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## The Retinal Pigment Epithelium: Development, Injury Responses, and Regenerative Potential in Mammalian and non-Mammalian Systems

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### Abstract

Diseases that result in retinal pigment epithelium (RPE) degeneration, such as age-related macular degeneration (AMD), are among the leading causes of blindness worldwide. Atrophic (dry) AMD is the most prevalent form of AMD and there are currently no effective therapies to prevent RPE cell death or restore RPE cells lost from AMD. An intriguing approach to treat AMD and other RPE degenerative diseases is to develop therapies focused on stimulating endogenous RPE regeneration. For this to become feasible, a deeper understanding of the mechanisms underlying RPE development, injury responses and regenerative potential is needed. In mammals, RPE regeneration is extremely limited; small lesions can be repaired by the expansion of adjacent RPE cells, but large lesions cannot be repaired as remaining RPE cells are unable to functionally replace lost RPE tissue. In some injury paradigms, RPE cells proliferate but do not regenerate a morphologically normal monolayer, while in others, proliferation is pathogenic and results in further disruption to the retina. This is in contrast to non-mammalian vertebrates, which possess tremendous RPE regenerative potential. Here, we discuss what is known about RPE formation during development in mammalian and non-mammalian vertebrates, we detail the processes by which RPE cells respond to injury, and we describe examples of RPE-to-retina and RPE-to-RPE regeneration in non-mammalian vertebrates. Finally, we outline barriers to RPE-dependent regeneration in mammals that could potentially be overcome to stimulate a regenerative response from the RPE.

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## Keywords

retinal pigment epithelium (RPE); regeneration; development; age-related macular degeneration (AMD); zebrafish

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## 1. INTRODUCTION

The retinal pigment epithelium (RPE) is a monolayer of cells located at the posterior of the eye, between the retina and choroid, which serves a number of important roles in vision (reviewed in Strauss, 2005; Lakkaraju et al., 2020; Fig. 1). The RPE is a major component of the blood-retinal-barrier (BRB) due to tight cell-cell junctions that form between RPE cells. On their apical side, RPE cells interdigitate with photoreceptor outer segments (POS) and play a crucial role in the function and maintenance of photoreceptor cells. For example, transport of oxygen, nutrients and ions from the choroid to the retina and back is mediated by the RPE, and the RPE functions in degrading or recycling components of the visual cascade to maintain the visual cycle, amongst other critical functions in supporting the retina. Evolutionarily, the association between pigmentation and vision also appears to be essential; across phyla, image-forming visual organs contain either a pigmented organelle located within a photoreceptive cell, or a pigmented cell tightly associated with a photoreceptive cell, underscoring the likely importance of pigmentation/RPE function and the evolution of visual function (reviewed in Koenig and Gross, 2020).

Not surprisingly, diseases that affect the RPE also severely affect vision. Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in developed countries (Klein et al., 1999; Congdon et al., 2004), and primarily affects the RPE. Other RPE-associated diseases include Stargardt disease and some forms of retinitis pigmentosa. Atrophic (dry) AMD is the most prevalent form of AMD, affecting approximately 90% of patients. In atrophic AMD, parafoveal RPE first degenerates and over time, RPE degeneration progresses centrally to the fovea, resulting in death of foveal cones and consequent loss of high-acuity vision (Scholl et al., 2004; Holz et al., 2007; Steinberg et al., 2015; Curcio et al., 1996; Lim et al., 2012; Jackson et al., 2002). There are currently no FDA-approved therapies for atrophic AMD and with incidences of AMD projected to continue to rapidly rise globally (Wong et al., 2014), new strategies must be developed to curb RPE cell death and subsequent retinal degeneration associated with AMD progression. A number of such strategies are in various phases of development and testing, and these include: pharmacological interventions (reviewed in Ammar et al., 2020); cell-based therapies (reviewed in Ben M'Barek et al., 2019); gene therapy (reviewed in Bordet and Behar-Cohen, 2019); stem cell therapies (reviewed in Nazari et al., 2015; Leach and Clegg, 2015); and stem cell-derived transplantation of RPE patches or sheets (reviewed in Sharma et al., 2020), amongst others. While all of these approaches are exciting and some may yield effective therapies, a complementary approach is to stimulate intrinsic regenerative pathways in RPE cells such that tissue damage can be repaired endogenously. Indeed, such a strategy has gained traction over the last few years as a possible treatment for retinal degenerations and optic neuropathies, which have been the focus of several National Eye Institute initiatives (Vetter and Hitchcock, 2017; Burns and Stevens, 2018). Non-mammalian

vertebrates possess remarkable regenerative abilities to repair and regenerate ocular tissues after injury (reviewed in Ail and Perron, 2017; Wilken and Reh, 2016; Wan and Goldman, 2016; Barbosa-Sabanero, 2012; Chiba, 2014), and identification of the molecular and cellular mechanisms underlying these regenerative strategies can serve as the foundation for developing approaches to potentially stimulate such responses in humans to treat currently intractable diseases like AMD.

In this review, we first summarize what is known about RPE development in mammals as well as non-mammalian vertebrate systems used for RPE development, injury and regeneration studies. We then define the molecular and cellular responses to acute and chronic RPE injury in these systems and discuss how they can contribute to further pathology in mammals, which cannot regenerate tissues from their RPE, and are therefore termed regeneration-incompetent. Further, we discuss pro-regenerative pathways in non-mammalian vertebrate systems like chickens, newts, frogs and zebrafish, which are regeneration-competent and possess the ability to regenerate retinal and/or RPE cells from native RPE after RPE or retinal injury. Finally, we delve into potential barriers to RPE reprogramming and regeneration. Our focus in this review is also on *in vivo* studies, whenever possible; while *in vitro* work on RPE cells has most certainly contributed to our understanding of RPE disease and injury responses, cultured systems do not adequately recapitulate the *in vivo* physiology of the RPE after injury, nor do they model the three-dimensional architecture of the eye, where RPE cells are intimately associated with photoreceptors, Bruch's membrane, the choroid, as well as other cell types (e.g. components of the immune system) that collectively modulate their behavior after injury and during regeneration.

## 2. RPE DEVELOPMENT

Here, we highlight key phases of RPE development and known molecular regulators of RPE specification, differentiation and morphogenesis, with an eye towards their reiterative deployment during RPE injury responses in mammalian systems and injury/regenerative responses in non-mammalian systems. There are a number of additional, excellent reviews on RPE development to which we direct interested readers (e.g. Martinez-Morales and Wittbrodt, 2009; Fuhrmann, 2010; Fuhrmann et al., 2014; Moreno-Marmol et al., 2018).

### 2.1 Eye Field Specification, Optic Vesicle, and Optic Cup Formation

RPE, retina, and optic stalk progenitors are derived from neuroepithelial tissue of the diencephalon (reviewed in Chow and Lang, 2001). Eye field transcription factors including Rx, Six3, Lhx2, and Pax6, along with cell-cell signaling pathways, collectively initiate eye field formation in mice, frogs and other vertebrates (Mathers et al., 1997; Lagutin et al., 2003; Porter et al., 1997; Grindley et al., 1995; Zuber et al., 2003). In all vertebrates, bilateral optic primordia evaginate from the diencephalon, forming the optic vesicles (OVs). The distal portion of the OV elongates outward, while the proximal portion constricts to form the optic stalk. Once the distal OV reaches the surface ectoderm, the OV invaginates to form a bilayered optic cup (OC) (reviewed in Fuhrmann, 2010). The distal layer of the OC forms the retina, and the proximal layer wraps around the retina to form the RPE (reviewed

in Fuhrmann et al., 2014). Teleosts (e.g. zebrafish and medaka) have become increasingly useful in studies of RPE development and regeneration in the last decade and importantly, the stages of OC morphogenesis in teleosts are largely similar to those in mammals and birds; however, each OV is flattened, as compared to the spherical presumptive OC in mammals, and extends posteriorly during elongation stages (Kwan et al., 2012; reviewed in Martinez-Morales and Wittbrodt, 2009). OC formation in teleosts also includes unique cell migration patterns not observed during mammalian OC formation; however, RPE progenitors in teleosts ultimately occupy the same region as RPE progenitors in amniotes, in the dorsal region of the eye behind the presumptive retina (Kwan et al., 2012). A comparison of eye development between teleosts and amniotes is summarized in Fig. 2.

## 2.2 RPE Specification/Differentiation

RPE specification begins upon OV evagination and is marked by the expression of *microphthalmia-associated transcription factor (Mitf)* and *orthodenticle homeobox 2 (Otx2)* (Hodgkinson et al., 1993; Bovolenta et al., 1997; Ma et al., 2019). *Mitf* is initially expressed throughout the early eye field, but later restricted to the presumptive RPE through the action of *Vsx2* (Chx10) (Horsford et al., 2005; Rowan et al., 2004) and *Otx2* (Martinez-Morales et al., 2003; Martinez-Morales et al., 2001), the latter of which promotes RPE fate over retina. Both *Mitf* and *Otx2* are directly required for normal RPE formation in mice (Matsuo et al., 1995; Martinez-Morales et al., 2001; Nguyen and Arnheiter, 2000; Hodgkinson et al., 1993; Hero et al., 1991). Similarly, *Otx* paralogs are required for RPE formation in zebrafish; however, the two zebrafish *Mitf* paralogs (*mitfa* and *mitfb*) are dispensable for RPE formation (Lane and Lister, 2012). It is thought that loss of *mitfa/b* function in zebrafish is compensated for by the expression of a related MITF family member, *tfec* (Lister et al., 2011; Sinagoga et al., 2020). Once the OV reaches the overlying surface ectoderm, *Mitf* and *Otx2* expression is identifiable solely in the dorsal OV, where the RPE begins to develop (Nguyen and Arnheiter, 2000; Bovolenta et al., 1997). *Otx2* remains as a marker of the RPE throughout the lifespan of many animals (Bovolenta et al., 1997; Baas et al., 2000; Martinez-Morales et al., 2001), while *Mitf* is only expressed during early development in the chick and mouse RPE (Mochii et al., 1998; Nakayama et al., 1998). *Mitf* exists in at least nine isoforms in mice, with MITF-D and MITF-H being the dominant isoforms required for normal RPE development (Bharti et al., 2008; Hallsson et al., 2000; Takeda et al., 2002), whereas MITF-M, MITF-H, and MITF-A predominate in adult human RPE tissues (Maruotti et al., 2012). In addition to *Otx2* and *Vsx2*, known regulators of *Mitf* expression in mice and humans include: *Lhx2*, *Pax2* and *Pax6* (Martinez-Morales et al., 2003; Yun et al., 2009; Bharti et al., 2012; Bharti et al., 2008; Bäumer et al., 2003). *Pax6* encodes a multifunctional transcription factor specifying both the eye field and specific tissues within the eye, including the RPE, retina, lens placode, iris, and cornea (Bharti et al., 2012; Singh et al., 2002; Ashery-Padan et al., 2000; Bäumer et al., 2003). *Pax6*, *Mitf*, and *Tfec* interact to promote RPE development through down-regulation of the Wnt inhibitor, *Dkk13*, and the proretinogenic growth factor, *Fgf15* (Bharti et al., 2012). *Six6*, *Lhx2* and *Vsx2* activities also stimulate *Pax6* expression in the prospective retina where it facilitates a variety of events during retina formation (e.g. Zuber et al., 2003; Marquardt et al., 2001; Philips et al., 2005; Grindley et al., 1995; Oron-Karni et al., 2008), highlighting the context-dependent and spatially-restricted roles that *Pax6* plays in driving development of both the

RPE and retina. Canonical Wnt/ $\beta$ -catenin signaling also modulates *Mitf* expression and more broadly, RPE development (Westenskow et al., 2009, Fujimura et al., 2009). Like *Mitf* and *Otx2*,  $\beta$ -catenin is expressed in the presumptive RPE and further modulates *Mitf-D* and *Otx2* through TCF/LEF binding within respective enhancer regions (Westenskow et al., 2009). An additional regulator of RPE-specific expression is the Hippo pathway, whose activity is modulated by the cellular localization of the transcriptional co-activators, Yap and Taz (Yu and Guan, 2013). Loss of Yap and Taz activity results in aberrant RPE specification and development in zebrafish and mice (Miesfeld et al., 2015; Kim et al., 2016). Moreover, Neurofibromin 2 (NF2) is an upstream activator of the Hippo pathway that is strongly expressed in the RPE and required for normal RPE development in a Yap/Taz-dependent manner (Moon et al., 2018).

After invagination of the OVs and early RPE specification events, the RPE becomes distinguishable through increased pigmentation and transition from a pseudostratified epithelium to a cuboidal monolayer (reviewed in Moreno-Marmol et al., 2018). Here, studies in a variety of animal models have contributed to our understanding of RPE development, including in non-human primates. For example, in rhesus macaques, the morphology of RPE differs remarkably between peripheral and central RPE at embryonic day (E) 45. The peripheral RPE appears multi-stratified, while the central RPE forms a mono-stratified layer of cuboidal epithelium, and it is thought that this change in morphology proceeds in a central to peripheral fashion (Rappaport et al., 1995). RPE cell proliferation occurs from ~E27 to E85; postnatally, RPE proliferation continues, but to a far more limited extent (Rappaport, et al., 1995). While multiple histological studies of RPE formation have been performed like the above in other animals models, our understanding of RPE morphogenesis in mammalian systems remains quite limited and much of our knowledge on this topic has come from *in vivo* and fixed sample imaging studies in zebrafish (Kwan et al., 2012; reviewed in Moreno-Marmol, 2018). In zebrafish, RPE morphogenesis occurs in two phases as the OV invaginates to form the OC. In the first phase, the presumptive RPE progenitor domain expands anteriorly and RPE progenitors initially exhibit a cuboidal shape, concomitant with increased cell number caused by *de novo* specification. This is followed by a second phase in which the cuboidal RPE cells elongate to adopt an RPE-like morphology and stretch around the retina along the dorsoventral and posterior axes (Li et al., 2000; Cechmanek and McFarlane, 2017). The molecular and cellular mechanisms underlying RPE cell flattening/stretching are not well-understood in any system and need to be further studied.

Finally, the RPE does not develop independently; rather, it interacts with surrounding extraocular mesenchyme, which sends signals that are required for RPE development (Fuhrmann et al., 2000). The transforming growth factor-beta (TGF- $\beta$ ) superfamily member, activin A, is a candidate signaling molecule mediating extraocular mesenchyme-dependent RPE formation. In chick RPE explants lacking extraocular mesenchyme, addition of activin A resulted in an upregulation of RPE markers and a downregulation of retinogenic factors, demonstrating that it was sufficient to enable RPE formation (Fuhrmann et al., 2000). While these data are exciting, this remains to be demonstrated *in vivo*. Bone morphogenetic proteins (BMPs), another critical member of the TGF- $\beta$  superfamily, are also involved in RPE development. In chick, BMPs released from the surface ectoderm inhibit retina

and optic stalk development and specify the RPE in dorsal OV cells by inducing *Mitf* expression (Müller et al., 2000). Wnt proteins are also released from the surface ectoderm and stabilize BMP signaling to initiate RPE specification via a Wnt/ $\beta$ -catenin-independent signaling pathway (Steinfeld et al., 2013). Surface ectoderm signals to the developing OC via fibroblast growth factor (FGF) to promote retinogenesis rather than RPE fates (Nguyen and Arnheiter, 2000), and therefore the extraocular mesenchyme appears to be the primary source of pro-RPE signals during early eye development. Finally, retinoic acid (RA), a derivative from vitamin A, is synthesized from multiple sites in and around the developing eye, including the RPE, and contributes to OC morphogenesis and retinal development (Prabhudesai et al., 2005; Hyatt et al., 1997; Molotkov et al., 2006). In human RPE cells *in vitro*, exposure to exogenous RA prevented cellular overgrowth and maintained a mature RPE-like morphology, suggesting a possible role for RA in the development and/or maintenance of RPE *in vivo* (Campochiaro et al., 1991).

### 2.3 RPE maturation

After early stages of specification, the retina and RPE are separated by the interphotoreceptor matrix (IPM), which also contributes to RPE maturation. The IPM acts as the intermediary between the two tissues during development, as well as throughout life (reviewed in Strauss, 2005; Halilagic et al., 2007; Ishikawa et al., 2015). Pigmentation, achieved through synthesis of melanin, is an obvious readout of differentiated RPE tissue (Beermann et al., 1992). Cell biologically, during maturation, the RPE attains a near-complete polarization along the apical-basal axis, as apical microvilli emerge and rudimentary basal infoldings make contact with the developing Bruch's membrane (Fig. 1). The outer aspect of the RPE basal membrane becomes incorporated into the developing Bruch's membrane, which matures into a pentalaminar structure upon incorporation of the basement membrane of the choriocapillaris and deposition of collagen and elastin proteins, regulated by the choroid and RPE (reviewed in Strauss, 2005; Lakkaraju et al., 2020; Booi et al., 2010). The RPE starts to express mature markers, including the adhesion molecule, N-CAM-140 (Gundersen et al., 1993), and apical microvilli protein, Ezrin, which promotes elongation of microvilli (Bonilha et al., 1999). Junctional proteins form tight connections between adjacent RPE cells, contributing to the completion of RPE apical-basal polarization and formation of the BRB (reviewed in Strauss 2005; Williams and Rizzolo, 1997). Indeed, the RPE tightly controls both basal nutrient entry from the fenestrated choriocapillaris and apical growth factor secretion to the retina (reviewed in Rizzolo, 2007), and these functions require the acquisition of precise apical-basal cell polarity during RPE development and maturation. During ocular morphogenesis, junctional protein composition in RPE cells is dynamic with protein expression changing over time as a function of retinal secretions (Williams and Rizzolo, 1997; Rahner et al., 2004). Initially, leaky tight junctions form discontinuities around developing RPE cells, permitting paracellular transport (Williams and Rizzolo, 1997). Later developmental stages elicit minimal transport between cells and ion selectivity, indicating mature barrier formation (Williams and Rizzolo, 1997; Ban and Rizzolo, 1999). Any remaining leaks between cells are resolved, resulting in a functional, continuous barrier (reviewed in Rizzolo et al., 2007).

As the RPE matures, its ability to regulate and secrete growth factors, extracellular matrix (ECM), and ECM-associated proteins is critical for maintaining the functional integrity of adjacent tissues. For example, pigment epithelium-derived factor (PEDF) is secreted apically from the RPE and provides neurotrophic support to photoreceptors (Becerra et al., 2004; Barnstable and Tombran-Tink, 2004). PEDF also acts as an angiogenic inhibitor (Dawson et al., 1999), which is important because pro-angiogenic vascular endothelial growth factor (VEGF) is secreted basally from RPE cells and acts as a paracrine signal to the underlying choroid (Campochiaro et al., 1989, Blaauwgeers et al., 1999, Marneros et al., 2005; Saint-Geniez et al., 2009). Indeed, VEGF secretion from the RPE is required for choroidal vasculature development (Goto et al., 2018), highlighting the importance of RPE-derived PEDF in modulating VEGF activity to prevent retinal neovascularization. TGF- $\beta$ 1 and TGF- $\beta$ 2 are secreted by cultured human RPE cells, with TGF- $\beta$ 2 as the dominant isoform (Hirsch et al., 2015). Apical secretion of active TGF- $\beta$  might play a role in maintaining the RPE immune microenvironment (Hirsch et al., 2015, Sugita et al., 2006). Studies in cultured RPE also suggest functions for RPE-derived FGFs in mitogenesis, angiogenesis and cell survival. For example, bFGF (FGF2) stimulates mitogenesis, possibly in an autocrine fashion (Schwegler et al., 1997), while FGF5 is secreted basally and serves to maintain choroid function (Dunn et al., 1998). Various other proteins including brain-derived growth factor (BDNF) (Kolomeyer et al., 2011), ciliary neurotrophic factor (CNTF) (Li et al., 2011), insulin-like growth factor-I (IGF-I) (Moriarty et al., 1994), nerve growth factor (NGF) (Kolomeyer et al., 2011), tissue inhibitor of matrix metalloproteinase (TIMP-1) (Padgett et al., 1997), and adhesion molecules (Aisenbrey et al., 2006) are also secreted from the RPE, some in a directional manner, further highlighting the importance of correct RPE polarization as the RPE matures and assumes its functional roles (reviewed in Kay et al., 2013).

As mentioned above, RPE polarity is plastic and dependent on interactions with the developing retina. For example, the elongation of apical microvilli is related to the extension of photoreceptor outer segments and the expression of adhesion proteins, including N-CAM and certain integrins, in the basolateral membranes of RPE that change progressively as the neural retina develops (reviewed in Rizzolo, 1997). As intercellular permeability decreases as a result of tight junction development, the RPE expresses GLUT transporters to supply the retina with glucose, which is critical for maintaining the health of the retina (Ban and Rizzolo, 2000), as well as other solutes and nutrients (reviewed in Strauss, 2005). The RPE also functions as a key metabolic component of a succinate shuttle that serves to transfer electrons from the hypoxic retina to the oxygen-rich RPE (Bisbach et al., 2020). In the final step of maturation, the RPE and retina interdigitate permanently, as the apical microvilli extend to interact with the POS in the subretinal space; this process is accompanied by the formation of complete basal infoldings (reviewed in Strauss, 2005). Mature RPE markers include: visual cycle proteins, RPE65 (Mata et al., 2004) and CRALBP (Xue et al., 2015); integrin  $\alpha$ v $\beta$ 5 (Anderson et al., 1995); the basolateral RPE protein, bestrophin (Bakall et al., 2003); PEDF (Wu et al., 1995); MerTK (Feng et al., 2002); pre-melanosomal protein PMEL17 (Raposo et al., 2001); tyrosinase (Dryja et al., 1978); apically localized Na<sup>+</sup> K<sup>+</sup>-ATPase (Hu et al., 1994); extracellular matrix metalloproteinase inducer (EMMPRIN) (Marmostein et al., 1998); in addition to N-CAM-140 (Gundersen et al., 1993) and Ezrin

(Bonilha et al., 1999). Gene expression profiling and single-cell sequencing of fetal and adult human RPE have also been completed, identifying a suite of RPE-enriched genes (Strunnikova et al., 2010; Hu et al., 2019; Schumacker et al., 2020; Voigt et al., 2019).

### 3. RPE INJURY RESPONSES

Once developed, the RPE becomes a structure vital to maintaining the health of photoreceptors and the function of the retina as a whole. Given the prevalence of RPE-related diseases that have deleterious effects on visual function, RPE injury paradigms have been developed in mammalian and non-mammalian systems to identify aspects of the injury response and to determine whether modulation of injury-responsive pathways might be leveraged to develop therapies to treat RPE disorders (Table 1).

Despite biological similarities, many different experimental methods have been utilized to reproduce degenerative ocular phenotypes, including, but not limited to, induction of oxidative stress, genetic ablation, and laser-induced choroidal neovascularization (CNV), which adds a level of complexity to studying RPE injury responses. Many traditional RPE injury studies have been conducted in mammalian model systems through the use of sodium iodate (NaIO<sub>3</sub>) injection, which induces oxidative stress in RPE cells that results in cell death (Noell, 1953; Grignolo et al., 1966). Depending on the dosage, animal, and method of administration, RPE degeneration and injury responses vary. For example, high-dose NaIO<sub>3</sub> injections have been widely characterized and implicated in primary degeneration of RPE, followed by secondary photoreceptor dysfunction (Kiuchi et al., 2002). However, low-dose administration of NaIO<sub>3</sub> in rats causes retinal disruption prior to any RPE degeneration, suggesting that NaIO<sub>3</sub> is not entirely specific to RPE (Wang et al., 2014). Other methods include AAV injection of a *Sod2* ribozyme and Ceruloplasmin/Hephaestin knockout mouse models to elevate reactive oxygen species (ROS), resulting in acute destruction of RPE cells (Seo et al., 2012; Hadziahmetovic et al., 2008). On the other hand, genetic ablation models utilize transgenic cassettes targeting a variety of proteins that stimulate programmed cell death as a means to recapitulate the loss of RPE cells that occurs during AMD. Like models increasing oxidative stress, genetic ablation causes acute damage to the RPE with secondary damage to the retina (Hanovice et al., 2019; Longbottom et al., 2009). Similarly, laser photocoagulation injures the RPE and/or retina, providing instant damage and stimulation of rapid wound healing responses (Roeder et al., 1992; Tso et al., 1973). Targeted induction of CNV is also an established methodology that utilizes laser burns to model exudative AMD (reviewed in Grossniklaus et al., 2010; Ryan, 1979). Here, immediately post-injury, the RPE and Bruch's membrane lose association, which leads to vascular invasion and leukocyte infiltration. Subsequently, fibrovascular tissue and proliferative RPE repair the disrupted BRB (Zhou et al., 2017; Sakurai et al., 2003; Miller et al., 1990). These models seek to emulate common late stages of AMD when choroidal vessels invade the retina (reviewed in Das and McGuire, 2003).

As the aforementioned systems provide insight into the biological response after acute injury, other models are used to study the responses following chronic RPE dysfunction/death in degenerative disease. Aged mice with human *APOE-4* targeted replacement that were provided with a high fat diet to stimulate amyloid beta (A $\beta$ ) accumulation showed an



AMD-like phenotype as A $\beta$  is present in drusen (Anderson et al., 2004) and co-localizes with complement proteins (Wang et al., 2009). These data suggest that A $\beta$  contributes to pathology by perpetuating chronic inflammation (Ding et al., 2011). This model demonstrates chronic presentations of the disease (e.g. basal RPE deposits, thickening of Bruch's membrane, and loss of visual function); however, acute presentations still remained (e.g. vacuole formation, multinucleate and enlarged RPE, and variable pigmentation) (Ding et al., 2011). Thus, although AMD progresses chronically, some stages of the disease are acute, highlighting the complexity of pathogenesis and a need to develop stage-specific therapeutic options.

Despite the existence of numerous methods to recapitulate RPE degenerative diseases, common injury responses suggest a trend in cellular behavior. Here, we discuss RPE injury responses, with a particular emphasis on those pathways that are also involved in RPE development and/or regeneration.

### 3.1 Acute injury responses

Cellular responses immediately following RPE injury have been widely characterized in mammalian and non-mammalian vertebrate model systems, although for many of these responses, how they lead to wound healing, RPE cell proliferation or repair remains ambiguous. RPE injury studies demonstrate a trend in biological responses that includes: loss of pigmentation (Hanovice et al., 2019; Hanus et al., 2016; Korte et al., 1984; Tso, 1973), loss of apical microvilli (Liu et al., 2019a; Korte et al., 1984) and basal infoldings (Liu et al., 2019a; Korte et al., 1984; Tso, 1973); followed by RPE cell enlargement (Hanus et al., 2016; Seo et al., 2012; Longbottom et al., 2009; Roider et al., 1992), the formation of multinucleate RPE cells (Seo et al., 2012; Ding et al., 2011; Hadziahmetovic et al., 2008), accumulation of intracellular vacuoles (Seo et al., 2012; Ding et al., 2011; Roider et al., 1992); collectively culminating in overall disruption of the RPE monolayer and photoreceptor disorganization (Hanovice et al., 2019; Moriguchi et al., 2018).

One common RPE phenotype resulting from injury, regardless of injury type, is cell enlargement, which could occur in an attempt to preserve the association between surrounding RPE cells, as well as RPE cells and underlying photoreceptors, to maintain physiological function. Multinucleate RPE exist physiologically in the human eye, specifically in the perifovea and near-periphery regions (Starnes et al., 2016). Multinucleation and cell enlargement becomes more pronounced after injury in rodent models (Seo et al., 2012; Ding et al., 2011; Hadziahmetovic et al., 2008). It is unknown if multinucleate RPE exist in non-mammalian systems. The function, if any, of these multinucleate cells in mammals is also not clear; however, it is possible that RPE re-enter the cell cycle in an attempt to proliferate and restore the monolayer, but are unable to complete cytokinesis, resulting in enlarged multinucleate cells. As the RPE is responsible for managing oxidative stress in the eye, targeted oxidative damage also results in enlargement and damage to mitochondria (Seo et al., 2012). Bruch's membrane breaks down in response to selective RPE insult in several animal model systems (Hanovice et al., 2019; Seo et al., 2012) and in AMD patients (Chong et al., 2005). Atrophy of the choriocapillaris following RPE degeneration (Korte et al., 1984) suggests the integrity of the RPE layer is necessary to

maintain structure and function of the underlying blood supply. Unlike biological responses observed in mammalian models, targeted ablation of zebrafish RPE resulted in degradation of Bruch's membrane, with gaps permeating the normally confluent structure, resulting in physical breakage in the BRB (Hanovice et al., 2019).

Typically, the confluent monolayer of RPE cells is held together through cadherins and epithelial junctional proteins, resulting in a rigid structure. Post-injury, there is a loss of the mature RPE marker, RPE65, as well as decreased cadherin and junctional protein, ZO-1, expression (Moriguchi et al., 2018; Hanus et al., 2016; Longbottom et al., 2009). These changes are often accompanied by a visible loss of cell-cell contact (Roider et al., 1992), dismantling the anchored monolayer. Most epithelial cells predominantly express E-cadherin (Halbieb and Nelson, 2006), but the dominant cadherin in RPE cells is disputed, as results differ between species and tissue origin (e.g. *in vivo* vs. *in vitro*). Recently, P-cadherin was found to be the dominant cadherin in physiological conditions in human and mouse RPE cells *in situ* (Yang et al., 2018). Following targeted oxidative stress, P-cadherin distribution decreases at RPE cell junctions and increases intracellularly. The localization of  $\beta$ -catenin follows that of P-cadherin, decreasing at cell-cell junctions and increasing in the nucleus, suggesting potential activation of the Wnt signaling pathway (Yang et al., 2018). Canonical Wnt/ $\beta$ -signaling has been implicated in injury responses and tissue regeneration in a variety of contexts (reviewed in Whyte et al., 2012) and may be activated post-RPE injury in attempts to repair the epithelial layer. As opposed to the typical hexagonal phenotype, RPE cells become rounded post-injury, which might signify functional changes, such as epithelial-mesenchymal-transition (EMT) (see the following section on chronic injury responses for further discussion of EMT). Several other signaling pathways have been proposed to be activated rapidly after an RPE insult, including PI3K/Akt (Liao et al., 2018; Yang et al., 2006), but more data are needed to understand the roles that these pathways play post-injury. Similarly, RPE cells responding to various modes of injury secrete signaling factors that can further modulate RPE or retinal physiology. One striking example of this is from experiments in the Royal College of Surgeons (RCS) rat model of photoreceptor degeneration, where subretinal injection of bFGF (FGF2) was shown to rescue photoreceptor survival (Faktorovich et al., 1990). However, in these experiments, the surgical needle control or injection of phosphate-buffered saline (PBS) also rescued photoreceptors, albeit to a lesser extent, suggesting that acute injury responses in the RPE triggered the release of trophic factors that affected surrounding tissues. In addition to FGF, other known RPE-derived factors, including PEDF, BDNF, and CNTF (discussed in section 2.3), have the potential to preserve photoreceptors when added exogenously to animal models of acute injury and inherited degeneration (Faktorovich et al., 1990; Cayouette et al., 1999; Imai et al., 2005; LaVail et al., 1992; LaVail et al., 1998; reviewed in Kolomeyer and Zarbin, 2014). Some of these factors are being pursued clinically as possible treatments for human patients with ocular disease (reviewed in Kolomeyer and Zarbin, 2014).

In physiological conditions, the apically-localized RPE receptors, integrin  $\alpha_5\beta_5$  and MerTK, promote phagocytosis of POS and recycling of their components; critically, this is achieved without initiating an inflammatory response (Finneman and Nandrot, 2006; Finneman and Rodriguez-Boulan, 1999). Likewise, the space between the RPE and the photoreceptors, called the subretinal space (SRS), is a zone normally devoid of immune cells due to the

stable resident microglia niche in the neural parenchyma, the presence of an outer BRB, and immunosuppressive factors secreted by the RPE (Karlstetter et al., 2015). There is evidence showing that accumulation of mononuclear phagocytes (MPs), including resident microglia and systemic monocyte-derived macrophages, occurs in several animal models of retinal and/or RPE damage (Sennlaub et al., 2013; Combadière et al., 2007; Leach et al., 2020; Moriguchi et al., 2018; O’Koren et al., 2019); however, the protective and/or pathogenic contribution of subretinal MPs appears to be context dependent. In the NaIO<sub>3</sub>-induced mouse injury model, pro-inflammatory macrophages have been shown to accumulate in the RPE layer, and depletion of macrophages by intraperitoneal injection of clodronate liposomes attenuated ONL thinning; however, macrophage depletion alone did not protect the RPE from damage (Moriguchi et al., 2018). While these observations may be specific to the NaIO<sub>3</sub> injury and/or macrophage depletion paradigms, variations in cell-specific requirements for macrophages and/or microglia post-injury are also possible. For instance, a recent study in mice revealed a potential RPE-protective feature of MPs in the SRS. Genetic depletion of microglia, which relocate toward the RPE layer in a model of photoreceptor degeneration, resulted in RPE disease-associated morphological defects, such as dysmorphic microvilli and disrupted interdigitation with retinal photoreceptors (O’Koren et al., 2019). Similarly, we have shown pharmacological and genetic perturbation of macrophages/microglia impaired RPE regeneration post-ablation in zebrafish (Leach et al., 2020). Collectively, these studies and others highlight the complex role subretinal MPs play in different ocular injury contexts. MicroRNAs (miRNAs) might also feed into the crosstalk between the immune system and injury/regenerative responses. Aging RPE appear to possess intrinsic protective mechanisms to maintain homeostasis; for example, miR-146a, was found to be upregulated in aged mouse RPE (Hao et al., 2016) and has been shown to repress pro-inflammatory signals (Kutty et al., 2013; Taganov et al., 2006). miRNAs could be potential targets to harness and deliver to damaged RPE to enhance repair and/or stimulate a regenerative response in mammals. Further understanding how signals from infiltrating MPs and miRNAs impact disease progression or attenuation will be critical for harnessing the therapeutic potential of the immune response in treating diseases involving the RPE.

Finally, the mechanism underlying RPE cell death can also provide insight into inflammatory responses and how they affect the RPE post-injury and conversely, how the inflammatory environment post-RPE injury could shape the degree of tissue damage and cell death. In a zebrafish RPE ablation model (see section 5.2 for details of this model), TUNEL staining revealed that programmed cell death began at 3 hours post-injury (hpi), peaking at 24hpi (Hanovice et al., 2019) and that macrophages and microglia infiltrated the injury site post-ablation (Leach et al., 2020), pointing to a potential reparative role of the immune system. Apoptosis is classically thought to be immunologically quiescent as the microenvironment surrounding apoptotic cells remains immunosuppressive through the release of anti-inflammatory cytokines, packaging of intracellular contents, and phagocytic cleanup of debris (reviewed in Campisi et al., 2014). However, other forms of cell death such as necroptosis and pyroptosis are associated with robust inflammation and diminish the regenerative potential of damaged cells. For example, in a model of RPE photooxidative damage where primary RPE cells were exposed to lipid-modified POS

then irradiated with blue light, inflammasome priming resulted in increased cell death by pyroptosis (Brandstetter et al., 2016), which is characterized by caspase 1 activation, membrane swelling, splitting and vesicle formation, and the release of pro-inflammatory cytokines (reviewed in Lamkanfi, 2011). Inflammatory cascades could easily counteract mechanisms that facilitate tissue regeneration and thereby stimulate fibrosis, scarring, or additional pathology. A recent study in mice demonstrated that RPE cells die through a regulated form of necrosis, or necroptosis, following damage from NaIO<sub>3</sub> injection, as demonstrated through the release of HMGB1, a necroptosis specific cytokine, and elevated RIPK3 expression, which is a downstream effector of a necroptotic response (Hanus et al., 2016). Although necroptosis shares features of apoptosis and necrosis, necroptosis results in the release of pro-inflammatory cytokines, priming a potent immune response (Dhuriya and Sharma, 2018). Other studies have suggested that there are additional mechanisms through which RPE cell death occurs, such as autophagy (Mitter et al., 2014). Indeed, it has been shown that autophagy might play an important role in physiological RPE cell death, as inhibition of autophagy results in accumulation of inflammatory monocytes contributing to pathology (Liu et al., 2016). It is important to note that the process of autophagy also has complex interactions with the immune system, where it contributes to leukocyte development and cytokine production, and may playing a critical role in perpetuating the pathogenesis of chronic inflammatory diseases (reviewed in Qian and Fang, 2017) Overall, the mechanisms underlying RPE cell death in human patients with ocular disease remain uncertain and are probably influenced by the mode of RPE injury or nature of RPE disease, amongst other variables. Regardless, the mechanism of RPE death could also contribute variable inflammatory responses and immune cell infiltration, which could then further affect disease progression as well as modulate the ability of the RPE to repair itself or regenerate.

### 3.2 Chronic injury responses

Failure to resolve inflammation and responses to RPE insult invariably results in functional deficits in vision, depending on the severity of the injury, and can lead to further degeneration and pathology. In the case of AMD, disease etiology is thought to be rooted in chronic, unresolved inflammation (reviewed in Hageman et al., 2001). Inflammation is multifaceted and characterized by complement activation, inflammasome assembly/activation, pro-inflammatory cytokine secretion, and MP recruitment, among other things (reviewed in Akhtar-Schäfer et al., 2018, Hageman et al., 2001; Ardeljan and Chan, 2013). Some of these characteristics (e.g. production of complement factors and MP accumulation and activation) are a consequence of normal homeostatic and aging processes (reviewed in Chen et al., 2019; Datta et al., 2017; Ardeljan and Chan, 2013). Another result of normal aging is the accumulation of extracellular drusen deposits between the RPE and Bruch's membrane; however, depending on their size and morphology, drusen are also a primary indicator of AMD (reviewed in Ardeljan and Chan, 2013). Complement components have been identified in drusen by proteomic and histological studies (Mullins et al., 2000; Crabb et al., 2002; Anderson et al., 2002; Johnson et al., 2000; Johnson et al., 2001; Hageman et al., 2005; reviewed in Toomey et al., 2018), and may exacerbate inflammation and expedite pathology by serving as a signal for MP recruitment, inflammasome activation, and subsequent secretion of pro-inflammatory cytokines (reviewed in Akhtar-Schafer et

al., 2018). Indeed, there is strong evidence showing that MPs accumulate in AMD lesions (reviewed in Guillonneau et al., 2017). As outlined in the acute injury responses (section 3.1), MP recruitment can be protective in some contexts, however, many studies have reported MPs contribute to pathology in AMD. For example, in donor eyes with AMD- and non-AMD-related CNV, stronger VEGF expression was found in lesions showing evidence of active inflammation (e.g. increased presence of macrophages); and some CNV-associated macrophages were the source of VEGF (Grossniklaus et al., 2002). It is thought that macrophages do not serve a reparative function in this context and instead may contribute to pathological blood vessel growth (Grossniklaus et al., 2002). Another study from humans showed activated microglia were present in the SRS and also the outer nuclear layer (ONL) of adult donor eyes presenting with a range of retinal degenerative diseases, including AMD (Gupta et al., 2003). Activated microglia contained membrane-bound rhodopsin, suggesting phagocytosis of rod photoreceptor debris; however, given the localization of microglia to regions of rod photoreceptor cell death, coupled with their capacity to produce cytotoxic secretions, the authors speculated that effects of activated microglia were likely deleterious, leading to further photoreceptor degeneration (Gupta et al., 2003). While evidence for the pathological role of immune-related components and signaling pathways is abundant, protective effects have also been shown. In a mouse model of laser-induced CNV, MP-specific genetic perturbation of interferon (IFN) signaling resulted in increased MP activation and angiogenic growth at the lesion site, while mice treated intravenously with IFN- $\beta$  showed improvements in resolution of laser lesions when compared to controls (Lückoff et al., 2016). IFN- $\beta$ -dependent neuroprotection has been shown in other disease contexts and may work through several downstream pathways to resolve inflammation (reviewed in Rashid et al., 2019). These immune-related components, some of which appear to exacerbate the progression of AMD, are likely inextricably linked (reviewed in Akhtar-Schafer et al., 2018), thus therapeutic intervention, like the roots of the disease, may require a multifaceted approach.

Proliferative responses are critical for tissue repair, but, similar to inflammation, can become pathological when left unresolved. This is the case in proliferative vitreoretinopathy (PVR), where damaged RPE cells lose contact with their neighbors and undergo EMT, a reversible process initiated by TGF- $\beta$  in which cells lose their epithelial morphology and shift to a mesenchymal-like phenotype (Miettinen et al., 1994; reviewed in Xu et al., 2009). Although EMT is necessary in a variety of contexts during early development and tissue formation, in PVR, RPE cells undergo type II EMT, where cell injury triggers RPE proliferation and fibrosis (reviewed in Dongre and Weinberg., 2019). EMT is a common sequela in other RPE diseases like AMD, where it is thought that RPE-derived mesenchymal cells contribute to subretinal fibrotic lesions in late stages of the disease (reviewed in Shu et al., 2020; Zhou et al., 2020). Disruption of cell-cell contact occurs during EMT to permit morphological changes and enable cell migration (Ozdamar et al., 2005). During EMT, the transcription factor Snail is often upregulated, which results in decreased expression of junctional proteins like occludins and claudin (Ozdamar et al., 2005; Ikenouchi et al., 2003). These changes lead to loss of apical-basal polarity within RPE cells and breakdown of the confluent RPE monolayer. The repression of E-cadherin is necessary for the onset of EMT and modulated through Zeb-1, Snail family transcription factors, and E47 (Liu et al., 2008; Cano et al.,

2000; Perez-Moreno et al., 2001). However, instances of type II EMT in PVR might require the repression of P-cadherin rather than E-cadherin as low levels of E-cadherin exist in human RPE cells (Yang et al., 2018). The repression of epithelial markers is accompanied by an increase in mesenchymal and myofibroblast gene expression, including N-cadherin, vimentin, and alpha-smooth muscle actin ( $\alpha$ -SMA) (Islam et al., 1996; Kim et al., 2000; Gilles et al., 1999; Kim et al., 2006).

During EMT, cells progressively reorganize intracellular actin filaments and front-rear polarity develops to enable migration (Ridley et al., 2003). These rounded cells migrate through the retina and invade the vitreous, where they settle, proliferate, and phenotypically appear as fibroblasts (Tamiya et al., 2010). Migratory RPE cells also deposit collagen and other ECM proteins that contribute to the formation of contractile scar tissue, leading to retinal detachment and vision loss (Morino et al., 1990). Basement membrane destruction and changes in ECM composition accompany the cellular changes, likely facilitating the events leading to pathology. After retinal injury or detachment, and during PVR, RPE cells can undergo EMT and contribute to the formation of an epiretinal membrane, which is a thin layer of fibrotic tissue overlying the retina (Wang et al., 2015; Tamiya and Kaplan, 2016; Boles et al., 2020). Numerous components of the immune system, including macrophages, microglia, thrombin, and T-helper cells contribute to the large inflammatory response that continues in the later phases of the RPE injury response (reviewed in Chaudhary et al., 2020). Downstream of these, cytokines, chemokines, and growth factors (including FGF and TGF- $\beta$ ) are released (reviewed in Chaudhary et al., 2020). Multiple *in vitro* studies highlight the role of TGF- $\beta$  signaling on EMT and promoting proliferation of the fibroblast-like RPE cells, and several recent studies have identified a novel role for miRNAs, including miR-124, miR-302d, and miR-93, in inhibiting TGF- $\beta$ -mediated EMT and the maintenance of fibroblastic phenotypes in cultured RPE cells (Jun and Joo, 2016; Fuchs et al., 2020). Importantly, transfection of miR-302d and miR-93 retransformed fibroblast-like RPE cells back to an epithelial-like state, regardless of TGF- $\beta$  exposure, as well as inhibited the TGF- $\beta$ -mediated secretion of VEGF (Fuchs et al., 2020). Beyond PVR, TGF- $\beta$ -mediated EMT also contributes to the pathogenesis of exudative AMD and VEGF-stimulated neovascularization. Thus, miRNAs could be novel therapeutic targets for PVR and AMD treatments. Finally, as discussed above, RA might also contribute to RPE proliferation and morphological changes during EMT. The lack of retinoid input due to retinal detachment could induce additional RPE cells to undergo EMT, or exacerbate EMT phenotypes (Campochiaro et al., 1991).

RPE proliferation holds many implications for understanding disease pathogenesis as well as the potential for RPE repair (reviewed in Stern and Temple, 2015). *In vitro* studies have extensively characterized RPE proliferation in a variety of contexts; however, support for the physiological relevance of many of these pathways *in vivo* is limited. Although adult RPE cells are known to be post-mitotic, RPE cells have the intrinsic capacity to re-enter the cell cycle in both injury and non-injury contexts (Hanovice et al., 2019; Grierson et al., 1994; von Leithner et al., 2010; Al-Hussaini et al., 2016; reviewed in Stern and Temple, 2015). Breakdown of Bruch's membrane is implicated in facilitating a proliferative response in RPE (Grierson et al., 1994). Lesions created in Bruch's membrane result in choroidal neovascularization (CNV), followed by RPE proliferation and a corresponding regression

of blood vessel growth, which suggests a possible link between RPE proliferation and amelioration of CNV (Miller et al., 1990; reviewed in Stern and Temple, 2015). RPE cell location within the eye might also be critical in modulating proliferative potential; for example, selective RPE ablation by low-energy laser-photocoagulation results in peripheral RPE re-entering the cell cycle to a larger extent than central RPE, regardless of the location of the lesion (von Leithner et al., 2010). Indeed, it has been demonstrated that peripheral RPE have a higher proliferative capacity than central RPE in a variety of contexts (Al-Hussaini et al., 2008; Kiilgaard et al., 2007; Kokkinopoulos et al., 2011). It could be that contact inhibition in central RPE prevents cell-cycle reentry, so peripheral RPE, which are more able to migrate to repair damaged tissue, become proliferative in response to injury. Co-localization of RPE-specific markers and cell-cycle markers also demonstrate that peripheral and equatorial RPE cells have the capacity to divide in contrast to central RPE (Al-Hussaini et al., 2008). In a zebrafish RPE ablation and regeneration model, proliferative cells were present in the injury site, first appearing in the peripheral RPE, and at later time-points, in the central RPE (Hanovice et al., 2019). Conceivably, protein expression levels also differ between peripheral and central RPE, as markers for cell proliferation, Wnt and BMP signaling, and cell cycle modulators are all upregulated in peripheral RPE cells under physiological conditions (Al-Hussaini et al., 2016). These and other studies highlight the dual significance of RPE proliferation: it can be detrimental to vision by contributing to EMT or it can potentially be beneficial, by contributing to the capacity to endogenously repair damaged RPE tissue.

#### 4. RPE AGING

Age-related changes of the RPE are physiological and on their own, do not denote pathogenesis; however, age-related changes might contribute to the development of disease states in the RPE (reviewed in Ardeljan and Chan, 2013). Aged rat RPE cells proliferate, form vacuoles, and possess abnormal basal infoldings in the peripheral eye *in vivo* (Fan et al., 1996; Lai et al., 1978). These changes, although not pathological, could contribute to increased susceptibility to developing ocular diseases, such as AMD (reviewed in Bonilha, 2008; Sarks, 1976). Lipofuscin is a lipid-derived pigment characteristic of aged RPE and exists as autofluorescent residual molecules from POS phagocytosis (reviewed in Bonilha, 2008; Kennedy et al., 1995). Clumping of lipofuscin bodies results in intracellular accumulation, increased oxidative stress, and melanin depletion through the disruption of antioxidation (reviewed in Bonila, 2008). Lipofuscin can contribute to autofluorescence visible in unconventional subretinal drusenoid deposits (Lee and Ham, 2014). Conventional drusen, discussed above, are located between the RPE and Bruch's membrane and are composed of lipoprotein deposits and complement factors that collect with age (reviewed in Khan et al., 2016); these are not shown to directly cause AMD, but rather pose a risk factor as extracellular deposits accumulate. Regardless, progressive build-up of debris can lead to RPE cell detachment and disruption of RPE cell junctions (reviewed in Al-Hussaini et al., 2008). While drusen and lipofuscin are often present in pathological contexts, both are visible in aged mouse eyes and considered a physiological component of the aging process (Liu et al., 2019b; Xu et al., 2008). Expression of the tight junction protein, ZO-1, decreases in aged mouse RPE (Chen et al., 2019), suggesting that RPE undergo morphological

changes throughout aging. Microglia also accumulate in the SRS of aging mice suggesting that they may be involved in aging or the progression of age-related diseases that affect the RPE (Xu et al., 2008; Combadière et al., 2007),

Management of oxidative stress could be compromised in aging RPE. Differential protein expression exists between young and aged rats, and fewer proteins that reduce oxidative stress are detected in aged RPE (Gu et al., 2012). RPE mitochondria in aged mice have elevated levels of glycolytic intermediates and decreased lipid levels, suggesting a decrease in mitochondrial efficiency and transition to fatty-acid metabolism (Wang et al., 2018). RPE cells rely on oxidative metabolism (reviewed in Lakkaraju et al., 2020), so any imbalance could affect normal function. Interestingly, aged monkey RPE have fewer mitochondria, and the ones that remain are abnormally elongated (Gouras et al., 2016). It has been previously shown that mitochondrial enlargement/elongation is associated with senescence and oxidative stress (Mai et al., 2010; Yoon et al., 2006), as aged RPE cells produce a high amount of reactive oxygen species (ROS) (reviewed in Bellezza, 2018). While ROS are required for redox signaling that is vital to RPE health, physiological levels can become pathogenic in aged RPE, although the transition point between maintaining RPE health and pathogenesis has yet to be determined (reviewed in Datta et al., 2017). Critical antioxidative enzymes in the RPE include members of the superoxide dismutase (SOD) family, catalase, and glutathione enzymes (reviewed in Newsome et al., 1994).

Results from rodent model systems corroborate many phenotypes observed in aged human RPE: accumulation of lipofuscin and drusen and enlarged RPE cell cytoplasm have all been reported (Feeney-Burns et al., 1984). *In vivo* studies also highlight the characteristic increase in Bruch's membrane thickness in older patients (Harris et al., 2017). Bruch's membrane thickens as a result of increased protein and lipid deposition and decreased degradation of ECM (reviewed in Zarbin, 2004). Bruch's membrane thickening leads to decreased permeability and flow of nutrients from the choriocapillaris to RPE, and decreased export of RPE waste, contributing to RPE stress. This phenotype seems to be largely limited to aging RPE (reviewed in Ardeljan and Chan., 2013) and has been reported in aging mouse and rat models (Ivert et al., 2005, Fan et al., 1996). However, some mammalian models of AMD have also reported increased thickness of Bruch's membrane as a result of disease progression (Seo et al., 2012; Ding et al., 2011). Nonetheless, Bruch's membrane thickening remains an aging phenotype, as advanced stages of AMD involve choroidal vasculature invasion through Bruch's membrane, resulting in Bruch's membrane thinning and deterioration (Bird et al., 1995).

Increased ratios of multinucleate RPE are observed in aged mice (Chen et al., 2016) and aged human eyes, specifically in the perifoveal region of the latter (Starnes et al., 2016). As discussed previously, the implications of multinucleation in human eyes are still under debate; multinucleation could contribute to enlarged cytoplasmic space to compensate for RPE cell loss. Together, these data suggest that mature mammalian RPE might maintain some proliferative potential, and if this ability could be harnessed, endogenous tissue repair might be possible.



## 5. REGENERATIVE RESPONSES IN RPE

As discussed above, a range of cellular and systemic responses are triggered by RPE injury, including signaling cascades (e.g. leading to EMT), changes in the cell biology of RPE and surrounding tissues, activation of an immune response, and RPE proliferation, among others. Resolution of these injury responses is critical to restoring tissue homeostasis, thus many of the same injury responses also contribute to RPE repair and regeneration. Here, we discuss what is known about RPE repair and regenerative responses in mammals and non-mammalian vertebrates, some of which possess remarkable abilities to regenerate retina and/or RPE after injury (Fig. 3).

### 5.1 RPE repair and regeneration in mammals

Mammalian RPE is normally non-self-renewing, but as discussed above, peripheral RPE cells do have intrinsic proliferative capacity in mice, rats, and pigs (Al-Hussaini et al., 2008; Kokkinopoulos et al., 2011; von Leithner et al., 2010; Kiilgaard et al., 2007). Indeed, RPE repair in mammals depends much on the severity of injury (Table 2). The RPE heals after debridement in animal models like pig and rabbit, but data suggest that only small lesions can be repaired by migration and proliferation of injury-adjacent RPE (Del Priore et al., 1995, Grierson et al., 1994, Lopez et al., 1995). In particular, in MRL/MpJ mice, a mammalian model with extraordinary regeneration abilities (Clark et al., 1998), robust RPE regeneration and full recovery of retinal function were observed one month after NaIO<sub>3</sub>-induced RPE injury at low-dose NaIO<sub>3</sub> injection, while little RPE regeneration occurred after high-dose NaIO<sub>3</sub> injection (Xia et al., 2011). Intravenous, low-dose NaIO<sub>3</sub> treatments have been shown to induce patchy RPE loss or central RPE damage with preservation of the peripheral RPE, while high-dose treatments cause complete RPE ablation and progressive retinal degeneration (Franco et al., 2009, Machali ska et al., 2010). This suggests that peripheral RPE are vital for repair and regeneration, but the roles that they play in this process remain to be defined. Consistently, Machali ska et al. (2014) observed complete RPE regeneration in mice 3 months post-injury in low-dose NaIO<sub>3</sub>-treated animals, while irreversible degeneration occurred at high doses (Machali ska et al., 2014). Transgene-mediated RPE ablation in mice has also been utilized to study RPE degeneration, repair, and regeneration. In adult RPE<sup>CreER</sup>/DTA mice, two weeks post-tamoxifen administration, surrounding RPE cells enlarged to fill space vacated by lost RPE but no proliferation or regeneration was observed (Longbottom et al., 2009). The RPE does have the potential to proliferate and regenerate, however, as subretinal injection of a viral vector containing E2F2 induced limited RPE proliferation and regeneration after genetic ablation in adult RPE<sup>CreER</sup>/DTA mice, indicating that mammalian RPE are capable of overcoming intrinsic barriers to regeneration (Kampik et al., 2017).

Interestingly, a study investigating the efficacy of human central nervous system stem cell (HuCNS-SC) replacement therapy in the RCS rat model of retinal degeneration found that host RPE proliferated when in proximity to transplanted HuCNS-SCs (McGill et al., 2012). Subsequent investigations demonstrated that proliferation occurred in RPE cells adjacent to HuCNS-SCs that were injected into the SRS of a rat model of retinitis pigmentosa, further supporting the ability of HuCNS-SCs to stimulate host RPE proliferation (McGill

et al., 2019). In humans, under physiological conditions, the RPE remains non-proliferative and RPE loss cannot be compensated by cellular regeneration. Nevertheless, when human cadaver-derived adult RPE cells were cultured *in vitro* with appropriate growth conditions, RPE cells proliferated and were able to generate an RPE monolayer, in addition to neural and mesenchymal cells types, suggesting the presence of an RPE stem cell in the human eye (Salero et al., 2012; reviewed in Saini et al., 2016).

## 5.2 RPE regeneration and RPE-dependent retinal regeneration in non-mammalian systems

The regenerative capacity of RPE has been studied in amniotes (mammals and birds) and anamniotes (amphibians and fish). Unlike in mammals, there is a range of regenerative responses in the RPE of non-mammalian systems. Most studies, however, have experimentally characterized the ability of the RPE to transdifferentiate into retina after retinal injury or retinectomy, so-called RPE-to-retina regeneration. By contrast, examples of intrinsic RPE regeneration (RPE-to-RPE) have thus far been limited to studies in zebrafish (Hanovice et al., 2019). Here we discuss what is known about RPE-dependent regenerative responses, both RPE-to-RPE and RPE-to-retina; for the latter, we focus on how it relates to the ability of RPE cells to reprogram, transdifferentiate, and repair retinal damage. Indeed, the inability of RPE to reprogram and transdifferentiate could be an intrinsic barrier to endogenous RPE regeneration in mammals. We also point interested readers to several excellent reviews that delve further into RPE-to-retina regeneration in non-mammalian systems (reviewed in Ail and Perron, 2017; Grigoryan, 2016; Araki, 2007; Chiba, 2014; Wang et al., 2010; Barbosa-Sabanero, 2012).

**Amniotes (Birds)**—RPE-to-retina regeneration has been extensively studied in the chicken, a non-mammalian amniote. Unlike the adult chicken, embryonic chicks can regenerate all of the retinal layers after retinectomy during a limited window in their development, around E4 (Hamburger and Hamilton stages 22–24.5) (Coulombre and Coulombre, 1965). The sources of regenerated retina are transdifferentiated RPE and the ciliary body/ciliary marginal zone (CB/CMZ) at the periphery of the retina (Coulombre and Coulombre, 1965, Spence et al., 2004). Members of the FGF family stimulate transdifferentiation of RPE cells through MEK–ERK signaling to induce retinal regeneration after retinectomy, while Shh and activin signaling may impair the transdifferentiation process (Park and Hollenberg, 1989; Park and Hollenberg, 1991; Spence et al., 2004; Spence et al., 2007; Sakami et al., 2008). During transdifferentiation, chick RPE cells lose their epithelial characteristics, depigment, and proliferate. RPE-derived cells form a neuroepithelial layer that then differentiates into retinal tissue (Spence et al., 2007; Spence et al., 2004). The transdifferentiated RPE regenerates a retina with reversed polarity which occurs primarily in the posterior part of the OC (Coulombre and Coulombre, 1965). As the retinal layers are reversed, the rod and cone photoreceptors are located in the innermost layer of the retina, closest to the lens. Thus, the RPE transdifferentiates to form a retina, but does not self-regenerate, resulting in an eye that lacks the RPE layer (Coulombre and Coulombre, 1965). However, regenerated retinal tissue derived from the CB/CMZ develops with correct polarity and in the presence of a pigmented RPE layer (Luz-Madrigal et al., 2014; Luz-Madrigal et al., 2020; Spence et al., 2004). Based on these studies, intrinsic

RPE-to-RPE regeneration cannot be studied in chicks, at least using current models and injury paradigms.

## Anamniotes (Amphibians and Fish)

### Amphibians:

*Urodele (Newt)* –: Newts are capable of regenerating a completely functional retina after traumatic eye injury or retinectomy. In adult newts, the primary source of regenerating retina is RPE cells while retinal stem cells and retinal progenitor cells present in the CMZ regenerate only peripheral aspects of the retina (Chiba et al., 2006, Ikegami et al., 2002). In the adult Japanese fire-bellied newt, *Cynops pyrrhogaster*, RPE cells reprogram after retinectomy to produce RPE cells and all cell types of the retina (Chiba et al., 2006; Islam et al., 2014). While definitive lineage tracing has not been performed in this system, the source of regenerating retinal cells in the posterior eye of *C. pyrrhogaster* is likely to be the RPE as the peripheral retina is removed during retinectomy.

The molecular mechanisms underlying the ability of RPE cells to transdifferentiate and re-enter the cell cycle after retinal injury have been studied *in vitro* and *in vivo* and are beginning to be understood. Upon retinectomy, RPE cells reprogram and transdifferentiate in a process that involves detachment from each other and from Bruch's membrane, loss of epithelial characteristics, and reentry into the cell cycle (Stage E-1; between days 5 and 10 post-retinectomy) (Chiba et al., 2006). In amphibians, it is thought that upon injury, mature RPE cells re-enter the cell cycle due to loss of cell-cell contacts (reviewed in Chiba, 2014). As discussed above, in humans and other mammalian models, loss of RPE cell-cell contact contributes to EMT and subsequent pathology, suggesting that events downstream of cell-cell contact attenuation could also contribute to the different RPE repair/regenerative responses in amphibians and mammals. The MEK–ERK pathway has also been shown to play a role in re-entry of mitotically quiescent RPE cells into the cell-cycle both *in vivo* and *in vitro*. MEK–ERK pathway activity is rapidly upregulated (within 30 min) after retinectomy in RPE cells (Mizuno et al., 2012).

During RPE reprogramming and transdifferentiation, RPE cells enter a multipotent state (defined as RPE stem cells; RPESC) (Islam et al., 2014). RPESCs uniformly express the neuronal stem cell marker, Musashi-1 (Kaneko and Chiba, 2009), multipotency factors *c-Myc* and *Klf4*, and early eye development markers such as *Sox2*, *Pax6*, and *Mitf* (Islam et al., 2014); however, RPESCs maintain RPE65 protein expression (Chiba et al., 2006). Transcriptome analyses of early transdifferentiating RPE cells (Stage E-0 to E-2) also demonstrated that the expression of genes associated with differentiated RPE cells such as *RPE65*, *CRALBP/RLBP1*, *ZO1*, *Otx2*, and *Musashi1a/c* gradually decrease between E-0 and E-2 post-retinectomy, while genes associated with cell-cycle progression such as *Cyclin D1*, *CDK4*, *Histone H3*, and those associated with growth factor signaling like *FGFR1* and *FGFR3* are upregulated (Nakamura et al., 2014). RPESCs subsequently re-differentiate to form two epithelial layers that have correct polarity and partial pigmentation, a “pro-RPE layer” and “pro-retina layer”, which renew the RPE and regenerate the retina, respectively (Islam et al., 2014). *Pax6* appears to play a critical role during RPE cell reprogramming. RPESCs from juvenile newts (around 7 months old) in which *Pax6* was knocked down were

unable to self-organize into two epithelial layers and did not differentiate into pro-retina or pro-RPE layers; instead, RPESCs underwent EMT and formed myofibroblasts, similar to what occurs in RPE injury and disease in mammals (Casco-Robles et al., 2016; Chaudhary et al., 2020; Yang et al., 2018). The pro-RPE layer exits the cell cycle and reinitiates pigmentation, while the pro-retina layer continues to proliferate (Stage E-3; day 19 post retinectomy). RPE65 immunoreactivity decreases in both layers at Stage E-3 but increases as the pro-RPE layer matures. Between days 45 and 65 after retinectomy, regeneration is complete and the regenerated retina and RPE have identical morphology and characteristics to those in intact eyes (Islam et al., 2014).

The mechanisms underlying RPE cell proliferation and cell cycle re-entry have also been studied *in vitro* using a retina-less eyecup (RLEC) culture system (Susaki and Chiba, 2007). In the RLEC system, under serum-free culture, the retina is removed from the posterior half of the eyeball and after 10 days in culture, RPE cells become mitotically active and behave as they do *in vivo* during retinal regeneration (Yoshikawa et al., 2012). In RLEC, MEK-ERK signaling is activated within 1 hour post-retinectomy (Yoshikawa et al., 2012) and  $\beta$ -catenin-positive nuclei increase in RPE cells when cell-cell contacts are disrupted by incision or treatment with ethylene glycol tetraacetic acid (EGTA) (Yasumuro et al., 2017), similar to what is observed in mouse RPE after  $\text{NaIO}_3$  injection (Yang et al., 2018). Presumably,  $\beta$ -catenin nuclear translocation contributes to transcriptional regulation of injury-responsive genes in RPE cells, but this has not yet been addressed experimentally. *In vitro* studies also demonstrate that interaction between the RPE and choroid are likely to play a critical role during RPE-to-retina regeneration. Under organotypic culture conditions, explanted adult RPE and surrounding connective tissue, including the choroid, were co-cultured to understand mechanisms involving RPE proliferation and transdifferentiation (Ikegami et al., 2002). When the choroid was included in these organ cultures, RPE transdifferentiated into retinal tissue, but not when the choroid was absent (Mitsuda et al., 2005). FGF2 and IGF-1, amongst other factors, were shown to be involved in this inductive process.

**Anurans (Frogs) –:** Adult *Xenopus laevis* (3 to 9 months after metamorphosis), regenerate their retina after retinectomy through RPE-to-retina transdifferentiation, like newts (Yoshii et al., 2007; reviewed in Ail and Perron, 2017). RPE-to-retina transdifferentiation during regeneration is not uniform across frog species; however, as *Xenopus tropicalis* regenerates its retina after retinectomy from the CMZ through a process that does not involve RPE transdifferentiation (Miyake and Araki, 2014). In *X. laevis*, RPE transdifferentiation only occurs if the retinal vascular membrane (RVM) (consisting of capillary rich basal membrane bounding the inner margin of the retina) is retained after retinectomy (Yoshii et al., 2007). After retinectomy, RPE cells migrate to the RVM, which facilitates RPE transdifferentiation, although the inducing factors and molecular mechanisms present in the RVM are not fully understood. However, retinectomy that also includes removal of the RVM, followed by FGF2 induction, successfully results in a regenerated retina indicating that FGF2 is sufficient in this regard (Vergara and Del Rio-Tsonis, 2009). FGF2 activates the MEK-ERK pathway and similar to newt RPE-to-retina regeneration (Yoshikawa et al., 2012), the MEK-ERK pathway is activated during the early stages of the regenerative response.

Moreover, inhibition of MEK signaling significantly impairs RPE-to-retina regeneration in frogs (Vergara and Del Rio-Tsonis, 2009), again highlighting the potential importance of MEK-ERK activity in modulating RPE-derived injury and regenerative responses.

Upon retinectomy, it is Pax6-positive pigmented RPE cells that detach from Bruch's membrane and migrate towards the RVM (Kuriyama et al., 2009; Yoshii et al., 2007). At the RVM, RPE-derived cells undergo transdifferentiation, become stem-cell-like, and form a neuroepithelium (Kuriyama et al., 2009). Loss of cell-cell contact between RPE cells and underlying Bruch's membrane results in upregulation of Rax expression followed by Pax6 expression (Martinez-De Luna et al., 2011; Nabeshima et al., 2013). The Pax6-positive RPE-derived epithelial layer transdifferentiates into retinal tissue along the RVM. RPE cells that remain attached to Bruch's membrane are Pax6-negative and subsequently repair and renew the RPE itself; however, the molecular mechanisms underlying this process are not known (Kuriyama et al., 2009; Yoshii et al., 2007). Loss of contact between RPE cells and the choroid appears to play a role in triggering Pax6 expression. RPE cells become *Pax6*-positive once they migrate away from choroid, although the choroid could also be inductive in this regard (Kuriyama et al., 2009). FGF2 is essential for sustained Pax6 expression, as it promotes differentiation of RPE-derived proliferating progenitors into retina (Kuriyama et al., 2009). In comparison, in humans and other mammals, loss of contact between RPE cells and the choroid triggers EMT, leading to PVR (reviewed in Chiba, 2014).

**Zebrafish** –: Zebrafish have a robust intrinsic potential to regenerate multiple tissue and organ types after damage, including the retina (reviewed in Goldman, 2014; Karl and Reh, 2010; Gao et al., 2021). Recent work demonstrates that both larval and adult zebrafish are also able to fully regenerate RPE after widespread RPE injury (Hanovice et al., 2019). Experimentally, a transgenic system was developed in zebrafish through which large swathes of mature RPE could be ablated and regeneration assessed (Fig. 4). RPE ablation resulted in rapid apoptosis of RPE, followed by degeneration of photoreceptors (Fig. 5) and Bruch's membrane, and loss of visual function (Hanovice et al., 2019). After ablation, the RPE regenerates in a peripheral-to-central manner (Fig. 6), driven by proliferative cells that first appear in the periphery and over the duration of the regenerative response are detected more centrally as the injury site is repopulated (Fig. 7). The cellular source of the regenerated RPE is not yet known but two candidates are peripheral RPE that is spared from ablation or cells of the CMZ, which are known to generate both retina and RPE progenitor cells (Reinhardt et al., 2015).

This zebrafish RPE ablation and regeneration paradigm is a powerful *in vivo* system through which the molecular underpinnings of endogenous RPE regeneration can be identified. Wnt signaling was shown to play a role in RPE regeneration as the Wnt target gene *lef1* was upregulated post-ablation and disruption of Wnt pathway activity reduced proliferation post-injury and impaired the regenerative response (Fig. 8; Hanovice et al., 2019). Interestingly, Wnt/ $\beta$ -catenin signaling was activated after laser photocoagulation in RPE cells of the mouse eye and the Wnt targets, *Otx2* and *Mitf*, were upregulated (Han et al., 2015). Moreover, inhibition of Wnt pathway activity impaired RPE cell proliferation and EMT, suggesting that Wnt activity may be a common mechanism involved in RPE proliferation and repair. Indeed, nuclear  $\beta$ -catenin accumulates in RPE cells in mouse and newt models

when RPE cell-cell adhesions are perturbed supporting a potential role in the injury and RPE-to-retina transdifferentiation pathways, respectively, in these systems (Han et al., 2015, Yasumuro et al., 2017). Results in the chicken, however, indicate that  $\beta$ -catenin in the RPE suppresses the ability of RPE to regenerate retinal tissue after retinectomy (Zhu et al., 2014), so there are also context-dependent roles for Wnt/ $\beta$ -catenin activity in various models of RPE injury and regeneration that could modulate cellular responses. Finally, recent genomic analyses of the regenerative response in zebrafish identified a number of immune-related signaling pathways activated in RPE after injury and a direct role for macrophages and microglia as key mediators of intrinsic RPE regeneration (Leach et al., 2020). While much more needs to be done in this system, these early results point to an interplay between components of the immune system and RPE after injury and during regeneration, a link that has also been made during retinal regeneration (Silva et al., 2020; White et al., 2017) as well as regeneration in other tissues and organs (reviewed in Mescher, 2017; Mescher et al., 2017).

## 6. MECHANISMS INVOLVED IN REPROGRAMMING AND REGENERATION

A common theme in non-mammalian systems that possess the capacity to regenerate lost or damaged tissues is the reprogramming of differentiated cells to a progenitor-like state, which then proliferate and regenerate lost tissue (Tai et al., 2020; Fang et al., 2018). Reprogramming refers to the process through which a differentiated somatic cell is converted into a multipotent or pluripotent state, so called induced pluripotent stem cells (iPSCs) (Gurdon, 1962; Takahashi and Yamanaka, 2006), or converted directly into an unrelated terminally differentiated cell type (reviewed in Srivastava and DeWitt, 2016; Fang et al., 2018). The reprogramming process has been studied extensively in many cell types and organs (reviewed Chen et al., 2020), including the retina, where Müller glia of zebrafish and *Xenopus* are able to regenerate damaged retinal neurons (reviewed in Lahne et al., 2020; Garcia-Garcia et al., 2020). Comparatively less is known about reprogramming in the RPE, but recent studies in the chicken have begun to shed some light on this process during RPE-to-retina regeneration (Luz-Madrigal, et al., 2014; Luz-Madrigal, et al., 2020). Here, the differential expression of genes associated with epigenetic modification, histone markers, and DNA methylation/demethylation in reprogramming-competent RPE before injury and at different phases post-retinectomy was quantified. Significant changes of bivalent chromatin marks (decrease of H3K27me3 and increase of H3K4me3) and in DNA demethylation activity during early phase of reprogramming of intact RPE to transient reprogrammed RPE were detected, as well as during late phases of reprogramming of RPE to neuroepithelium in response to retinectomy and FGF2 addition. Ten-eleven translocation 3 (TET3), one of the TET family of enzymes that catalyze DNA hydroxymethylation, and potentially contributing to DNA demethylation, was found to be significantly upregulated at 24hpi in RPE cells. Overexpression of TET3 was sufficient to reprogram RPE by 3 days post-retinectomy without the addition of exogenous FGF2, suggesting that DNA hydroxymethylation and/or demethylation might play a role in the reprogramming of RPE cells during RPE-to-retina transdifferentiation (Luz-Madrigal, et al., 2014; Luz-Madrigal, et al., 2020). Consistently in zebrafish, DNA demethylation events predominate at early times during MG reprogramming and fin regeneration after retinal injury or fin amputation,

although the functional importance of these changes remains unknown (Hirose et al., 2013, Powell et al., 2013).

## 7. BARRIERS TO RPE-DEPENDENT REGENERATION

As discussed above, newts, *X. laevis*, zebrafish, and chickens are regeneration-competent non-mammalian systems in which RPE have the capacity to regenerate lost retina or RPE tissue. In newts, *X. laevis*, and chickens, RPE undergo reprogramming and transdifferentiation after retinal injury to regenerate lost retinal tissue. However, RPE-to-RPE regeneration either does not occur (in chickens) or is limited to solely replenishing RPE cells that undergo transdifferentiation to regenerate retinal tissue (in *X. laevis* and newts). Zebrafish do not regenerate retinal tissue from the RPE after injury, instead doing so through Müller glia, but they are able to intrinsically regenerate RPE through what appears to be an RPE-to-RPE dependent process. Reprogramming and transdifferentiation of RPE are not observed in mammalian systems and RPE-to-RPE or RPE-to-retina regeneration do not occur, with the exception of in the MRL/MPJ mouse when peripheral RPE cells are spared from injury and are therefore able to repair minor RPE lesions (Xia et al., 2011). What then can we learn about RPE regeneration from these regeneration-competent systems that could identify potential barriers to RPE-to-RPE or RPE-to-retina regeneration in mammals and, most importantly, in humans suffering from ocular degenerative diseases? Three potential barriers to regeneration in mammals are: i) differences in the activities or functions of pro-regenerative cell-cell signaling pathways and proliferative potential of RPE cells; ii) epigenetic barriers to reprogramming; and iii) differing pro- and anti-regenerative functions of the immune system.

### 7.1 Differences in cell-cell signaling pathway activities and proliferative potential

MEK-ERK signaling is required for RPE-to-retina regeneration in newts (Mizuno et al., 2012) and chickens (Spence et al., 2007). MEK-ERK signaling is involved in other regenerative processes in non-mammalian vertebrates, such as cardiac regeneration after heart resection in zebrafish (Liu and Zhong, 2017), blastema formation after limb amputation in *X. laevis* (Suzuki et al., 2007), and muscle (myotube) formation in cultured salamander cells (Yun et al., 2014). In the salamander *in vitro* myotube culture system, MEK-ERK signaling was activated in response to serum stimulation, and sustained ERK activity was found to be necessary for reprogramming and cell cycle re-entry of salamander myotubes. In contrast, only transient activation of ERK signaling, was observed and no change of proliferative genes like cyclins and PCNA were found in a murine myogenic cell line, which was unable to dedifferentiate or regenerate upon serum stimulation (Yun et al., 2014). These data suggest that the duration or magnitude of MEK-ERK activation could influence the regenerative process and that this differs between mammals and regeneration-competent animals like salamanders. In the eye, retina, or RPE, injury may stimulate MEK-ERK activity and thereby enable or enhance the reprogramming and/or proliferative responses in RPE cells in these regeneration-competent animals, but not in mammals, which do not regenerate.

FGF and Wnt/ $\beta$ -catenin signaling also play roles in RPE-dependent regenerative responses in regeneration-competent non-mammalian vertebrates. FGF is known to function in maintaining pluripotency in a variety of stem cells (reviewed in Mossahebi-Mohammadi et al., 2020) and in facilitating regenerative responses in a number of organ systems and animal models (reviewed in Maddaluno et al., 2017). In the eye, pro-regenerative roles include during Müller glia-derived retinal regeneration in chickens (Fischer et al., 2002) and zebrafish (Wan et al., 2014), rod regeneration in zebrafish (Qin et al., 2011), and lens regeneration in *X. laevis* (Fukui and Henry, 2011), amongst others, in addition to its role during the RPE-to-retina transdifferentiation process. In mammals, FGF2 secretion is elevated in human iPSC-derived RPE cells in an *in vitro* scratch assay (Greene et al., 2016) and FGFs are upregulated in mouse and rat retina after mechanical injury (Cao et al., 1997; Cao et al., 2001). Interestingly, FGF2 addition to culture medium enables human RPE stem cell self-renewal *in vitro* (Salero et al., 2012). Although the ability of FGF activation to stimulate human RPE stem cell proliferation *in vivo*, or in response to injury, has yet to be established, given the role of FGF in facilitating regenerative responses in the eyes of many regeneration-competent non-mammalian systems, this is an interesting avenue for future studies. Wnt/ $\beta$ -catenin signaling is required for zebrafish RPE-to-RPE regeneration (Hanovice et al., 2019); however, in mammals, Wnt/ $\beta$ -catenin activity is linked to pathological proliferation and EMT in PVR (Han et al., 2015; reviewed in Tamiya and Kaplan, 2016). Moreover, in chicken,  $\beta$ -catenin negatively regulates RPE-dependent retina regeneration (Zhu et al., 2014), so  $\beta$ -catenin roles in modulating RPE-dependent repair and regenerative processes are also context dependent, differing between regeneration-competent non-mammalian systems and mammals, which do not regenerate.

Taken together, these data suggest species-specific differences in signaling pathway activity during the injury and regenerative responses. One common thread in these pathways is the downstream activation of proliferative responses in RPE cells. Some of these pathways are also activated in mammalian RPE after injury; however, they do not stimulate proliferative responses that contribute to repair or regeneration. Rather, RPE cells that do proliferate after injury often undergo EMT and lead to additional pathology. Thus, the cellular response to signaling pathway activity also differs between regeneration-competent and regeneration-incompetent animals. With that in mind, one approach towards enhancing the regenerative ability of mammalian RPE might be to modulate pro-regenerative signaling pathways that are either inactive in injured mammalian RPE or only modestly activated upon injury, or to somehow modulate the proliferative responses of mammalian RPE cells in such a way that pushes them away from EMT and towards a pro-regenerative response. While this strategy may be appealing, it is however not likely to stimulate a regenerative response *de novo* after injury and would most certainly have to be combined with parallel strategies to reprogram RPE cells towards a pro-regenerative state.

## 7.2 Epigenetic barriers to reprogramming:

Direct reprogramming of somatic cells has gained traction as a potential mechanism to stimulate regenerative responses in non-regenerating cells and tissues, including the retina (Yao et al., 2018). RPE-to-retina transdifferentiation in chickens and newts is an *in vivo* exemplar of the direct reprogramming process whereby differentiated RPE cells



reprogram after retinectomy and differentiate into retinal neurons; as such, it can serve as a useful model through which physiologically relevant mechanisms that modulate cell reprogramming can be identified. One such mechanism is the epigenetic regulation of gene expression, through which genes required for reprogramming and/or repair and regeneration are held in an epigenetically silenced state in mammals and therefore unable to respond to injury. Studies in the chicken support this notion and identified significant changes in bivalent chromatin marks and in DNA demethylation activity in RPE cells during RPE-to-retina regeneration post-retinectomy (Luz-Madrigal, et al., 2014; Luz-Madrigal, et al., 2020). Beyond this, no studies have been reported that identify changes in the expression of genes associated with epigenetic modifications or the modifications themselves during RPE-to-RPE or RPE-to-retina regeneration in other regenerating systems. However, a recent study characterized the epigenetic profile of adult mouse RPE cells using ChIP-seq and whole-genome bisulfite sequencing, and data therein provide some insight into potential epigenetic constraints in mammals that might limit the regenerative capacity of RPE (Dvorianchikova et al., 2019). In this study, a large portion of genes associated with optic vesicle development, retinal progenitor cells, and Müller glia possessed chromatin in an open/active state in adult RPE and their surrounding genomic regions were unmethylated or hypomethylated. Genes associated with retinal neuron functions were in a repressed chromatin state, with the exception of photoreceptor genes, which were in a permissive state, but associated with methylated regions of the genome. Taken together, these data suggest that RPE cells could be stimulated to express retinal neuron genes, but this would still require them to first reprogram and transdifferentiate.

As mentioned above, direct reprogramming of somatic cells is an exciting approach for stimulating regenerative responses in normally non-regenerative tissues, but there are as-yet no reports of the efficacy of this approach in the RPE. However, also as noted above, viral delivery of E2F2 can induce limited RPE proliferation and regeneration after genetic ablation in mice, supporting the notion that mammalian RPE are capable of overcoming intrinsic barriers to regeneration (Kampik et al., 2017). As noted by Dvorianchikova et al., (2019), it could be that the addition of specific pioneer transcription factors to RPE cells that open the repressive chromatin state, and/or mechanisms that promote epigenetic changes at pro-regenerative loci after injury, could be an effective strategy to stimulate a regenerative response in mammals. Indeed, such a strategy has proven effective based on knowledge of the regenerative response in zebrafish Müller glia where a number of key regulators of reprogramming have been identified, including *Ascl1*, a master regulator of neuron specification (Lahne et al., 2020; Wan and Goldman, 2016). *Ascl1*, when expressed in mammalian Müller glia in culture or *in vivo*, stimulates the production of new neurons from the Müller glia (Pollak et al., 2013; Ueki et al., 2015). Moreover, this effect can be dramatically enhanced *in vivo* when combined with a histone deacetylase inhibitor (Jorstad et al., 2017), highlighting the potential role for epigenetic regulation of the regenerative process, as well as the likelihood that combinatorial approaches will be needed to simulate such responses from normally non-regenerative cell types. The ability of zebrafish to endogenously regenerate RPE after widespread injury also presents a unique opportunity to identify epigenetic factors that regulate this process, which may also be potential targets for modulation in non-regenerating mammals. Here, the process may be even simpler in the

sense that no transdifferentiation is required; RPE need only to be stimulated to proliferate again, differentiate, and re-establish epithelial architecture. It will be of interest to identify the epigenetic signature of RPE cells in zebrafish and mammals before and after injury, as well as to screen for potential epigenetic regulators whose expression changes in response to injury, as these factors could be good targets for further studies.

### 7.3 Differences in immune responses:

It has been postulated that the evolutionary complexity of the immune system is a direct contributor to the capacity for an organism to regenerate tissues; wherein animals with primitive immune responses (e.g. teleosts and amphibians) are robust regenerators and phylogenetic acquisition of a sophisticated immune system (as in mammals) comes at the cost of losing the ability to regenerate damaged tissues (reviewed in Aurora and Olson, 2014; Mescher and Neff, 2005; Mescher et al., 2017). Some immune-related elements thought to contribute to these differences include the composition of immune cells at the injury site and the duration, strength, and specificity of the immune/inflammatory response (reviewed in Aurora and Olson, 2014; Mescher and Neff, 2005; Mescher et al., 2017). A stunning example of this exists in *X. laevis*, where larvae are able to undergo epimorphic regeneration (where new tissues regrow from a blastema) of limbs and tails at early developmental stages, but regenerative capacity decreases as animals approach metamorphosis (reviewed in Mescher and Neff, 2005; Mescher et al., 2017). Importantly, in this context, anuran amphibians at metamorphosis undergo a complete restructuring of the immune system, which increases in complexity and mature lymphocyte pools when compared to the immune system present at larval stages (reviewed in Rollins-Smith, 1998). Adult anurans (e.g. *Xenopus*) are part of the earliest evolutionary class of vertebrates capable of canonical antibody class switching (CSR), a DNA recombination mechanism also present in mammals that improves the diversity and effectiveness of antibody responses; this and other components of post-metamorphic immunity are considered analogous to the mammalian response, but like mammals, these animals are unable to achieve epimorphic regeneration (reviewed in Flajnik, 2018; Robert and Ohta, 2009; Stavnezer and Amemiya, 2004; Stavnezer et al., 2008). Pre-metamorphic larval anurans possess epimorphic regeneration capacity but have poor CSR and weaker lymphocyte responses than their adult counterparts (reviewed in Robert and Ohta, 2009; Mescher and Neff, 2005). Comparatively, zebrafish (which are phylogenetically “lower” than amphibians) are epimorphic regeneration-competent as adults, for example, regeneration of the tail fin (Poss et al., 2000), but are incapable of canonical CSR and may be more reliant on innate rather than adaptive immunity (reviewed in Iwanami, 2014; Lewis et al., 2014). In *X. laevis*, the switch from epimorphic regeneration competence to incompetence occurs at a stage in development (stage 57) where T cell markers become expressed; likewise, at stage 57 post-hindlimb amputation, markers of inflammation resolution persist, and markers indicative of tissue patterning are decreased in comparison to younger (stage 53) epimorphic regeneration-competent larvae (Mescher et al., 2013). Indeed, chemical induction of inflammation in stage 53 larvae recapitulated stage 57 expression patterns (e.g. persistent inflammatory markers and loss of blastema patterning markers) and epimorphic regeneration capacity was compromised (Mescher et al., 2013). Similarly, *X. laevis* possess a transient loss of regenerative capacity during development, termed the “refractory period” (stages

45–47), and this capacity can be restored by chemical immune suppression (Fukazawa et al., 2009), indicating that dampening the immune/inflammatory response in some contexts may be permissive to regeneration.

The above examples demonstrate a link between modulating the immune response to affect regenerative capacity, and also highlight how acute inflammation may present a barrier to regeneration in animals with highly evolved immune responses. Other examples exist in fetal mammalian tissues, such as skin, which is able to undergo scar-free wound healing early in development and the transition to fibrotic wound healing (scarring) appears to occur with maturation of elements of the immune response (reviewed in Mescher and Neff, 2005; Colwell et al., 2005). In the adult human eye, chronic inflammation has been linked to AMD as leukocytes (Gupta et al., 2003) and immune-related components have been identified in patient tissues (reviewed in Guillonnet al., 2017). Indeed, immunosuppression has been assessed as an adjuvant therapy for AMD treatment (Nussenblatt et al., 2010). As onset of RPE degenerative diseases such as AMD can be difficult to pinpoint and tissue loss may be significant by the time of diagnosis, dampening inflammation in conjunction with stimulating pro-regenerative factors may be a feasible therapeutic approach to restoring RPE in mammals.

## 8. FUTURE DIRECTIONS

Herein we have summarized known mechanisms involved in RPE injury, regenerative capacity of the RPE in mammalian and non-mammalian model systems, and discussed likely barriers to RPE regeneration in mammals. Additionally, we have highlighted an existing theme in regenerative biology, which is that utilizing regeneration-competent non-mammalian organisms to understand and identify pro-regenerative cues is a viable translational strategy for treating degenerative diseases, like AMD, in mammalian systems incapable of robust tissue regeneration. In the context of RPE-based treatments, specifically, this approach is feasible and may only require stimulating the intrinsic regenerative capacity of a subset of RPE to greatly impact severity of vision loss and quell disease progression. Indeed, with each foveal RPE cell supporting many cones in the human fovea (e.g. ~16:1 (Granger et al., 2018), ~19:1 (Liu et al., 2017), and ~24:1 (Gao and Hollyfield, 1992) reported cone-to-RPE ratios), minimal RPE regeneration or even maintenance of existing RPE without further degeneration could lead to sufficient photoreceptor preservation to, in turn, preserve central visual acuity and patient quality of life. Looking to the future, modulating pro-RPE regenerative cues in humans could be complementary to stem cell-based RPE and/or retina replacement therapies in patients with advanced disease and tissue loss, or used in combination with other therapeutic approaches, such as immune suppression or anti-VEGF treatments (in the case of exudative AMD), which may help promote a regeneration-permissive microenvironment in the eye.

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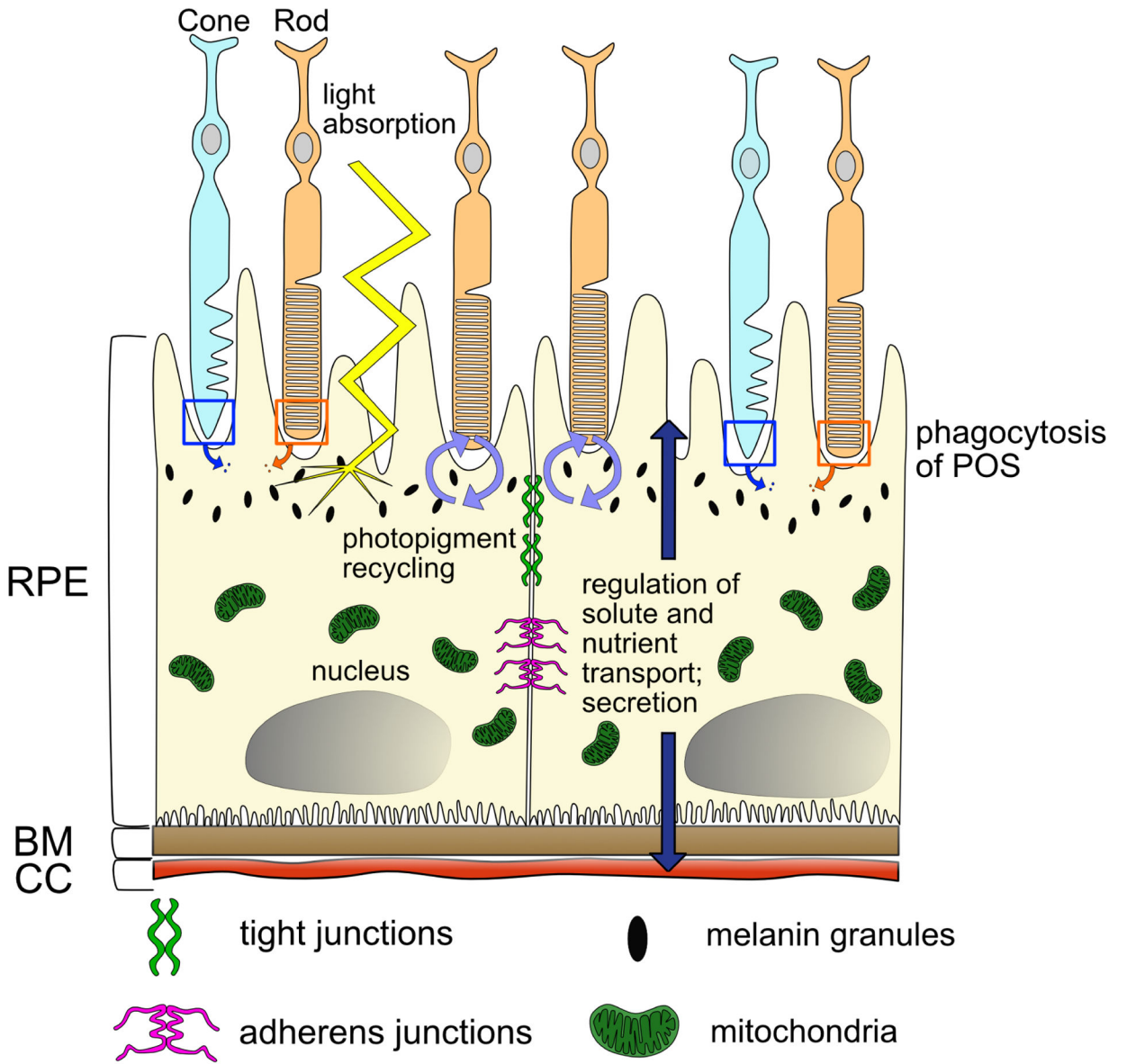
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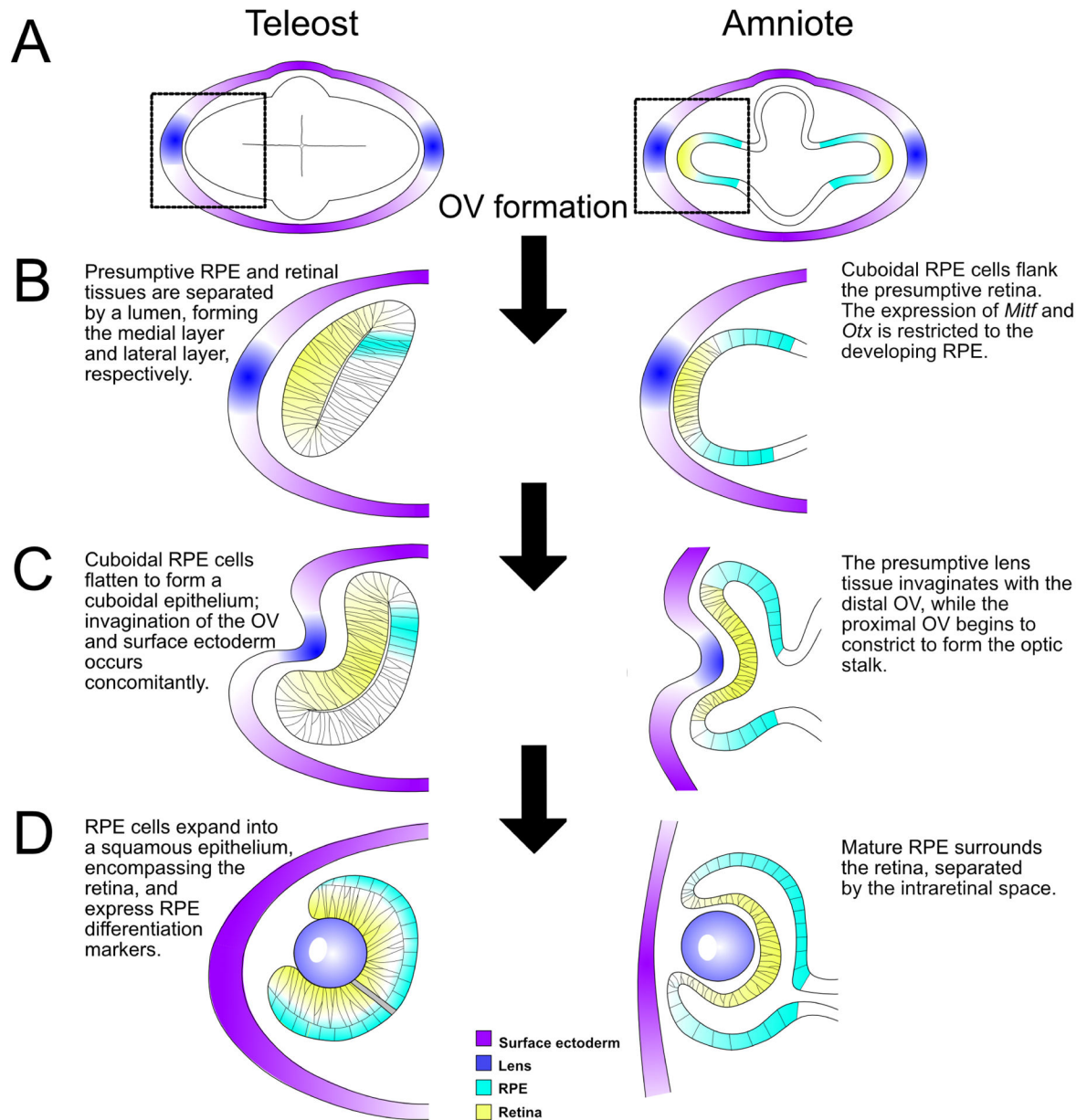
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**Figure 1. RPE biology and generalized functions.**

Cartoon illustrating the mature, polarized RPE monolayer and its interactions with the rod and cone PRs, BM, and CC, as well as the diversity of functions mediated by the RPE.

Abbreviations: PRs, photoreceptors; RPE, retinal pigment epithelium; CC, choriocapillaris; BM, Bruch's membrane; POS, photoreceptor outer segments.



**Figure 2. Eye morphogenesis in amniotes and teleosts.**

(A) In teleosts (e.g. zebrafish and medaka) and amniotes (e.g. mammals, birds), the OVs evaginate from the presumptive eye field in the forebrain. Enlargement of the left OV (*black dashed boxes, A*) to highlight subsequent steps of eye development (**B-D**). (B) OV elongation occurs posteriorly in teleosts, where RPE progenitors occupy a small portion of the medial (dorsal) layer as a pseudostratified epithelium. In amniotes, the OV extends outward, and the future RPE cells are cuboidal and occupy the region surrounding the presumptive retina. (C) Invagination of the surface ectoderm and distal OV occurs simultaneously; RPE in both teleosts and amniotes are cuboidal epithelial cells. (D) Eye morphogenesis concludes with the formation of a bilayered optic cup: RPE cells in teleosts are squamous and directly surround/interdigitate with the retina with no intervening space,

while RPE cells in amniotes remain cuboidal and interdigitate apically with the retina, but remain separated from it by the intraretinal space, which disappears before birth. Abbreviations: OV, optic vesicle; RPE; retinal pigment epithelium.

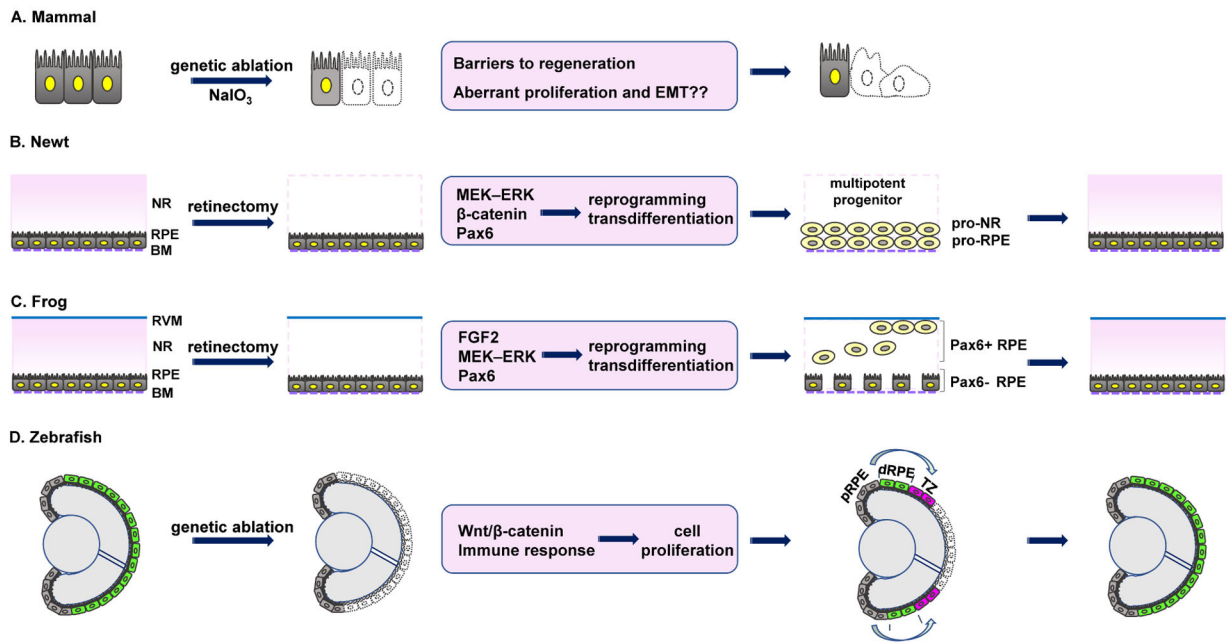
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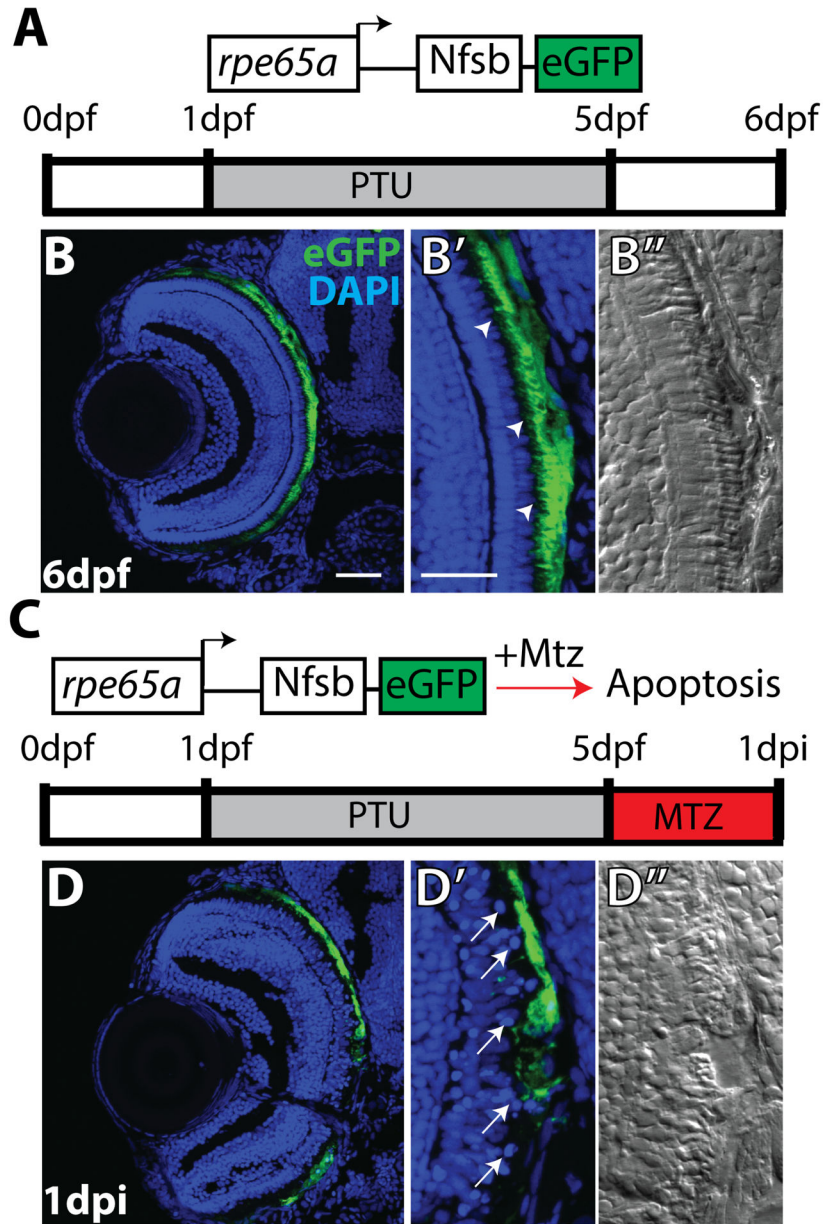
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**Figure 3. RPE-dependent repair and regeneration in mammalian and non-mammalian systems.** Models of repair and regenerative responses in mammals (**A**); the newt, *Cynops pyrrhogaster* (**B**); the frog, *Xenopus laevis* (**C**); and zebrafish (**D**). (**A**) In mammals, after transgenic ablation or pharmacological injury of the RPE, remaining RPE cells are unable to regenerate. (**B**) In adult *C. pyrrhogaster*, upon retinectomy RPE cells undergo reprogramming, re-enter the cell cycle, and convert into a multipotent state through the regulation of MEK/ERK/ $\beta$ -catenin and Pax6 signaling after retinectomy. The multipotent cells segregate into two layers; the outer layer renews the RPE while the inner layer regenerates NR. (**C**) In adult *X. laevis*, upon retinectomy, with RVM present, RPE cells detach from each other and BM, express Pax6, and migrate towards the RVM to regenerate NR. RPE cells that remain attached to BM replenish the RPE layer. (**D**) In zebrafish, *rpe65a:nfsB-eGFP*-mediated transgenic ablation of large swathes of RPE results in the proliferation of injury-adjacent RPE cells that subsequently regenerate lost RPE tissue in a peripheral to central fashion. Abbreviations: NR, neural retina; BM, Bruch's Membrane; RVM, retinal vascular membrane; RPE, retinal pigment epithelium; pRPE, peripheral retinal pigment epithelium; dRPE: dedifferentiated retinal pigment epithelium; TZ: transition zone. (**B**, modified from Chiba, 2014; **D**, modified from Hanovice et al., 2019).



**Figure 4. RPE ablation paradigm in zebrafish:**

(A) Cartoon depicting the *rpe65a:nfsB-eGFP* transgene and treatment course of unablated embryos. (B) Transverse cryosections of an unablated 6 dpf larva. (B,B') After exposure to PTU between 1–5 dpf, transgene expression is specifically restricted to mature RPE cells, with the brightest expression confined to the central two-thirds of the RPE. Arrowheads indicate apical microvilli. (B'') DIC images reveal RPE repigmentation and normal photoreceptor layer architecture. (C) Cartoon depicting the nitroreductase-mediated ablation paradigm: after washing out PTU, larvae were treated with MTZ for 24 hours. Within cells expressing the transgene, nfsB converts MTZ into a potent DNA crosslinking agent and induces cell death. (D,D') Transverse cryosections of a 1 dpi larva reveal significant disruption of eGFP<sup>+</sup> cell morphology and disorganization in INL nuclear lamination.

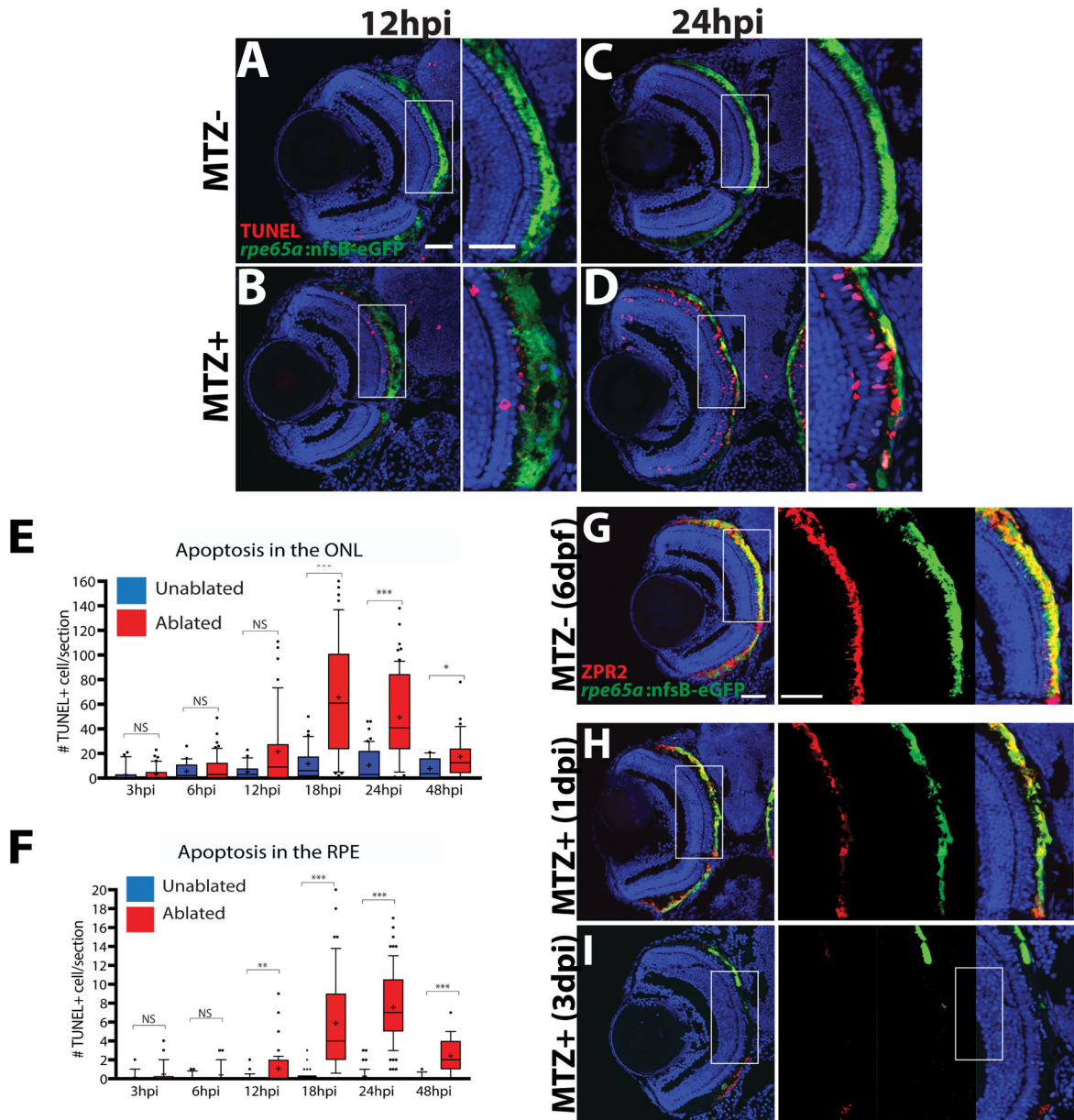
Arrows indicate delaminated and pyknotic nuclei. **(D'')** DIC images reveal a lack of RPE pigmentation and the marked disruption of photoreceptor layer architecture. Abbreviations: dpf, days post-fertilization; PTU, phenylthiourea; RPE, retinal pigment epithelium; MTZ, metronidazole; nfsB, nitroreductase; dpi, days post-injury; INL, inner nuclear layer. Green=eGFP, blue=nuclei. Dorsal is up and distal is left. Scale bar = 40mm. Figure taken from Hanovice et al., 2019.

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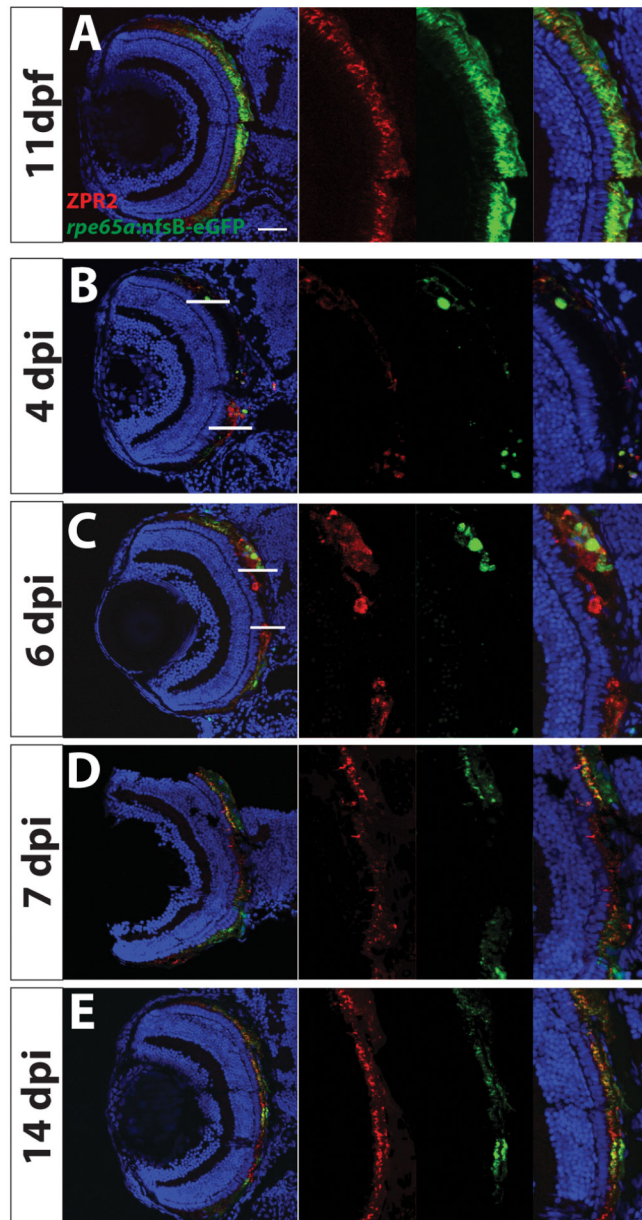
blue=nuclei. Dorsal is up and distal is left. Scale bar = 40mm. Figure modified from Hanovice et al., 2019.

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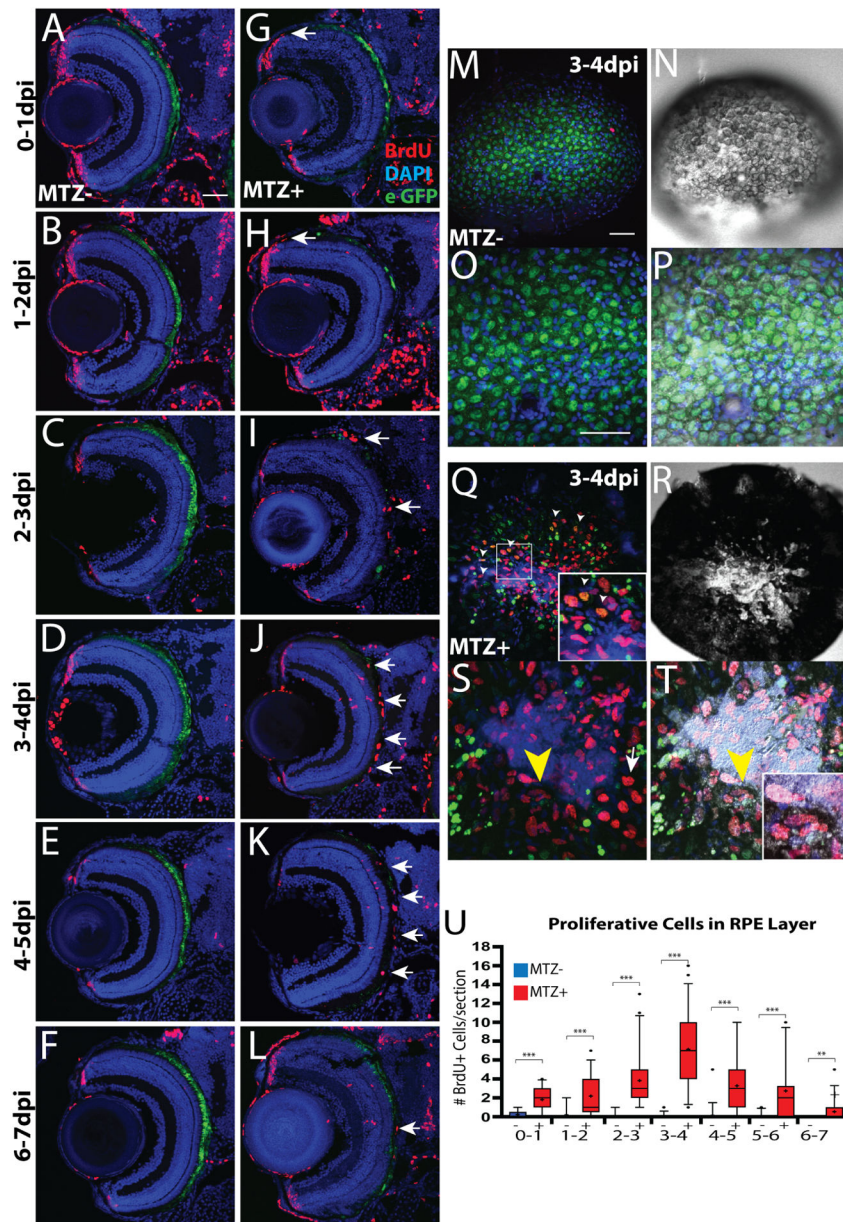
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**Figure 6. RPE regeneration in zebrafish initiates in the periphery and proceeds inward.** Transverse sections of unablated larvae stained for the RPE marker ZPR2 (A) at 11 dpf. Ablated eyes stained for ZPR2 at 4, 6, 7 and 14 dpi (B-E). Green=eGFP, blue=nuclei, red=ZPR2. eGFP<sup>+</sup> RPE appears in the periphery at 4dpi (marked by lines at dorsal and ventral margins in B,C). As regeneration proceeds, eGFP<sup>+</sup> RPE extends further toward the eye center, and the leading tip of the regenerated monolayer often consists of both immature and mature RPE (ZPR2<sup>+</sup>/eGFP<sup>-</sup> cells in C). By 7dpi, ZPR2<sup>+</sup> RPE is present throughout the RPE (D). By 14dpi, mature eGFP<sup>+</sup>/ZPR2<sup>+</sup> RPE cells are present throughout the RPE (E). Dorsal is up and distal is left. Abbreviations: RPE, retinal pigment epithelium; dpf, days post-fertilization; dpi, days post-injury. Scale bar = 40mm. Figure modified from Hanovice et al., 2019.



**Figure 7. RPE regeneration in zebrafish involves a robust proliferative response.** (A-L) Transverse retinal sections of unablated (A-F) and ablated (G-L) larvae exposed to 24-hour BrdU pulses at various days post-injury. BrdU<sup>+</sup> cells first appear in the periphery as early as 0–1dpi (arrow, G), and 1–2dpi (arrow, H). As regeneration proceeds, BrdU<sup>+</sup> cells appear closer to the central injury site and at the inner tip of the regenerating RPE layer (arrows, I). BrdU<sup>+</sup> cells then populate the injury site (arrows, J-L). (M-T) *en face* wholemount images of unablated (M-P) and ablated (Q-T) eyes from larvae exposed to BrdU between 3–4dpi. White arrowheads in (Q) and (inset, Q) indicate BrdU<sup>+</sup>/eGFP<sup>+</sup> cells near the injury site. Yellow arrowhead in (S) and (T) indicate BrdU<sup>+</sup> cells proximal to the injury site that are beginning to become pigmented. (Inset, T) Magnified image of BrdU<sup>+</sup>, pigmented cells. (U) Quantification of total number of BrdU<sup>+</sup> cells/section in

the RPE reveals an increase of BrdU<sup>+</sup> cells in the RPE starting at 0–1dpi and peaking at 3–4dpi. Mann-Whitney U Test, \* p<0.05, \*\* p<0.005, \*\*\* p<0.0005. Dorsal is up and distal is left. Abbreviations: dpi, days post-injury; RPE, retinal pigment epithelium; BrdU, bromodeoxyuridine. Scale bar = 40mm. Figure modified from Hanovice et al., 2019.

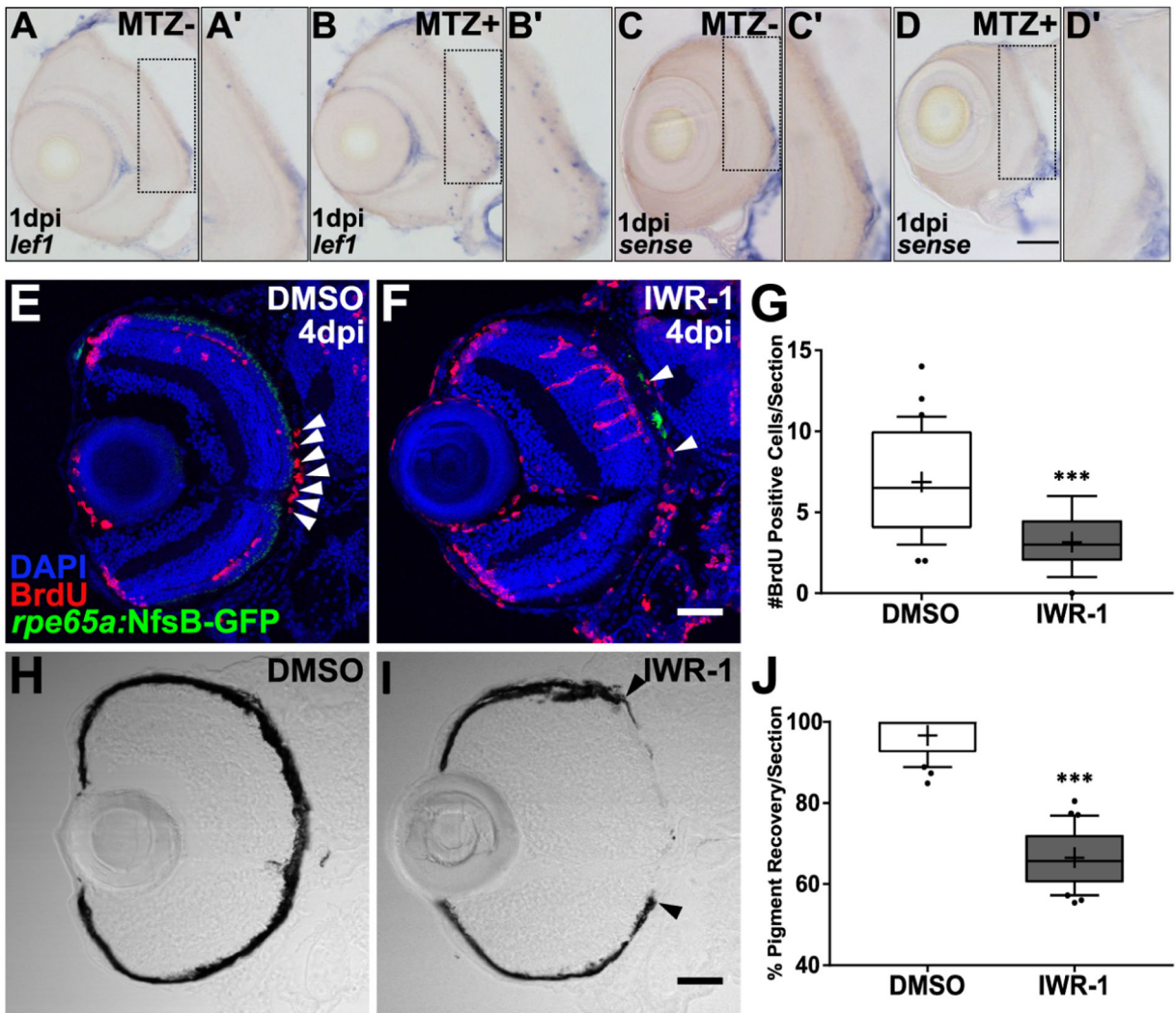
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**Figure 8. Pharmacological inhibition of Wnt pathway activity using IWR-1 impairs RPE regeneration in zebrafish.**

(A-D) Transverse sections of *lef1*, a marker of Wnt pathway activity, or sense RNA expression in unablated 6dpf (MTZ-) and ablated 1dpi (MTZ+) larvae. *lef1* is detected in and around the RPE in MTZ+ (B') but not MTZ- larvae (A'). *lef1*:  $n>5$ ; *lef1* sense:  $n=4$ . (E-J) Transverse sections of 4dpi ablated DMSO- (E,H;  $n=10$ ) and 15 $\mu$ M IWR-1-treated (F,I;  $n=11$ ) larvae exposed to a 24-hour pulse of BrdU from 3–4dpi. (E,F) Green=eGFP, blue=DNA, red=BrdU; white arrowheads highlight BrdU+ cells in the RPE. (G) Quantification of BrdU+ cells/section reveals that IWR-1 treatment significantly decreases the number of proliferative cells in the RPE at 4dpi (Student's unpaired t-test, \*\*\*  $p<0.0001$ ). Brightfield images (H,I) and quantification of percent RPE recovery/section (J) shows a significant delay in recovery of a pigmented monolayer in IWR-1 treated larvae (Student's unpaired t-test, \*\*\*  $p<0.0001$ ). (I) Black arrowheads indicate the central-most edge of the regenerating RPE. Abbreviations: dpf, days post-fertilization; MTZ, metronidazole; dpi, days post-injury; RPE, retinal pigment epithelium; BrdU,

bromodeoxyuridine; DMSO, dimethylsulfoxide. Dorsal is up and distal is left. Scale bars = 40 $\mu$ m. Figure modified from Hanovice et al., 2019.

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Table 1.

## RPE injury responses

Injury type	Model System	Biological Response	References
NaIO <sub>3</sub> injection	Mice	Loss of pigmentation, RPE cell enlargement, vacuolization, loss of cell-to-cell contact	Hanus et al., 2016
NaIO <sub>3</sub> injection	MRL/MPJ mice	Loss of RPE65 expression	Xia et al., 2011
NaIO <sub>3</sub> injection	Rabbit	Loss of pigmentation, apical microvilli, basal infolds	Korte et al., 1984
NaIO <sub>3</sub> injection	Rat	Loss of apical microvilli, basal infoldings, and tight junctions	Liu et al., 2019a
Genetic ablation	Zebrafish	Loss of pigmentation, shortening of apical microvilli, BM thinning and degradation	Hanovice et al., 2019
Genetic ablation	Mice	RPE cell enlargement, abnormal shape	Longbottom et al., 2009
Selective photocoagulation of RPE	Rabbit	Loss of cell-to-cell contact, RPE cell enlargement, vacuolization	Roider et al., 1992
Adeno-associated viral injection of Rz432	Mice	RPE cell enlargement, multinucleation, vacuolization	Seo et al., 2012
APOE-4 targeted replacement with high-fat, cholesterol-enriched diet	Mice	RPE cell enlargement, multinucleation, vacuolization	Ding et al., 2011
Ceruloplasmin/Hephaestin knock-out (KO)	Mice	Multinucleation	Hadziahmetovic et al., 2008
RPE debridement	Pig	Abnormal cell morphology, loss of pigmentation, formation of mono- or multi-layered epithelium apical to BM	Del Priore et al., 1995
Photic maculopathy	Monkey	Morphologically disrupted pigment granules, vacuolization, RPE pyknosis, migration to subretinal space, loss of pigmentation, loss of basal infoldings, RPE proliferation, abnormal morphology	Tso, 1973
Retinectomy	Chick	Loss of epithelial characteristics and pigmentation, proliferation	Coulombre & Coulombre, 1965
Retinectomy	Frog	Partial loss of pigmentation and BM detachment	Yoshii et al., 2007
Retinectomy	Newt	Loss of epithelial characteristics and pigmentation, BM detachment, RPE cells gain multipotency	Chiba et al., 2006
Traction retinal detachment	Rabbit	Pigmented granules on retinal surface	Cleary & Ryan, 1979a
Traction retinal detachment	Rabbit	RPE proliferation, formation of epiretinal membrane, partial loss of pigmentation, abnormal morphology	Cleary & Ryan, 1979b

Abbreviations: RPE, retinal pigment epithelium; BM, Bruch's membrane; NaIO<sub>3</sub>, sodium iodate

Table 2.

## RPE-dependent repair and regenerative responses

Injury Type	Model System	Age	Regenerative Ability		RPE regenerative Responses	References
			RPE-to-RPE	RPE-to-Retina		
Genetic RPE ablation	Zebrafish	Larvae and adult	yes	no	Lose epithelia characteristic; RPE proliferates in a peripheral to central fashion to regenerate a functional RPE layer	Hanovice et al., 2019
	Mouse	Infants and adult	no	no	Regenerative capacity in RPE <sup>Cx36</sup> ER/DTA mice was not observed.	Longbottom et al., 2009
NaIO <sub>3</sub> injection	Mouse	Adult	yes	no	Low-dose NaIO <sub>3</sub> treated mice display RPE regeneration.	Machalinska et al., 2014; Machalinska et al., 2010
	Mouse	Adult	yes	no	RPE regenerates with complete retinal function upon low dose of NaIO <sub>3</sub> in MRL/MpJ mice.	Xia et al., 2011;
Retinectomy	Newt	Juvenile and adult	yes	yes	Lose epithelial characteristic; RPE cells enter a multipotent state, form “pro-RPE layer” and “pro-NR layer” and subsequently regenerate NR and RPE	Chiba et al., 2006; Islam et al., 2014
	Frogs	Adult	yes	yes	Transdifferentiated RPE cells migrate onto RVM to generate NR, RPE cells that remain attached to BM renew RPE layer	Yoshii et al., 2007; Kuriyama et al., 2009
E2F2 injection using lentiviral vector	Chicken	E4-4.5	no	yes	Transdifferentiated RPE does not produce RPE cells	Coulombre & Coulombre, 1965
	Mouse	Juvenile and adult	limited	no	Limited RPE proliferation and regeneration	Kampik et al., 2017

Abbreviations: RPE, retinal pigment epithelium; NR, neural retina; DTA, diphtheria toxin subunit A, NaIO<sub>3</sub>, sodium iodate; BM, Bruch's membrane