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Regenerative Potential of the Zebrafish Corneal Endothelium

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Abstract

Corneal transparency, critical for clear vision, is maintained in part by the pump function of the corneal endothelial cells that are arrested in G₁ phase of the cell cycle in adult humans. Thus loss of endothelial cells leads to a decrease in endothelial cell density. A decrease below a critical threshold results in corneal edema and subsequent vision loss. Corneal edema due to endothelial dysfunction is a common indication for transplantation in developed countries. The zebrafish has emerged as a model for vertebrate regeneration due to its ease of genetic manipulation and remarkable regenerative capacity. The purpose of this study was to investigate the response and regenerative potential of the zebrafish corneal endothelium to pharmacological and mechanical injury. Similar to the human cornea, Na⁺/K⁺ ATPase activity is necessary to maintain the pump function as intracameral injection of ouabain resulted in an increase in central corneal thickness. Surgical removal of the majority of the central corneal endothelium resulted in a similar increase in corneal thickness. Remarkably, by just one week post-injury the central corneal endothelium had largely re-formed. Immunofluorescence of phosphorylated histone H3 indicated that this recovery correlated with corneal endothelial cells re-entering the cell cycle. In conclusion, our results establish zebrafish as a useful model of corneal injury and repair that may offer insights into the mechanism of cell cycle arrest in human corneal endothelial cells.

Cornea, the anterior-most structure of the eye, is the only tissue in the human body that is completely transparent due to several exceptional properties.(Jester et al., 1999) Proper corneal hydration, mediated in part through the pump function of the corneal endothelium, is necessary for maintenance of transparency and clear vision. Damage to the corneal endothelium from aging, infection or surgical injury can result in corneal edema leading to vision loss, a common indication for corneal transplants in developed countries. Adult human corneal endothelial cells, which are arranged as a monolayer on Descemet's membrane, are arrested in the G₁ phase of the cell cycle and remain so even in response to various insults.(Joyce et al., 1996) Due to the cell cycle arrest, loss of endothelial cells due to injury, infection, aging, and/or disease, results in migration and enlargement of surrounding cells resulting in decreased cell density. A decrease in cell density below a critical threshold, approximately 500 cells/mm², results in corneal edema that leads to loss of transparency and subsequent vision loss, a common indication for corneal transplantation.

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Although there currently is no shortage of donor corneas in developed nations, there is a global shortage of donor corneas as evidenced by approximately 8 million untreated corneal blindness patients worldwide.(Whitcher et al., 2001) The demand for donor corneas is expected to rise due to the increasing age of the baby boomers and more patients undergoing intraocular surgery, such as cataract surgery, and this will necessitate alternative therapeutic options, such as the use of stem-cell-derived endothelial tissue or the stimulation of endogenous endothelial repair.

Previous studies have shown limited regenerative potential of the corneal endothelium in rabbits and cats, yet further studies in these model systems have been hindered by a dearth of molecular and genetic tools.(Nakahori et al., 1996; Schubert and Trokel, 1984) Alternatively, the zebrafish has emerged as an excellent organism for modeling human disease, including diseases of the eye.(Bibliowicz et al., 2011; Bohnsack et al., 2012; Gupta et al., 2011; Kleinjan et al., 2008; Neumann et al., 2009; Sun et al., 2004) The zebrafish cornea is structurally similar to the human cornea and shares a similar developmental gene expression repertoire, including BIGH3, keratin 3, and keratan sulfate, whose mutations are associated with human corneal dystrophies.(Hasegawa et al., 2000; Irvine et al., 1997; Munier et al., 2002; Tanhehco et al., 2006; Zhao et al., 2006) Zebrafish also has a remarkable regenerative capacity compared to mammals, with substantial molecular and cellular insights having been made into the regeneration of the heart, retina, and many other organs.(Chablais and Jazwinska, 2012; Wan et al., 2012) Prior to our study, it was not known whether the zebrafish corneal endothelium also had regenerative potential in response to insults known to damage the human cornea. Here, we present evidence that the injury response of the endothelium in zebrafish corneas is similar to human corneas, but unlike in humans, corneal endothelial cells in zebrafish are able to reenter the cell cycle following surgical injury and largely repair the wound.

For all studies, zebrafish were handled per protocols approved by the University of Southern California Institutional Animal Care and Use Committee and maintained under previously described conditions.(Kimmel et al., 1995) We first sought to determine whether, like in human corneas, the zebrafish corneal endothelium served a critical pump function in maintaining deturgescence of the cornea. In order to do so, a bent 30-gauge needle was introduced into the anterior chamber and used to scrape away the endothelial cells in the central 0.785mm^2 of the corneas, representing approximately 25% of the entire endothelial surface area, in anesthetized adult wild-type zebrafish. We also specifically blocked the Na^+/K^+ ATPase activity of the corneal endothelium by injecting $10\text{ }\mu\text{l}$ of $100\text{ }\mu\text{M}$ ouabain (Sigma-Aldrich Corp., St. Louis, MO) into the anterior chamber, with injection of the balanced salt solution (BSS) carrier serving as a sham injection control. In both experiments, only the right eyes were surgically injured or injected while left eyes served as internal controls. Zebrafish were recovered immediately after injury or injection and euthanized 1 week later for imaging. Corneas of the euthanized zebrafish were imaged using a bench-top spectral domain optical coherence tomography (SD-OCT) system with $6\text{ }\mu\text{m}$ resolution and an imaging speed of 24,000 A-lines/second. Corneal thickness was calculated by measuring the pixel numbers between the front and back boundaries of the cornea in the SD-OCT image, as shown by the brackets in Figure 1, relative to the full imaging depth and dividing by the refractive index of the cornea. The average refractive index of the zebrafish cornea in air was assumed to be 1.4 for the purpose of calculating the corneal thickness. Average central corneal thickness (CCT) was significantly increased in injured corneas, $77.0 \pm 27.0\text{ }\mu\text{m}$ ($n = 6$), versus control corneas, $46.5 \pm 11.5\text{ }\mu\text{m}$ ($n = 6$), $p = 0.044$ in a paired t-test (Figure 1, A – C). Ouabain injection resulted in a similar increase in average CCT: $79.3 \pm 19.6\text{ }\mu\text{m}$ ($n = 4$) compared to $48.0 \pm 2.5\text{ }\mu\text{m}$ ($n = 4$) for the uninjected control eyes, $p = 0.048$ (Figure 1, G – I). There was no difference in average CCT of eyes that were sham injected with BSS, $51.6 \pm 10.6\text{ }\mu\text{m}$ ($n = 4$), versus uninjected control eyes, $52.7 \pm 8.4\text{ }\mu\text{m}$ ($n = 4$), $p =$

0.61 (Figure 1, D – F). Therefore, like in humans, the pump function of corneal endothelial cells is required for maintenance of corneal deturgescence in zebrafish.

We next investigated the response of the zebrafish cornea to the surgical injury to its central endothelial cells. To do so, endothelial cells in the central 0.785mm^2 of the right corneas in anesthetized adult zebrafish were removed using a bent 30-gauge needle while the left eyes were uninjured and served as internal controls ($n = 49$). The zebrafish were then recovered and randomly euthanized either immediately ($n = 15$), at 1 day ($n = 15$), or at 7 days ($n = 19$) after injury. Following euthanasia, an eye bank specular microscope (Model EB-2000XYZ, HAI Labs, Lexington, MA) was used to image the central 0.085mm^2 of the control (Figure 2A), immediate (Figure 2B), 1-day (Figure 2C), and 7-days (Figure 2D) postop corneas, and endothelial cell densities were determined by counting the number of endothelial cells in the imaged area (Figure 2E). Immediately following injury, the average cell density decreased to 103.4 ± 30.6 cells/ mm^2 from 2223.5 ± 290.0 cells/ mm^2 observed in control corneas. One day following injury, the average cell density was determined to be 183.7 ± 40.3 cells/ mm^2 , and 7 days following injury, it had risen to 1306.4 ± 325.6 cells/ mm^2 . Differences in average endothelial cell densities between all groups were found to be statistically significant. It was remarkable that the zebrafish were able to recover approximately 60% of the lost endothelial cells within 7 days of injury.

The inability of human corneal endothelial cells to reenter the cell cycle following injury has been proposed to underlie the poor wound healing capability of the human corneal endothelium. Hence, we investigated whether the reappearance of central corneal endothelial cells following surgical injury in zebrafish correlated with cell cycle progression in the remaining peripheral endothelial cells. The right eyes of adult zebrafish were surgically injured as previously described while the left eyes served as internal controls. The corneas were harvested 1, 2, and 7 days after surgical injury, and immunofluorescence was performed using rabbit anti-phospho-histone H3 (pH3) antibody (Millipore, Billerica, MA) to label the condensed chromatin of mitotic cells.(Hendzel et al., 1997) Mouse anti-zonula occludens 1 (ZO-1) antibody (Life Technologies, Grand Island, NY) was used to label the corneal endothelial cell membranes. Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG (Life Technologies, Grand Island, NY) were used as secondary antibodies. Whole corneas were cut radially and flat mounted on slides using Vectashield containing DAPI (Vector Laboratories, Inc., Burlingame, CA) to counterstain the cell nuclei, and images were captured using a Zeiss LSM 5 confocal fluorescence microscope (Carl Zeiss MicroImaging, LLC, Thornwood, NY). No cells in the control cornea (Figure 2F) were observed to be positive for pH3, indicating that they were mitotically quiescent. Numerous cells were observed to be double positive for ZO-1 and pH3 at 1 day (Figure 2G) and 2 days (Figure 2H) post injury, consistent with corneal endothelial cells reentering the cell cycle after injury. No cells were observed to be in mitosis at 7 days post injury (Figure 2I). These results suggest that there is a burst of proliferative activity at 1 and 2 days following injury with return to mitotic quiescence at 7 days in zebrafish corneal endothelial cells. There was significant polymegethism following surgical injury as remaining endothelial cells following injury were larger and had lost the regular polygonal shape (Figure 2G & 2H). The expression of ZO-1 was also not as robust following injury when compared to controls.

Powerful imaging techniques and relative ease of genetic manipulation have made zebrafish an attractive model system for understanding the molecular and cellular basis of vertebrate regeneration. Corneal edema following surgical injury and ouabain injection indicates that, like its human counterpart, the zebrafish corneal endothelium has an essential pump function in maintaining corneal deturgescence. In contrast, the rapid repopulation and cell cycle reentry of endothelial cells following surgical injury suggest that zebrafish have the capacity

to regenerate the majority of their corneal endothelial cells following surgical injury. At 7 days, the central cornea had recovered approximately 60% of the lost endothelial cells, however this recovery was not sufficient to restore full pump function of the endothelium as evidenced by persistent corneal edema at 1-week post injury. Future studies will be needed to determine the extent of functional recovery in the regenerated corneal endothelium, as well as the mechanism by which zebrafish corneal endothelial cells are able to sense injury and reenter the cell cycle, a process that may involve endothelial-mesenchymal transition. The inability of adult human corneal endothelial cells to reenter the cell cycle following injury has been proposed to involve contact inhibition mediated through p27^{Kip1} and inhibition of S-phase entry mediated through TGF- β 2 (Chen et al., 1999; Joyce et al., 2002; Yoshida et al., 2004), and thus it will be interesting to examine whether these pathways act differently in the injured zebrafish cornea. Ease of genetic manipulation of zebrafish will allow for investigation of the molecular mechanisms of corneal endothelial regeneration *in vivo* that is not possible in other model systems. In conclusion, we have established the first model of corneal endothelial regeneration in a genetically tractable organism, the long-term study of which may offer a means of treating vision loss secondary to corneal endothelial dysfunction without the need for donor corneas.

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Figure 1.

Effect of endothelial injury on central corneal thickness (CCT). Spectral domain OCT imaging shows a qualitative difference in CCT, as indicated by the brackets, between a control eye (A) and a surgically injured eye (B). C) Mean CCT ± SD was determined to be $77.0 \pm 27.0 \mu\text{m}$ (n = 6) in injured corneas versus $46.5 \pm 11.5 \mu\text{m}$ (n = 6) in control corneas, p = 0.044. Sham injection with balanced salt solution (BSS) did not result in a difference in CCT between control eye (D) and sham-injected eye (E). F) Mean CCT ± SD was determined to be $51.6 \pm 10.6 \mu\text{m}$ (n = 4) in BSS-injected eyes versus $52.7 \pm 8.4 \mu\text{m}$ (n = 4) in uninjected control eyes, p = 0.61. Ouabain injection resulted in a qualitative difference in CCT between a control eye (G) and an ouabain-injected eye (H). I) Mean CCT was determined to be $79.3 \pm 19.6 \mu\text{m}$ (n = 4) for ouabain injected eyes versus $48.0 \pm 2.5 \mu\text{m}$ (n = 4) for uninjected control eyes, p = 0.048. There was no difference in average CCT between uninjected eyes of ouabain and BSS groups.

Figure 2.

Endothelial regeneration following surgical injury. Specular microscopy images of the endothelium in central corneas of control (A), immediate postop (B), 1-day postop (C), and 7-day postop (D) zebrafish eyes following surgical injury. The cell count was determined in the injured areas by counting the cells in the central 0.085mm² area of the injured and control corneas. E) Mean endothelial cell density $\pm$ SD was determined to be 2223.5 $\pm$ 290.0 cells/mm² for control (n = 49), 103.4 $\pm$ 30.6 cells/mm² for immediate (n = 15), 183.7 $\pm$ 40.3 cells/mm² for 1 day (n = 15) and 1306.4 $\pm$ 325.6 cells/mm² for 7 day postop (n = 19) corneas. T tests showed statistically significant differences in endothelial cell counts between each groups: control vs immediate ($p = 3.3 \times 10^{-17}$), control vs 1 day ($p = 8.3 \times 10^{-17}$), control vs 7 days ($p = 0.00019$), immediate vs 1 day ($p = 1.3 \times 10^{-6}$), immediate vs 7 days ($p = 1.4 \times 10^{-13}$), and 1 day vs 7 days ($p = 7.5 \times 10^{-13}$). Immunofluorescence of whole-mount corneas from control (F), 1 day post injury (G), 2 days post injury (H) and 7 days post injury (I) corneas using antibodies against zonula occludens 1 (ZO-1), green, and phosphohistone H3 (pH3), red, was performed. F) The control cornea shows the absence of any cells in mitosis. G) One day following injury, border between injured and uninjured endothelium is visible in the lower right corner of the panel. Numerous cells, including some expressing ZO-1 can be seen containing pH3 in their nuclei. H) Two days following injury, there still are numerous ZO-1 and pH3 double positive cells. I) Seven days following injury, there no longer are cells that are positive for pH3. DAPI (blue) was used to counterstain the nuclei. Scale bars = 50 μ m.