic activity (Tanaka *et al.* 1999). The properties of this residual activity suggest that it is the ligand referred to as Fa (figure 2). This was tested on the iris PEC and gave a significant stimulation (table 1; Simon & Brockes 2002). If the FCS at 0.25% was treated with thrombin and then added to the cells after inactivation of the thrombin activity, this proved to be active, inducing a 5.6-fold increase in S-phase re-entry (table 1; figure 4). If the iris was divided into dorsal and ventral halves, the PEC derived from each half were equally responsive to the thrombin-derived activity in culture (table 1). These experiments establish that iris cells are responsive to the thrombin-derived ligand, and hence that local thrombin activation is a candidate for initiating re-entry in the context of lens regeneration (Simon & Brockes 2002).

4. ACTIVATION OF THROMBIN DURING REGENERATION

To detect thrombin activity at a tissue level we have overlaid frozen sections of tissue with a membrane impregnated with a fluorogenic thrombin substrate (Day & Neufeld 1997). This molecule is a coumarin ester of the canonical thrombin substrate phe-pro-arg. After cleavage the fluorescent product coumarin is readily detectable in relation to the section, although it diffuses quite rapidly thus giving resolution at the tissue rather than the single cell level. This method was initially applied in the context of limb regeneration (Tanaka *et al.* 1999). An overlay assay on a section of a newt limb blastema at 8 days post-amputation showed that thrombin activity was elevated in the mesenchymal tissue at the end of the limb, whereas in a parallel section preincubated with the thrombin inhibitor PPACK there was no detectable activity (figure 5*a-d*). This is at an early stage of regeneration when implanted myotubes re-enter S phase (Kumar *et al.* 2000). Limb regeneration is a context where the site of injury and clot formation overlaps with the location of

re-entry and regeneration, and it is therefore not surprising that there is thrombin activity at the end of the stump. Lens regeneration provides a more critical test of the association between thrombin and re-entry because of the displacement discussed earlier.

The newt iris was frozen and sectioned at 3 days after lensectomy and overlaid for the membrane assay (Imokawa & Brookes 2003). The reaction product was detected in the dorsal but not the ventral iris, and the signal was often particularly strong at the pupillary margin (figure 6*a,b*). No activity was detectable if the iris was frozen 1 min after lensectomy (figure 6*c*), but it was present on the dorsal margin at 20 and 30 min, and was then comparable up to 4 days. The activity diminished between days 4 and 7 and had completely disappeared by 15 days when the new lens was clearly visible (figure 6) (Imokawa & Brookes 2003). These observations are circumstantial with respect to any functional role of thrombin in regeneration, but they provide strong encouragement to attempt to block the activity on the dorsal iris and determine the functional consequences.

Before considering such experiments, it is interesting to consider the hypothesis that the blood vessels of the iris are a local source for PT. For example if the withdrawal of the lens resulted in an injury stimulus to the iris, and if there was a marked dorsoventral asymmetry in the iris vasculature, it is possible that this could account for the results above. The distribution of vessels in the newt iris was analysed after cardiac perfusion with the tracer fluorescein dextran, and analysed in whole mounts by fluorescence microscopy. Although the distribution was asymmetric, the density of vessels was comparable in dorsal and ventral iris, and interestingly no vessels were detectable in the zone around the pupillary margin (figure 6*o*). It seems most likely that precursor PT is released from a non-iris source, such as ruptured vessels on the ciliary muscle, and then selectively activated on the dorsal margin.

5. FUNCTIONAL CONSEQUENCES OF THROMBIN INACTIVATION

(a) *Effect on re-entry of pigment epithelial cells*

Our primary assay for the role of thrombin is the re-entry to S phase of PEC on the dorsal margin. This is a more direct assay than lens regeneration itself and it can readily be quantitated. An earlier investigation has demonstrated that after injection of tritiated thymidine at 5 days post-lensectomy, there is a selective labelling of PEC at the dorsal pupillary margin (Eguchi & Shingai 1971). If the label was chased, then the labelled cells were located in the new lens, and hence may be considered the founder population or functional 'stem cells' of the regenerate. An example of the labelling pattern in the iris is shown in figure 7*a*, with BrdU as the tracer rather than tritiated thymidine. The iris was whole mounted, and the cells that have entered S phase appear as white dots against a black background. The cohort at the dorsal margin is arrowed and stands out against the background labelling in the rest of the tissue. A higher power image of the pupillary margin is also shown (figure 7*b*) and clearly illustrates the difference in re-entry at this stage between the dorsal and ventral margins.

We have found that re-entry to S phase as scored by BrdU labelling correlates with phosphorylation of Rb. The 51B7 monoclonal antibody is directed against the phosphorylated epitope at serine 608 in the mammalian sequence. This is sufficiently conserved in newt Rb that the antibody reacts well with Rb in cultured newt cardiomyocytes or myotubes that re-enter S phase. It also labels the dorsal cohort of PEC at 5 days post-lensectomy (Thitoff *et al.* 2003; Y. Imokawa and J. P. Brookes, unpublished results).

To evaluate the function of selective thrombin activation for S-phase re-entry, we injected PPACK or vehicle control into the anterior chamber at several times post-lensectomy (Imokawa & Brookes 2003). This was followed by injection of BrdU at day 5 as described above. The PPACK injections were effective at inactivating thrombin on the dorsal iris as determined with the overlay assay. The irides were analysed by whole mounts as above, and in fact figure 7*a,b* is taken from the vehicle control series. By contrast, figure 7*c,d* shows that PPACK led to a dramatic inhibition of re-entry on the dorsal margin, although several examples of labelling in other locations on the iris are visible. The total numbers of labelled cells on the dorsal and ventral margins were determined for the PPACK and control groups and the results are shown in figure 8*a*. There is clearly a marked (greater than 90%) and selective inhibition of re-entry on the dorsal margin.

The mechanism of inhibition of thrombin by PPACK involves covalent modification of a histidine residue at the catalytic centre of the enzyme (Bode *et al.* 1989). To confirm that thrombin is the significant target for inhibition, we have repeated the analysis with ATIII, a protein inhibitor of thrombin which acts by forming a covalent complex with the nucleophilic serine residue at the active centre (Tsiang *et al.* 1997). ATIII is considered to be a physiological inhibitor of thrombin in the context of haemostasis. The injection of ATIII led to marked inhibition of S-phase re-entry relative to vehicle or serum albumin-injected controls (figure 8*b*). It can therefore be concluded from the action of the two inhibitors that thrombin activation on the dorsal iris after lensectomy is necessary for S-phase re-entry by PEC in this location.

(b) *Effect of thrombin inactivation for lens regeneration*

A control group (vehicle injected) of six animals was allowed to regenerate for one month, while an experimental group (PPACK injected as described above) of 29 animals was analysed in parallel. The results are shown in figure 9, where the different stages of regeneration are represented as 1 to 6. Whereas the control animals all regenerated to stages 5 or 6, the PPACK group were largely in stages 3 or 4. Although the PPACK group is clearly inhibited, the inhibition is not as clear cut as that observed for the more proximal step of S-phase re-entry. It is possible that some reactivation or *de novo* activation of thrombin may occur with time, or that some transdifferentiation to lens may occur in the absence of S-phase re-entry. These possibilities would require further work to evaluate them.

Figure 4. PEC from the newt iris re-enter S phase after thrombin stimulation. (a) A cluster of PEC dissociated from the adult newt iris and plated in culture. (b) The cluster from (a) is taken from a culture exposed to thrombin treated FCS as described (Simon & Brockes 2002). Two nuclei have re-entered S phase and taken up BrdU.

(c) *Lack of thrombin activation in the axolotl*

The axolotl is an example of a salamander species that is excellent at regeneration of several structures, but cannot regenerate the lens (Stone 1967). We were therefore interested to determine if thrombin was activated on the dorsal iris after lentectomy. As a positive control the limb blastema was analysed at 1–3 days post-amputation with the overlay assay. Thrombin activity was readily detectable at the end of the stump (figure 10*a,b*) and the results were comparable to those obtained with the regenerating newt limb. After lentectomy, however, there was no detectable thrombin activity at any of the time points analysed (figure 10*c,d*). This striking difference leads to the hypothesis that the failure of regeneration results from a failure to activate thrombin on the iris (Imokawa & Brockes 2003).

6. A MODEL FOR THROMBIN ACTIVATION IN LENS REGENERATION

Because of the detailed understanding of thrombin activation after injury, a plausible model can be suggested for the events following lentectomy (figure 11). Because the critical role of TF in determining the location for formation of the PT activation complex is well established (figure 3), we suggest that the newt iris has a marked dorsoventral difference in expression of this molecule. After removal of the lens, clotting factors could be released into the aqueous humour from ruptured vessels associated

Figure 5. Thrombin is locally activated after newt limb regeneration. Thrombin proteolytic activity was localized in sections of regenerating limbs using a membrane overlay procedure (Tanaka *et al.* 1999). (a,c) Overlay on a section of a day 8 regenerate in the (a) absence or (c) presence of the thrombin inhibitor PPACK. Note the strong fluorescent reaction product in (a) at the end of the stump. (b,d) Propidium iodide staining of the sections shown in (a,c) to show nuclei within the regenerate.

with the suspensory ligaments or with the surface of the ciliary muscle. These would then interact with the TF expressed in the dorsal iris to generate the prothrombinase complex containing factors Xa and Va. This in turn would lead to the local activation of thrombin at the cell surface in this location, as detected by the membrane overlay assay (figure 6). The thrombin activity, which is particularly high at the pupillary margin, would act on protein substrate(s) in the aqueous humour leading to extracellular generation of the activity referred to as Fa (figure 2), and localized S-phase re-entry of the dorsal cohort of PEC which are exposed to a sufficiently high concentration to activate the Rb pathway. The proposed sequence of events is illustrated in figure 11.

7. DISCUSSION

These experiments provide, to our knowledge, the first clearcut evidence that activation of thrombin is a significant signal for urodele regeneration. The inhibitory effect of PPACK and of ATIII on re-entry of the PEC at the dorsal margin is marked. Although neither reagent is of absolute specificity, it is unlikely that any enzyme other than thrombin would be activated under these circumstances and have a positive role for re-entry, as well as being a target for the two inhibitors. It is noteworthy that a variety of proteases were tested for their ability to generate activity on the myotubes in cell culture (Tanaka *et al.* 1999). These included trypsin and factors Xa and IX, but all were inactive. The other protease that was positive was

Figure 6. (Caption overleaf.)

Figure 6. Expression of thrombin activity in the dorsal iris after lentiectomy. (a) Enzyme overlay assay on a section of the anterior orbit showing the fluorescent reaction product associated with the dorsal but not ventral iris (Imokawa & Brockes 2003). Note the strong reaction at the dorsal pupillary margin. (b) A parallel section reacted in the presence of PPACK. (c) Assay at 1 min post-lentiectomy; (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 h; (f) and (j) 22 h; (k) and (m) 5 days; and (l) and (n) 15 days. (n) The new lens growing from the dorsal margin (arrowhead). (o) Distribution of blood vessels in a whole mount of the newt iris after perfusion with fluorescein-labelled 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 , (c)–(n) 200 μm and (o) 300 μm .

plasmin and this would not be expected to show inhibition by ATIII. Although the studies in culture involve serum or plasma as a source of the protein referred to as F, it is unclear if F is normally present in aqueous humour which is known to contain many plasma proteins. If clotting factors are released after lentiectomy it is possible that this is also the case for F but resolution of this issue must await its identification.

(a) *Thrombin as a regeneration signal in other urodele contexts*

If thrombin is a general signal for regeneration of different structures in urodeles, it is possible to review various phenomena in this light. The failure of thrombin activation to occur in the axolotl (figure 10) is probably not an intrinsic defect of the components of the activation pathway because it occurs normally after limb amputation. It could, however, be a result of a loss of expression of TF at the dorsal margin (Imokawa & Brockes 2003). Another example is that regeneration of a newt limb does not necessarily involve prior amputation. If a major peripheral nerve, for example the sciatic, is cut and the proximal branch is deflected into a superficial wound on the limb surface, or even on the flank around the limb, this is effective at evoking a supernumerary limb (Egar 1988). The role of the nerve in this context is primarily to stimulate division of blastemal cells during the early stages of regeneration (Brockes 1984), and hence it is plausible that the rather 'non-specific' stimulus of a superficial wound with attendant thrombin activation and clotting may be able to generate an initial cohort of precursor cells for regeneration. There may nonetheless be other signals involved in addition to thrombin because the appearance of blastemal cell markers can occur before S-phase re-entry (Gordon & Brockes 1988). However, it is possible that activation of the thrombin pathway has consequences for the competence of cells in addition to S-phase re-entry, a possibility that is mentioned below in the context of lens regeneration.

Thrombin activation also seems an attractive candidate for mediating division of cardiomyocytes after lesions of the newt heart. In this case it has been observed that most S-phase re-entry occurs within a zone of *ca.* 500 μm from the edge of the clot and falls away with increasing distance (McDonnell & Oberpriller 1984). In limb and heart

regeneration it is very difficult to envisage experimental approaches that interfere with thrombin activation because of its central role in the injury response, in particular the formation of a clot.

(b) *Relationship between re-entry and transdifferentiation*

Although the evidence for a functional role of thrombin activation after lentiectomy seems clear, the precise nature of this role in relation to the subsequent events of transdifferentiation and lens formation is unclear. One important context in which to confront these issues is the experiments on lens formation from reaggregates of iris cells. It has been clearly established that dissociated cultures of iris cells can be stimulated to transdifferentiate under the regimen established earlier. However, dissociated iris cells can be reaggregated in culture and then implanted into lentiectomized host eyes. Under these conditions dorsal aggregates form a lens but ventral ones do not, even though proliferation does occur in both (Okamoto *et al.* 1998). Interestingly, this is also true for implantation into a limb blastema (Ito *et al.* 1999). On the present hypothesis, the critical difference between dorsal and ventral cells would be in their expression of TF and hence the ability to nucleate the local activation of thrombin. One would correspondingly predict that fragments of axolotl iris would not be activated after transplantation into a newt eye.

A more problematic issue is the precise relationship between cell cycle re-entry and transdifferentiation. Re-entry on its own is unlikely to be a sufficient condition. There is evidence from the transdifferentiation of *Xenopus* corneal cells rendered quiescent by treatment with mitomycin C (Filoni *et al.* 1995), as well as from the conversion of chick PEC in culture, that some transdifferentiation might occur without re-entry. On the other hand, re-entry clearly occurs in ventral iris cells after lentiectomy but does not lead to transdifferentiation (Eguchi & Shingai 1971). One possibility mentioned above is that the thrombin-derived signal(s) may independently initiate both S-phase re-entry and the responsiveness to signals that promote transdifferentiation.

(c) *Evolutionary and phylogenetic issues*

It is striking that apparently all vertebrates possess PEC that are capable of transdifferentiating into lens under appropriate culture conditions, but the only adult vertebrates that can regenerate the lens are certain species of newts and fishes. The present work raises various possibilities in relation to the vexed issue of why some closely related species can regenerate and others apparently not. We have suggested that the axolotl has lost the ability to activate thrombin on the dorsal iris because it no longer expresses TF in this location (Imokawa & Brockes 2003). It is interesting that like haemostasis and platelet aggregation, lens regeneration is a thrombin-dependent response. Although lens regeneration is a relatively slow response of somewhat uncertain selective significance, haemostasis is a rapid response subject to the most stringent selective pressures. It is possible that certain changes in relation to haemostasis, for example in the promoter of the TF gene that regulates expression of this protein in time or space, might have a secondary effect such as diminishing expression in the dorsal iris. This would abrogate lens

Figure 7. S-phase labelling of cells in the iris at 6 days post-lentectomy. (a) Whole mount of an iris after vehicle control injection showing BrdU-labelled nuclei at the dorsal pupillary margin (arrowed) and elsewhere. (b) Higher magnification image of the pupillary margin from (a). (c) Whole mount of the iris after injections of PPACK, showing no labelled nuclei at the dorsal margin (arrowhead) but some labelling elsewhere. (d) Higher magnification of the pupillary margin from (c). The scale bar is 300 µm throughout.

Figure 8. S-phase labelling of cells at the dorsal and ventral margins after inhibition of thrombin activity. (a) The aggregate number of S-phase labelled cells on the dorsal (D) and ventral (V) margins is shown at 6 days post-lentectomy. The control group was injected with 10 mM of HCl (vehicle) and the experimental group with PPACK/HCl. Each group consisted of six newts. (b) The same as (a) except that the control group was injected with ATIII and each group consisted of five newts. Control groups injected with bovine serum albumin solution gave comparable results to buffer controls. Note the marked inhibition of S-phase labelling on the dorsal margin in both experimental groups (Imokawa & Brookes 2003).

regeneration but could be subject to strong positive selection in relation to the acute thrombin-dependent responses.

However, it may be that higher vertebrates have lost responsiveness to the thrombin-derived activity. This is known to have happened with skeletal myotubes (Tanaka *et al.* 1997; Velloso *et al.* 2001), and could well have happened with iris PEC. These various possibilities can now

be tested and may shed light on the evolutionary and phylogenetic issues.

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Figure 9. Inhibition of lens regeneration after inhibition of thrombin activity on the iris post-lentiectomy. A control group of six animals was injected with vehicle post-lentiectomy as for figure 8. The extent of lens regeneration at one month was scored in relation to the stages shown. A parallel experimental group of 29 animals was injected with PPACK and analysed as for the control group. The overall appearance of the regenerates is indicated by the set of five *en face* drawings at the top. This figure is based on one kindly supplied by Professor Goro Eguchi.

Figure 10. Thrombin is activated after limb amputation in the axolotl but not after lentiectomy. (a) Enzyme overlay assay on a section of axolotl limb at 1 day post-amputation. (b) A fluorescence image of nuclei in the section. Note the thrombin activity in the blastemal mesenchyme. Comparable results were obtained at 1–3 days post-amputation. (c) Overlay assay on a section of axolotl iris at 1 day post-lentiectomy. (d) A fluorescence image of nuclei in the section. No thrombin activity is detectable and comparable results were obtained at 1–4 days post-lentiectomy (Imokawa & Brockes 2003). Di, dorsal iris; Vi, ventral iris.

Figure 11. Schematic diagram of hypothesis for mechanism linking lens removal to lens regeneration (see also figure 3). After lens removal, PT and clotting factors (yellow circle) are released into the anterior chamber, possibly from ruptured vessels in the ciliary body (Imokawa & Brookes 2003). The site of PT activation is determined by the local expression of TF (green Y), which nucleates the conversion of PT to thrombin (red T), so that thrombin activity appears on the surface of PECs at the dorsal margin. Thrombin converts F to Fa as outlined in figure 2, which triggers re-entry of PEC in this location (shown as red nucleus) and depigmentation (grey cytoplasm), as shown for PEC in culture (Simon & Brookes 2002). Note the role of thrombin in coupling injury to regeneration as outlined in figure 3.

REFERENCES

- Agata, K., Kobayashi, H., Itoh, Y., Mochii, M., Sawada, K. & Eguchi, G. 1993 Genetic characterization of the multipotent dedifferentiated state of pigmented epithelial cells *in vitro*. *Development* **118**, 1025–1030.
- Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R. & Hofsteenge, J. 1989 The refined 1.9 Å crystal structure of human alpha-thrombin: interaction with D-Phe-Pro-Arg-chloromethylketone and significance of the Tyr-Pro-Pro-Trp insertion segment. *EMBO J.* **8**, 3467–3475.
- Brookes, J. P. 1984 Mitogenic growth factors and nerve dependence of limb regeneration. *Science* **225**, 1280–1287.
- Brookes, J. P. 1997 Amphibian limb regeneration: rebuilding a complex structure. *Science* **276**, 81–87.
- Brookes, J. P. & Kumar, A. 2002 Plasticity and reprogramming of differentiated cells in amphibian regeneration. *Nature Rev. Mol. Cell Biol.* **3**, 566–574.
- Brookes, J. P., Kumar, A. & Velloso, C. P. 2001 Regeneration as an evolutionary variable. *J. Anat.* **199**, 3–11.

- Bron, A. J., Tripathi, R. C. & Tripathi, B. J. 1997 *Wolff's anatomy of the eye and orbit*. London: Chapman & Hall Medical.
- Day, F. A. & Neufeld, D. A. 1997 Use of enzyme overlay membranes to survey proteinase activity in frozen sections: cathepsin-like and plasmin-like activity in regenerating newt limbs. *J. Histochem. Cytochem.* **45**, 779–783.
- Del Rio-Tsonis, K. & Tsonis, P. A. 2003 Eye regeneration at the molecular age. *Dev. Dyn.* **226**, 211–224.
- Del Rio-Tsonis, K., Washabaugh, C. H. & Tsonis, P. A. 1995 Expression of pax-6 during urodele eye development and lens regeneration. *Proc. Natl Acad. Sci. USA* **92**, 5092–5096.
- Del Rio-Tsonis, K., Jung, J. C., Chiu, I. M. & Tsonis, P. A. 1997 Conservation of fibroblast growth factor function in lens regeneration. *Proc. Natl Acad. Sci. USA* **94**, 13 701–13 706.
- Egar, M. W. 1988 Accessory limb production by nerve-induced cell proliferation. *Anat. Rec.* **221**, 550–564.
- Eguchi, G. 1998 Transdifferentiation as the basis of eye lens regeneration. In *Cellular and molecular basis of regeneration* (ed. J. Geraudie), pp. 207–228. Chichester, UK: Wiley.
- Eguchi, G. & Okada, T. S. 1973 Differentiation of lens tissue from the progeny of chick retinal pigment cells cultured *in vitro*: a demonstration of a switch of cell types in clonal cell culture. *Proc. Natl Acad. Sci. USA* **70**, 1495–1499.
- Eguchi, G. & Shingai, R. 1971 Cellular analysis on localization of lens forming potency in the newt iris epithelium. *Dev. Growth Differ.* **13**, 337–349.
- Eguchi, G., Abe, S. I. & Watanabe, K. 1974 Differentiation of lens-like structures from newt iris epithelial cells *in vitro*. *Proc. Natl Acad. Sci. USA* **71**, 5052–5056.
- Filoni, S., Cannata, S. M., Bernardini, S. & La Mesa, G. 1995 The inhibition of cell proliferation by mitomycin C does not prevent transdifferentiation of outer cornea into lens in larval *Xenopus laevis*. *Differentiation* **58**, 195–203.
- Gordon, H. & Brookes, J. P. 1988 Appearance and regulation of an antigen associated with limb regeneration in *Notophthalmus viridescens*. *J. Exp. Zool.* **247**, 232–243.
- Hutton, R. A., Laffan, M. A. & Tuddenham, E. G. D. 1999 Normal haemostasis. In *Postgraduate haematology* (ed. E. G. D. Tuddenham), pp. 550–580. Oxford: Butterworth-Heinemann.
- Imokawa, Y. & Brookes, J. P. 2003 Selective activation of thrombin is a critical determinant for vertebrate lens regeneration. *Curr. Biol.* **13**, 877–881.
- Ito, M., Hayashi, T., Kuroiwa, A. & Okamoto, M. 1999 Lens formation by pigmented epithelial cell reaggregate from dorsal iris implanted into limb blastema in the adult newt. *Dev. Growth Differ.* **41**, 429–440.
- Kumar, A., Velloso, C. P., Imokawa, Y. & Brookes, J. P. 2000 Plasticity of retrovirus-labelled myotubes in the newt limb regeneration blastema. *Dev. Biol.* **218**, 125–136.
- McDonnell, T. J. & Oberpriller, J. O. 1984 The response of the atrium to direct mechanical wounding in the adult heart of the newt, *Notophthalmus viridescens*. An electron-microscopic and autoradiographic study. *Cell Tissue Res.* **235**, 583–592.
- Mitashov, V. I. 1966 Comparative study of lens regeneration in cobitid fishes (*Misgurnus fossilis*, *Nemachilus barbatulus*, *Nemachilus dorsalis*). *Dokl. Akad. Nauk. SSSR* **170**, 1439–1442.
- Novitsch, B. G., Spicer, D. B., Kim, P. S., Cheung, W. L. & Lassar, A. B. 1999 pRb is required for MEF2-dependent gene expression as well as cell-cycle arrest during skeletal muscle differentiation. *Curr. Biol.* **9**, 449–459.
- Okada, T. S. 1991 *Transdifferentiation*. Oxford: Clarendon.
- Okamoto, M., Ito, M. & Owaribe, K. 1998 Difference between dorsal and ventral iris in lens producing potency in normal lens regeneration is maintained after dissociation and re-aggregation of cells from the adult newt, *Cynops pyrrhogaster*. *Dev. Growth Differ.* **40**, 11–18.

- Reyer, R. W. 1954 Regeneration of the lens in the amphibian eye. *Q. Rev. Biol.* **29**, 1–46.
- Reyer, R. W., Woolfitt, R. A. & Withersty, L. T. 1973 Stimulation of lens regeneration from the newt dorsal iris when implanted into the blastema of the regenerating limb. *Dev. Biol.* **32**, 258–281.
- Sato, T. 1961 Über die Linsen-Regeneration bei den Cobaliden Fischen *Misgurnus anguillicaudatus*. *Embryologia* **6**, 251–291.
- Schneider, J. W., Gu, W., Zhu, L., Mahdavi, V. & Nadal-Ginard, B. 1994 Reversal of terminal differentiation mediated by p107 in Rb^{-/-} muscle cells. *Science* **264**, 1467–1471.
- Simon, A. & Brockes, J. P. 2002 Thrombin activation of S-phase re-entry by cultured pigmented epithelial cells of adult newt iris. *Exp. Cell Res.* **281**, 101–106.
- Stone, L. S. 1967 An investigation recording all salamanders which can and cannot regenerate a lens from the dorsal iris. *J. Exp. Zool.* **164**, 87–103.
- Tanaka, E. M., Gann, A. A., Gates, P. B. & Brockes, J. P. 1997 Newt myotubes re-enter the cell cycle by phosphorylation of the retinoblastoma protein. *J. Cell Biol.* **136**, 155–165.
- Tanaka, E. M., Drechsel, D. N. & Brockes, J. P. 1999 Thrombin regulates S-phase re-entry by cultured newt myotubes. *Curr. Biol.* **9**, 792–799.
- Thitoff, A. R., Call, M. K., Del Rio-Tsonis, K. & Tsonis, P. A. 2003 Unique expression patterns of the retinoblastoma (Rb) gene in intact and lens regeneration-undergoing newt eyes. *Anat. Rec.* **271A**, 185–188.
- Tsiang, M., Jain, A. K. & Gibbs, C. S. 1997 Functional requirements for inhibition of thrombin by antithrombin III in the presence and absence of heparin. *J. Biol. Chem.* **272**, 12 024–12 029.
- Velloso, C. P., Simon, A. & Brockes, J. P. 2001 Mammalian postmitotic nuclei re-enter the cell cycle after serum stimulation in newt/mouse hybrid myotubes. *Curr. Biol.* **11**, 855–858.
- Yasuda, K., Okada, T. S., Eguchi, G. & Hayashi, M. 1978 A demonstration of a switch of cell type in human fetal eye tissues *in vitro*: pigmented cells of the iris or the retina can transdifferentiate into lens. *Exp. Eye Res.* **26**, 591–595.

GLOSSARY

- ATIII: antithrombin III
 BrdU: bromodeoxyuridine
 FCS: foetal calf serum
 FGF: fibroblast growth factor
 IX: factor 9
 PDGF: platelet-derived growth factor
 PEC: pigment epithelial cells
 PPACK: phe-pro-argchloromethyl ketone
 PT: prothrombin
 Rb: retinoblastoma protein
 TF: tissue factor
 Va: activated factor 5
 Xa: activated factor 10