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Expression of pluripotency factors in larval epithelia of the frog *Xenopus*: Evidence for the presence of cornea epithelial stem cells

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ABSTRACT

Understanding the biology of somatic stem cells in self renewing tissues represents an exciting field of study, especially given the potential to harness these cells for tissue regeneration and repair in treating injury and disease. The mammalian cornea contains a population of basal epithelial stem cells involved in cornea homeostasis and repair. Research has been restricted to mammalian systems and little is known about the presence or function of these stem cells in other vertebrates. Therefore, we carried out studies to characterize frog cornea epithelium. Careful examination shows that the *Xenopus* larval cornea epithelium consists of three distinct layers that include an outer epithelial layer and underlying basal epithelium, in addition to a deeper fibrous layer that contains the main sensory nerve trunks that give rise to numerous branches that extend into these epithelia. These nerves convey sensory and presumably also autonomic innervation to those tissues. The sensory nerves are all derived as branches of the trigeminal nerve/ganglion similar to the situation encountered in mammals, though there appear to be some potentially interesting differences, which are detailed in this paper. We show further that numerous pluripotency genes are expressed by cells in the cornea epithelium, including: *sox2*, *p63*, various *oct4* homologs, *c-myc*, *klf4* and many others. Antibody localization revealed that p63, a well known mammalian epithelial stem cell marker, was localized strictly to all cells in the basal cornea epithelium. c-myc, was visualized in a smaller subset of basal epithelial cells and adjacent stromal tissue predominately at the periphery of the cornea (limbal zone). Finally, sox2 protein was found to be present throughout all cells of both the outer and basal epithelia, but was much more intensely expressed in a distinct subset of cells that appeared to be either multinucleate or possessed multi-lobed nuclei that are normally located at the periphery of the cornea. Using a thymidine analog (EdU), we were able to label mitotically active cells, which revealed that cell proliferation takes place throughout the cornea epithelium, predominantly in the basal epithelial layer. Species of *Xenopus* and one other amphibian are unique in their ability to replace a missing lens from cells derived from the basal cornea epithelium. Using EdU we show, as others have previously, that proliferating cells within the cornea epithelium do contribute to the formation of these regenerated lenses. Furthermore, using qPCR we determined that representatives of various pluripotency genes (i.e., *sox2*, *p63* and *oct60*) are upregulated early during the process of lens regeneration. Antibody labeling showed that the number of sox2 expressing cells increased dramatically within 4 h following lens removal and these cells were scattered throughout the basal layer of the cornea epithelium. Historically, the process of lens regeneration in *Xenopus* had been described as one involving transdifferentiation of cornea epithelial cells (i.e., one involving cellular dedifferentiation followed by redifferentiation). Our combined observations provide evidence that a population of stem cells exists within the *Xenopus* cornea. We hypothesize that the basal epithelium contains oligopotent epithelial stem cells that also represent the source of regenerated lenses in the frog. Future studies will be required to clearly identify the source of these lenses.

Introduction

Much interest lies in understanding how adult cells replenish normal and damaged tissues, and how these cells may be harnessed to repair and regenerate intact organs in humans. For instance, this occurs in the mammalian eye where epithelial cells of the cornea are constantly sloughed off and replaced (Hanna and O'Brien, 1960; Thoft and Friend, 1983). Obviously, the proper maintenance of this tissue is essential for normal vision (Cotsarelis et al., 1989). The existence of a population of epithelial stem cells in the cornea was postulated years ago (Davanger and Evensen, 1971); however, the exact location of these cells is a controversial topic with respect to various mammals (Di Girolamo, 2011). Earlier research showed that corneal epithelial stem cells resided in the basal epithelium at the periphery of the

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cornea (in the so-called limbal region or Palisades of Vogt; Cotsarelis et al., 1989; Davanger and Evensen, 1971; Schermer et al., 1986). More recent studies indicate that these cells are not restricted to the limbus and may be found throughout the cornea (Chang et al., 2008; Majo et al., 2008). A different population of mesenchymal stem cells also resides within the corneal stroma (the corneal keratocytes; Du et al., 2005; Funderburgh et al., 2005). It is not known whether similar corneal stem cells reside in the eyes of other vertebrates including those of reptiles, birds, fish and amphibians.

Certain vertebrates exhibit the capacity to regenerate eye tissues, which far exceeds that seen in mammals. For instance, the frog *Xenopus* is known to exhibit remarkable powers of regeneration of various eye tissues, including the neural retina and the lens (reviewed by Henry, 2003; Henry et al., 2008; Henry and Tsonis, 2010). Intact regenerated lenses arise from cells of the basal layer of the cornea epithelium once the original lens is removed (Freeman, 1963; Waggoner, 1973). Historically, this process has been described as one involving transdifferentiation of cornea cells, although it is unclear if such a process involving cellular dedifferentiation and redifferentiation of cornea epithelial cells actually takes place (Bosco et al.,1980; Freeman, 1963). Could, in fact, these regenerated lenses be derived from a population of undifferentiated, somatic stem cells within the basal cornea epithelium?

Using a combination of reverse transcriptase PCR (RT-PCR) and real-time quantitative PCR (qPCR), as well as immunohistochemistry, we show that cells reside within the *Xenopus* cornea epithelium that express numerous pluripotency factors, including: sox2, Oct4 homologs, c-myc, and klf4. We show further that specific pluripotency factors are upregulated early during the process of lens regeneration once the original lens is removed. Antibody labeling shows that the proteins encoded by some of these genes (*sox2*, *p63* and *c-myc*) reside within unique subsets of cornea cells. We also show for the first time that the frog cornea is highly innervated and this network of nerves extends to the cornea from a deeper fibrillar layer that resides just below the basal cornea epithelium. Similar to mammals, these nerves are derived from branches of the trigeminal ganglion. Aside from providing a dense sensory network to the cornea (and possibly autonomic innervation), these nerves could represent a key element of a basal cornea stem cell niche. Finally, using EdU, we verified that cell proliferation takes place throughout the cornea, predominately in the basal epithelium, and these proliferating cells contribute to the formation of regenerated lenses. Together, these results indicate that the amphibian cornea exhibits many similarities to those of mammals in possessing a population of somatic epithelial stem cells. Though not conclusive, we hypothesize that these cells may represent oligopotent stem cells that also contribute to the formation of regenerated lenses in *Xenopus*.

Material and methods

Animals

Xenopus laevis adults were obtained from Nasco (Fort Atkinson, WI) and fertilized eggs were prepared according to Henry and Grainger (1987). Embryos and larvae were raised according to Henry and Mittleman (1995) and developmental staging was based on Nieuwkoop and Faber (1956). Animals were anesthetized in a 1:2000 dilution of MS222 (ethyl 3-aminobenzoate methanesulfonate, Sigma, St. Louis, MO) and lentectomies (lens removal) were performed using fine iridectomy scissors and forceps, as previously described (Henry and Mittleman, 1995; Waggoner, 1973). Each animal was allowed to recover in 1/20X normal amphibian media (NAM; Slack, 1984) prior to feeding.

RT-PCR

Various stages of embryonic material (stages 25–40) and corneas from control and regenerating animals (stage 50–52 at days 0–5 post-lentectomy) were collected and flash frozen in a dry-ice/ethanol bath (Henry et al., 2002). Total RNA was isolated from each representative sample using TriZol (Invitrogen, Carlsbad, CA). Samples were treated with DNaseI (Ambion, Grand Island, NY) and purified using NucAway columns (Ambion, Grand Island, NY). Embryonic RNA was pooled together from a range of embryonic stages (stages 25–40) for first strand cDNA synthesis. Larval corneas were also combined for sample sets of both unoperated control corneas and also regenerating corneas, which consisted of equal quantities of 1-, 3-, and 5-day regenerating cornea RNA. First strand cDNA was generated from total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). PCR fragments were verified by sequencing at the Roy J. Carver Biotechnology Center (University of Illinois, Urbana, Illinois) and all PCR primer pairs are listed in Supplementary Table 1.

qPCR

Corneas were harvested and combined from 50–75 (stages 50–52) animals at each time point of lens regeneration, including control unoperated corneas, as well as corneas undergoing lens regeneration at either 4 h, 12 h, 1 day, 3 days or 5 days post lentectomy. RNA was harvested using TriZol and each sample set was treated with DNaseI. Each RNA sample was amplified and first strand cDNA synthesized using the Message BOOSTER RNA amplification and cDNA synthesis kit (Epicentre, Madison, WI). *Xenopus* amplified cDNA was diluted 1:25 and 2 μl of the dilution was used with 1 μl of each primer (10 μM), 6 μl of water and 10 μl of SYBR green (kindly provided by Dr. William Brieher, University of Illinois). Triplicate runs were completed for each time point and condition, and gene specific primers are listed in Supplementary Table 1. Melting curve analysis was conducted for each sample and relative quantitation of expression levels (where control cornea expression levels were compared to regenerating expression levels) was determined using the ΔCt equation and values were normalized against ornithine decarboxylase (*odc*), a commonly accepted endogenous control for *Xenopus* (Christen et al., 2010; Heasman et al., 2000; Morrison and Brickman, 2006).

Immunohistochemistry

Histological analysis of whole corneas (referred to as cornea "pelts") began with fixation of the whole larval specimen in 4% paraformaldehyde (PFA) diluted in 1X PBS (1.86 mM NaH_2PO_4, 8.41 mM Na_2HPO_4, 175 mM NaCl, pH7.4), followed by 3 washes in 1X PBS. Eyes were then excised from each larval sample prior to immunostaining, taking care to keep the cornea attached to the eye. Eyes were washed in 0.5% Triton/1X PBS for 20 min, blocked in 0.5% Triton/1X PBS/10% goat serum for 2 h and then incubated with antibodies diluted in the blocking mixture. The antibodies used include a polyclonal rabbit anti-Sox2 antibody (1:300 dilution; ARP31737, Aviva Systems Biology, San Diego, CA), a monoclonal mouse anti-P63 antibody that targets the ΔNp63 isoform (1:300 dilution; P3362, Sigma), and a monoclonal mouse anti-c-Myc antibody (1:200 dilution; M4439, Sigma, St. Louis, MO). Antibodies to examine neuronal structures include a mouse anti-acetylated tubulin antibody (1:300; T7451, Sigma) and a polyclonal rabbit anti-Substance P antibody (1:250; NBP1-78326, Novus Biologicals, Littleton, CO). Several washes with 0.5% Triton/1X PBS were performed prior to secondary antibody incubation at a concentration of 1:300 for all secondaries (goat anti-mouse Alexafluor 488 or 546 from Molecular Probes, Eugene, OR; or goat-anti-rabbit FITC or TRITC from Jackson Immuno Research,