The role of semaphorin3fa in Retinal Pigment Epithelium regeneration in zebrafish

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INTRODUCTION

The Retinal Pigment Epithelium (RPE) is a monolayer of pigmented cells in the chambered eye that closely interacts with the outer segments of the photoreceptors of the outer retina to maintain visual function. Damage to the RPE, for instance in a disease such as age-related macular degeneration results in the degeneration of the underlying photoreceptors and subsequently, vision loss. In contrast to mammals, zebrafish can intrinsically regenerate a functional RPE layer if the RPE is injured. Some molecular pathways are known to regulate RPE proliferation in culture, but the molecular pathways that function in vivo to promote RPE regeneration remain largely unknown. My aim is to take the secreted ligand semaphorin 3F which is expressed in the RPE of both mammals and zebrafish and determine its importance in the RPE regeneration signaling cascade. Semaphorins are modulators of axonal sprouting and angiogenesis, but Sema3f specifically, has anti-angiogenic effects and acts as a vasorepulsive cue and since wet AMD is caused by abnormal choroidal neovascularization, I believe that they could be correlated.

PURPOSE

To refine the RPE injury model first derived by Hanovice *et al.* by working out the parameters for efficient RPE death and analysis of RPE regeneration. Fully working out this model will allow me to efficiently test the hypothesis that secreted extrinsic signals promote RPE regeneration after injury and death.

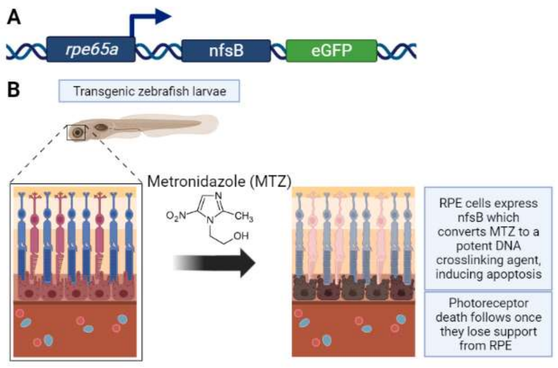

Figure 1. (A) Cartoon depicting *rpe65a*:nfsB-eGFP transgene where nitroreductase (nfsB) and eGFP are located downstream of the rpe65a promoter. (B) Cartoon cross-section of retina depicting theory behind MTZ-nitroreductase model where RPE cells express nitroreductase (nfsB) that will convert MTZ into a potent DNA crosslinking agent, inducing apoptosis. After RPE undergoes apoptosis, the photoreceptors lose their support, and also undergo apoptosis.

MATERIALS & METHODS

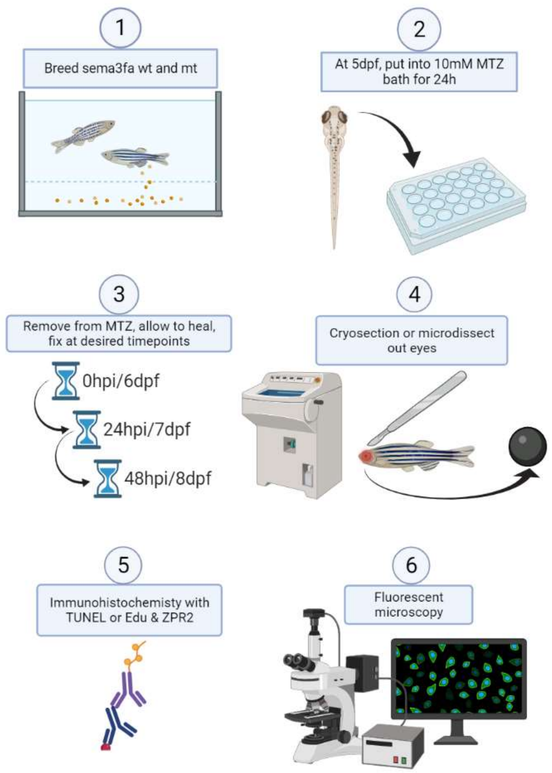

Figure 2. Zebrafish (*Danio rerio*) embryos were obtained by spawning of either wild-type (Wt) or transgenic mutant (Mt; Sema3fa Δ2 mutant) parents on *rpe65a*:nfsB-eGFP background. At 1 day post-fertilization (dpf), embryos were placed into a 1-phenyl 2-thiourea (PTU) bath to prevent formation of pigment for ease of imaging. At 5 dpf, the larvae were placed into a 10mM bath of metronidazole (MTZ) for 24h. After removal from MTZ bath and washed with E3, the larvae were either fixed immediately in 4% PFA at 0 hours post-injury (hpi) or left to heal in E3 and fixed at various time points. After fixation, the larvae were either cryosectioned or placed in a salt solution (Ringer's solution) before having their eyes microdissected, in which their lens, sclera, and iridophores were removed. Immunohistochemistry was then performed on the samples, which consisted of either TUNEL or Edu & ZPR2 labeling. Fluorescent microscopy was performed to gather images.

RESULTS

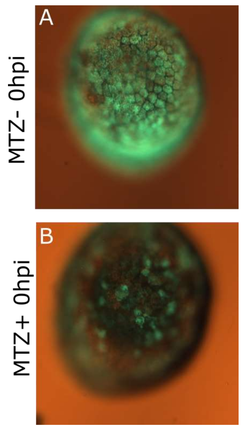

Figure 3. *En face* wholemount of microdissected eye of sema3fa mutant of both control (MTZ-) and experimental (MTZ+) samples. Labels include transgenic GFP (green) expressed by live RPE cells and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling; orange) which labels 3'-hydroxyl termini present during apoptosis. Although the TUNEL labeling was unsuccessful, we can still see widespread and consistent GFP fluorescence in a healthy RPE (A), while dim, sporadic GFP shows widespread RPE ablation (B).

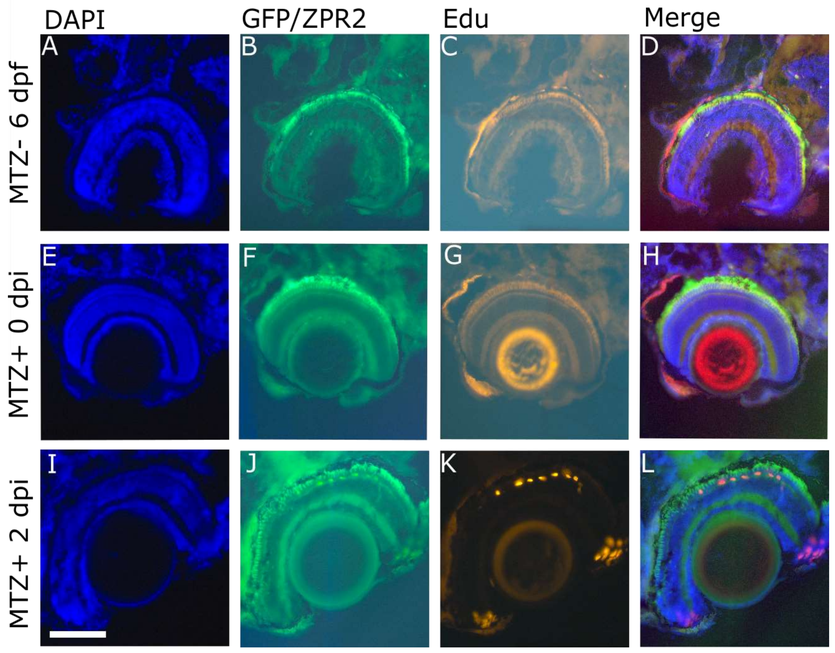

Figure 4. Transverse cryosections stained for DAPI (blue), GFP & ZPR2 (green), Edu (orange/red). Compared to untreated larvae (A-D), ablated RPE was disrupted (F & H), but no proliferation was observed until 2 dpi (K & L).
To further characterize RPE degeneration, ZPR2 (an RPE marker) was stained in addition to intrinsic GFP fluorescence. In the untreated/control eye (A-D), there is an intact RPE cell layer (B) and no Edu fluorescence besides the background autofluorescence (C). The section is not as precise as the other two and is missing the lens. Immediately following MTZ treatment, at 0 dpi, there is evidence of missing cells in the RPE layer (F) but proliferation has not yet begun.
At 2 dpi, there is still RPE disruption, however, there is now the appearance of Edu-labeled cells in the outer nuclear layer which is correlated with Edu-labeled cells in the CMZ as well (K).
Distal is down and ventral is left. Scale bar denotes 50 µm.

CONCLUSIONS

- The transgene is successful in expressing nitroreductase and labeling RPE with GFP.
- Loss of GFP signal is correlated with RPE death.
- 10mM MTZ is sufficient to induce RPE ablation without being lethal to larvae.
- *En face* wholemount is a suitable to image the RPE as a whole, although in older larvae peripheral focal planes become less clear.
- The TUNEL assay was indistinct, perhaps because the kit was too old. It should be repeated with a new kit.
- Edu labels cells that are proliferating, which start to be displayed at 2 dpi.
- Edu-labeled cells in the outer nuclear layer, which may be proliferating photoreceptors, are correlated with proliferative cells present in the ciliary marginal zone (CMZ).

REFERENCES

- Hanovice NJ, Leach LL, Slater K, et al. Regeneration of the zebrafish retinal pigment epithelium after widespread genetic ablation. *PLOS Genetics*. 2019;15(1). doi:10.1371/journal.pgen.1007939
- Figures 1 & 2 created with BioRender.com

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