Developmental Biology 353 (2011) 10–18

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

The Retinal Homeobox (Rx) gene is necessary for retinal regeneration

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ARTICLE INFO

Article history:
Received for publication 17 February 2010
Revised 4 February 2011
Accepted 10 February 2011
Available online 17 February 2011

Keywords:
Retinal Homeobox
Regeneration
Transdifferentiation
Retinal progenitor cells
shRNA

ABSTRACT

The Retinal Homeobox (Rx) gene is essential for vertebrate eye development. Rx function is required for the specification and maintenance of retinal progenitor cells (RPCs). Loss of Rx function leads to a lack of eye development in a variety of species. Here we show that Rx function is also necessary during retinal regeneration. We performed a thorough characterization of retinal regeneration after partial retinal resection in pre-metamorphic *Xenopus laevis*. We show that after injury the wound is repopulated with retinal progenitor cells (RPCs) that express Rx and other RPC marker genes. We used an shRNA-based approach to specifically silence Rx expression *in vivo* in tadpoles. We found that loss of Rx function results in impaired retinal regeneration, including defects in the cells that repopulate the wound and the RPE at the wound site. We show that the regeneration defects can be rescued by provision of exogenous Rx. These results demonstrate for the first time that Rx, in addition to being essential during retinal development, also functions during retinal regeneration.

Introduction

Retinal regeneration in vertebrates was first demonstrated in urodele amphibians over 100 years ago (Del Rio-Tsonis and Tsonis, 2003; Yoshii et al., 2007). Retinal regeneration has also been documented in frogs, embryonic and post-natal chickens, and fish (Araki, 2007; Bernardos et al., 2007; Del Rio-Tsonis and Tsonis, 2003; Fischer, 2005; Vergara and Del Rio-Tsonis, 2009; Yoshii et al., 2007). The mammalian retina can also initiate regeneration (Karl et al., 2008). The *Xenopus laevis* tadpole is capable of regenerating its retina after surgical removal of 2/3 of the eye (Ide et al., 1984, 1987). Similarly, studies in *Rana catesbiana* showed that tadpoles of this species could also regenerate the retina after damage induced by devascularization and severing the optic nerve (Reh and Nagy, 1987). Additionally, adult *Rana temporaria* and *X. laevis* can also regenerate the retina following partial resection (Levine, 1981; Lombardo, 1969). Recently, it was demonstrated that both tadpoles and adult *X. laevis* have the capacity to regenerate their retina even after complete retinectomy (Vergara and Del Rio-Tsonis, 2009; Yoshii et al., 2007).

In salamanders and newts, retinal regeneration occurs mostly through transdifferentiation of the retinal pigment epithelium (RPE) (Del Rio-Tsonis and Tsonis, 2003). RPE transdifferentiation is also a source of regenerating cells in embryonic chicks (Spence et al., 2007, 2004). Regeneration is also possible in post-natal chickens (Fischer and Reh, 2001). After neurotoxic damage, chickens can regenerate the retina by transdifferentiation of Müller glia (Fischer and Reh, 2001). Müller glia can also transdifferentiate and give rise to new photoreceptors after light-induced damage in fish (Bernardos et al., 2007). Similar to regeneration in newts, RPE transdifferentiation is considered to be a major source of regenerating cells in frogs. Transplantation of RPE into the eye showed that RPE could undergo metaplasia and produce new retinal tissue (Sologub, 1975; Arresta et al., 2005). RPE can differentiate into neural retina in post-metamorphic *X. laevis* as well (Yoshii et al., 2007). The process and molecular details of transdifferentiation of frog RPE into new retinal neurons have not been characterized. Another potential source of regenerating cells in frogs is the retinal progenitor cells (RPCs) located at the ciliary marginal zone (CMZ) (Moshiri et al., 2004; Reh and Fischer, 2001, 2006; Reh and Levine, 1998). These RPCs continually proliferate and give rise to most of the retinal growth that occurs in *X. laevis* larvae (Hollyfield, 1971).

Regeneration is said to recapitulate embryonic development. The Retinal Homeobox (Rx) gene is one of the earliest genes to be expressed during eye development (Casarosa et al., 1997; Chuang et al., 1999; Deschet et al., 1999; Furukawa et al., 1997; Mathers et al., 1997). It is expressed throughout retinal development, beginning at neural plate (Mathers et al., 1997). In the mature frog retina Rx is expressed in the photoreceptor layer (PRL), inner nuclear layer (INL) and throughout the CMZ (Pan et al., 2006). Loss of Rx function leads to a lack of eye structures in a variety of species including frogs, fish, mice and humans (Andreazzoli et al., 1999; Chen and Cepko, 2002;

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doi:10.1016/j.ydbio.2011.02.008

Chuang and Raymond, 2001; Loosli et al., 2003, 2001; Mathers et al., 1997; Voronina et al., 2004). Conversely, Rx overexpression results in the formation of extra retinal tissue (Andreazzoli et al., 1999; Chuang and Raymond, 2001; Mathers et al., 1997). Results from loss- and gain-of-function studies in *X. laevis* suggested that Rx function is essential for the specification and proliferation of RPCs. Subsequent studies then showed that Rx functions to maintain RPCs in a proliferative and multipotent state throughout development (Andreazzoli et al., 2003; Casarosa et al., 2003). Additionally, overexpression of Rx in the developing optic cup does not bias the fate of newly generated cells (Andreazzoli et al., 2003; Casarosa et al., 2003).

The purpose of this study is to characterize retinal regeneration in pre-metamorphic *X. laevis* both at a morphological and a molecular level. Here we show that pre-metamorphic *X. laevis* fully regenerates the retina by 30 days after surgical resection of 1/4 of the eye. We also show that retinal progenitor cells (RPCs) are induced at the site of resection after 1 week post-resection. Finally, we demonstrate that Rx is necessary for retinal regeneration and that the generation of RPCs during retinal regeneration may require Rx function.

Experimental procedures

Retinal resection

X. laevis tadpoles reared by *in vitro* fertilization (Sive et al., 2000) were raised to stage 44 (Nieuwkoop and Faber, 1994) and anesthetized in 0.1% MS-222 (ethyl-3-aminobenzoate methanesulfonate; Sigma) diluted in 0.1× MMR before resection. Tadpoles were placed in a small rectangular well made in 2.5% agarose dish for immobilization. The nasal-dorsal quarter of the eye was removed from the right eye of each tadpole using a pair of no. 5 forceps and a $27^{1/2}$-gage syringe or a Gastromaster. The left eye of the same tadpole was not resected and used for control experiments. Tadpoles were cultured at 16 °C and fed (Sera Micron) 6 days a week. Tadpoles in which the eye resorbed or collapsed over the first few days after resection were discarded and not used for further experiments. Under these conditions, tadpoles developed as follows: st 44 — day 1; st 45 — day 2; st 46 — day 3; st 47 — day 5; st 48 — day 10; st 49 — day 15; st 50 — day 18; and st 51 — day 22.

Histological staining and immunohistochemistry

For histology and immunohistochemistry, tadpoles were fixed in MEMPFA [MOPS-EGTA-$MgSO_4$-paraformaldehyde] at different time points after resection during a span of 30 days (Sive et al., 2000), dehydrated in methanol, and embedded in paraffin as previously described (Pan et al., 2006). Eyes were sectioned coronally at 8 μm. Immunohistochemistry was performed as described previously (El-Hodiri et al., 1997). The primary antibodies were used in the following dilutions: mouse anti-rhodopsin (RetP1; Biomeda, Foster City, CA) 1:50; mouse anti-islet 1 (clone 39.4D5; Developmental Studies Hybridoma Bank [DSHB], University of Iowa) 1:50; rabbit anti-CRALBP (courtesy of Dr. J. Saari), 1:1000; and mouse anti-BrdU (clone G3G4; DSHB), 1:50. For immunofluorescence, we used an Alexa-fluor 488-conjugated goat anti-mouse secondary antibody (Invitrogen/Molecular Probes), diluted 1:1000.

BrdU incorporation

BrdU crystals (Sigma) were diluted to 0.01% in 40% Holtfreter's from a stock solution of 0.1× Holtfreter's and injected intra-abdominally. After injection, tadpoles were incubated at 16 °C for 2 h, fixed in MEMPFA for 1 h and dehydrated in methanol. To analyze the incorporation of BrdU in proliferating cells, embryos were paraffinized, and 8 μm sections were prepared and subjected to immunohistochemistry as described above, but with an incubation in 4 M HCl for 7 min prior to the blocking step during immunostaining or immunofluorescence.

In situ hybridization of retinal sections

Section *in situ* hybridization was performed on 8 μm retinal sections processed using either digoxygenin or fluorescein-labeled antisense riboprobes as previously described (Shimamura et al., 1994; Viczian et al., 2003). Antisense riboprobes for Rx1A, Pax6, Sox2, Notch1, NeuroD, and Xic1 were generated as previously described (Mathers et al., 1997; Mizuseki et al., 1998; Ohnuma et al., 1999; Pan et al., 2006). Double section *in situ* hybridization was performed using digoxigenin-labeled Notch1 and fluorescein labeled NeuroD antisense riboprobes as described previously (Martinez-De Luna and El-Hodiri, 2007). Fast Red (Sigma) was used as the second chromogen in the double *in situ* hybridization experiments.

Transgenesis

Transgenic *Xenopus* embryos were generated by the intracytosolic sperm injection (ICSI) method (Sparrow et al., 2000). To make the Rx and control shRNA transgenes, the transgene DNA was released from the vector by restriction digestion with BglII, PstI and SalI, and purified from agarose gel using the Gene Clean kit (QBiogene). ICSI was performed as previously described (Sparrow et al., 2000), using snap frozen sperm nuclei. For the transgenesis reaction 400,000 sperm nuclei were incubated with 250 ng of transgene DNA and 2 μl of sperm dilution buffer (SDB) for 15 min at room temperature. The reaction was then diluted in 22.5 μl and 2.5 μl of this mixture was further diluted in 230 μl of SDB for injection. Cysteine dejellied eggs were injected with 10 nl of transgenesis reaction in 0.4× MMR (Marc's Modified Ringer's) + 6% Ficoll. Properly dividing embryos were transferred to 0.1× MMR + 6% Ficoll and changed to 0.1× MMR after 24 h. Embryos were raised in 0.1×MMR until the appropriate stage. Control and Rx shRNA and mRx rescue transgenes were prepared as described previously (Pan et al., 2010). Transgenic embryos were selected using a fluorescent microscope with a blue-green filter to detect coral GFP (cGFP) fluorescence derived from the cGFP cassette present in the transgene vector.

Counts of retinal progenitor cells

We counted RPCs using digital images of sectioned regenerating retinas stained with hematoxylin and eosin as described above. RPCs were identified and counted in electronic images of sections through the center of the wound site. Examples are shown in Fig. S1. RPCs were identified by shape and stain color. Abnormally-shaped RPCs, often observed in Rx shRNA transgenic tadpoles, were included in our counts. RPCs were counted from 5 different tadpoles (one section each) in each group. Counts were averaged and compared using a 2-tailed Student's *t*-test using Prism software (GraphPad, Inc.).

Results

Progression of retinal regeneration in X. laevis

We began our studies of *X. laevis* tadpole retinal regeneration with a histological and molecular characterization of retinal regeneration. To determine the time course of regeneration, we performed histology on regenerating retinas from 1 to 30 days after resection. We found that the retina is essentially regenerated by 30 days post-resection as evidenced by the reorganization of the RPE and the retinal laminae (Fig. 1). On day 1, resection of the nasal-dorsal quarter is quite evident because retinal lamination and RPE integrity are disrupted (Fig. 1A; asterisks). By 3 days post-resection the RPE begins to wrap around the wound and the wound begins to close (Fig. 1B; red