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A model for investigating developmental eye repair in *Xenopus laevis*

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ABSTRACT

Vertebrate eye development is complex and requires early interactions between neuroectoderm and surface ectoderm during embryogenesis. In the African clawed frog, *Xenopus laevis*, individual eye tissues such as the retina and lens can undergo regeneration. However, it has been reported that removal of either the specified eye field at the neurula stage or the eye during tadpole stage does not induce replacement. Here we describe a model for investigating *Xenopus* developmental eye repair. We found that tailbud embryos can readily regrow eyes after surgical removal of over 83% of the specified eye and lens tissues. The regrown eye reached a comparable size to the contralateral control by 5 days and overall animal development was normal. It contained the expected complement of eye cell types (including the pigmented epithelium, retina and lens), and is connected to the brain. Our data also demonstrate that apoptosis, an early mechanism that regulates appendage regeneration, is also required for eye regrowth. Treatment with apoptosis inhibitors (M50054 or NS3694) blocked eye regrowth by inhibiting caspase activation. Together, our findings indicate that frog embryos can undergo successful eye repair after considerable tissue loss and reveals a required role for apoptosis in this process. Furthermore, this *Xenopus* model allows for rapid comparisons of productive eye repair and developmental pathways. It can also facilitate the molecular dissection of signaling mechanisms necessary for initiating repair.

1. Introduction

Regeneration, the ability to replace lost body parts in response to injury, is found in diverse animals. The processes that regulate tissue and organ regeneration are beginning to be understood. However, the reasons for variable capabilities amongst even similar species remain unknown (Agata and Inoue, 2012). An organ that has been studied extensively for its regenerative potential is the eye. The eye is an excellent model especially since its structure is mostly similar between different vertebrate species. Understanding how regeneration occurs in different contexts can provide fundamental insights for stem cell biology, reprogramming, and cell plasticity.

An animal that has high regenerative capabilities is the South African clawed frog, *Xenopus laevis* (Beck, 2012). The *Xenopus* tadpole is a well-established model for studying eye regeneration (Barbosa-Sabanero et al., 2012; Del Rio-Tsonis and Tsonis, 2003; Henry et al., 2008; Tseng, 2017). Its eye has the same structures as found in other vertebrates, including the neural retina, lens, cornea, and pigmented epithelium. Several components of the *Xenopus* eye regenerate after injury. Surgical excision of the tadpole or adult neural retina induced regeneration through activation of retinal progenitor cells and/or transdifferentiation of the retinal pigmented epithelium (Martinez-De Luna et al., 2011; Vergara and Del Rio-Tsonis, 2009; Yoshii et al., 2007). Similarly, removal of the lens also resulted in regeneration through transdifferentiation of the corneal epithelium cells (Day and Beck, 2011; Freeman, 1963; Hamilton et al., 2016).

Most eye repair studies have focused on understanding the regeneration of mature tissues such as the retina and lens. However, the repair capabilities of *Xenopus* embryos have not been investigated in detail. This may be in part due to the complex coordination of tissue interactions that is required for eye formation. After eye field specification during *Xenopus* neurulation, interactions between two tissues, the neuroectoderm (an outgrowth of the developing brain) and the surface ectoderm are required to form the eye properly (Heavner and Pevny, 2012).

There have been examinations of the regrowth ability of the *Xenopus* eye in embryos and tadpoles. Several studies found that after partial eye removal during late embryonic and tadpole stages, eye size recovered during a span of several weeks prior to adulthood (Berman and Hunt, 1975; Feldman et al., 1975; Underwood and Ide, 1992; Wunsh and Ide, 1990). Other reports indicated that eye growth did not occur after surgical ablation in *Xenopus*, nor *Rana* (leopard frog) embryos (Constantine Paton and Ferrari-Eastman, 1981; Currie and Cowan, 1974; Wetts et al., 1993). More recently, it was found that if an eye

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anlagen is excised from the neurula (developmental stage (st.) 15) embryo, then no eye is made (Viczian et al., 2009; Zuber, 2010). Interestingly, if the excised eye field tissues were then transplanted into another region of the embryo or cultured *in vitro*, then they developed into an eye (Zuber, 2010). This result showed that the specified eye anlagen can form the correct structure independent of additional signals. The consequences of the removal of a late tailbud embryo eye have also been investigated at st. 33 and st. 40. The embryo developed normally but with a missing eye (Blackiston and Levin, 2013; Sedohara et al., 2003). Together, these recent reports indicated that excision of immature eye tissues results in a failure of the embryo to regrow the lost structures. However, a common view is that regenerative capacity is highest during early life stages and decreases with increasing age. To further assess the eye repair capacity of *Xenopus* during development, we tested st. 27 early tailbud embryos and found that there is successful regrowth of the eye after surgical loss of tissues.

2. Materials and methods

2.1. Embryo culture and surgery

Embryos were obtained via *in vitro* fertilization and were raised in 0.1X Marc's Modified Ringer (MMR, 0.1 M NaCl, 2.0 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES, pH 7.8) medium (Sive et al., 2000). The eye assay was based on published surgical techniques (Holt, 1980). Embryos at stage (st.) 27 (Nieuwkoop and Faber, 1994) were anaesthetized with MS222 (Sigma). Surgery was performed using fine surgical forceps (Dumont No. 5). An initial cut is first made in the skin surrounding the protruding eye cup and overlying lens placode. The cut is continued around the raised outline of the eye and the protruding tissues are removed. For sham surgery, incisions are made around the perimeter of the raised eye structure but tissues are not excised. After surgery, embryos were transferred into 0.1X MMR, allowed to recover, and then cultured at 22 °C for 1–5 days. The contralateral eye of the embryo served as an internal control. For transplantation assay, a small incision was made at the posterior end along the body axis. The removed eye tissues from the same embryo were grafted to the incision site. Embryos were cultured at 22 °C for 4–5 days.

For chemical treatment, M50054 and NS3694 (Millipore, EMD Biosciences) were used. After experimental surgery at st. 27 and a brief recovery time, embryos were transferred to medium containing the chemical for 24 h. After chemical treatment was completed, embryos were incubated in two changes of medium to remove the inhibitor. Eye regrowth was assayed at 5 days.

2.2. Embryo sectioning, histology and immunofluorescence microscopy

Animals were fixed overnight at 4 °C in MEMFA (100 mM MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, 3.7% (v/v) formaldehyde) (Sive et al., 2000). For histology or immunostaining, embryos were dehydrated in ethanol, and embedded in Paraplast X-TRA, deparaffinized in xylene, rehydrated in graded ethanol, and stained with hematoxylin and eosin according to (Liu and He, 2013). Paraffin or sucrose embedded tissues were sectioned at 10 μm thickness using a Tissue-Tek Accu-Cut Rotary Microtome. For agarose sectioning, embryos were fixed immediately after surgery in MEMFA and processed as according to (Blackiston et al., 2010). Embryos and tadpoles were embedded in 4–6% agarose and sectioned into 50 μm slices using a Leica vt1000s vibratome.

Wholemount embryos and sections were stained with primary antibodies including: Xen1 (pan-neural antibody, 1:100 dilution, Developmental Studies Hybridoma Bank), anti-Islet-1 (retinal ganglion cells and inner nuclear cell layer, 1:200 dilution, Developmental Studies Hybridoma Bank), anti-Glutamine Synthetase (Müller glia, 1:500 dilution, Sigma-Aldrich), anti-Laminin (basal lamina, 1:300 dilution, Sigma-Aldrich), anti-Calbindin-D-28K (cone photoreceptor, 1:500 dilution, Sigma-Aldrich), anti-Rhodopsin (rod photoreceptor, 1:200 dilution, EMD Millipore), anti-phospho Histone H3 (mitosis marker, 1:500 dilution, EMD Millipore), and anti-activated Caspase-3 antibody (cleaved Caspase-3, 1:300 dilution, Cell Signaling). Alexa fluor conjugated secondary antibodies were used at 1:1000 dilution (Thermo Fisher Scientific). DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride, Sigma-Aldrich) and TO-PRO-3 (Molecular Probes) were used for DNA staining.

2.3. Assessment of eye surgery and regrowth

To quantify tissues remaining after surgery, embryos were fixed and the entire head of the animal was sectioned. The embryonic eye was defined as the area immunostained with the neural marker Xen1 and the boundaries highlighted by anti-laminin antibody, indicating the basement membrane surrounding the eye. For each embryo, 2–3 agarose sections of 50 μm thickness were generated. The section containing the largest amount of eye tissues remaining after surgery was measured (in μm) and compared to the area of the contralateral control eye to calculate the percentage of tissue removed in the left eye. To quantify eye regrowth, paraffin sections through the head were stained with hematoxylin and eosin to visualize eye structures. The section containing the largest area of eye tissues was selected from each animal and the eye region on the left (surgery) and right (unoperated control) were measured.

For mitotic counts, all agarose sections were quantified to determine the number of phospho-Histone H3 positive cells in the eye tissues. Images were acquired at the same exposure. The total area for each eye was calculated by measuring the surface area for each eye section and summing the measurements. The total mitotic counts for each eye was then normalized to the total area. The same method was used to assess the number of activated Caspase-3 positive cells in the regrowing control and treatment groups.

To compare the quantity and quality of regrowth in operated eyes versus control eyes, we developed a Regrowth Index (RI) similar to one previously described for tail regeneration (Tseng et al., 2010) (Supplemental Fig. 1). Each regrown eye was scored based on four phenotype categories: full regrowth of an eye (with lens) comparable to an unoperated control eye in structure; partial – minor abnormalities including misshapen and reduction in eye size or incomplete closure of the choroid fissure; weak – no lens and severely reduced and/or malformed eye with most tissues missing; and none – no regenerated tissues visible (See Supplemental Fig. 1). Based on the calculation of the percentage of the number of individuals grouped to each category, each category is then multiplied by 3 (full), 2 (partial), 1 (weak), or 0 (none). The resulting number is a value ranging from 0 to 300, constituting the RI. A value of 0 denotes no regeneration in any of the individuals in the group, while a value of 300 denotes full regeneration in 100% of individuals in a dish.

2.4. Microscopy

Images of whole animals and histological sections were obtained using a Zeiss V20 stereomicroscope with an AxioCam MRc camera. Immunostained tissues were imaged on a Nikon A1R confocal laser scanning microscope (UNLV Confocal and Biological Imaging Core) or a Zeiss Axio Imager 2 microscope. All acquired images were analyzed and/or processed using ZEN Image Analysis software or the open-source FIJI imaging software (Schindelin et al., 2012).

2.5. Statistical analysis

To compare eye regrowth experiments, raw data from scoring was used. Comparison of two treatments was analyzed with Mann-Whitney U test for ordinal data with tied ranks, using normal approximation for large sample sizes. Multiple treatments were compared using a Kruskal-Wallis test, with Dunn's Q corrected for tied ranks. All other