Published in final edited form as:

Exp Eye Res. 2014 June ; 0: 141–150. doi:10.1016/j.exer.2013.09.003.

Retinal pigment epithelium development, plasticity, and tissue homeostasis (Invited review for *Experimental Eye Research*)

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Abstract

The retinal pigment epithelium (RPE) is a simple epithelium interposed between the neural retina and the choroid. Although only 1 cell-layer in thickness, the RPE is a virtual workhorse, acting in several capacities that are essential for visual function and preserving the structural and physiological integrities of neighboring tissues. Defects in RPE function, whether through chronic dysfunction or age-related decline, are associated with retinal degenerative diseases including age-related macular degeneration. As such, investigations are focused on developing techniques to replace RPE through stem cell-based methods, motivated primarily because of the seemingly limited regeneration or self-repair properties of mature RPE. Despite this, RPE cells have an unusual capacity to transdifferentiate into various cell types, with the particular fate choices being highly context-dependent. In this review, we describe recent findings elucidating the mechanisms and steps of RPE development and propose a developmental framework for understanding the apparent contradiction in the capacity for low self-repair versus high transdifferentiation.

Keywords

RPE; development; regeneration; retina; transdifferentiation; age-related macular degeneration; self-repair

1. Overview of early eye development and RPE specification

To truly understand RPE development, it is important to begin at the steps leading to the formation of the eye field. Like the neural retina, the RPE is a derivative of the optic neuroepithelium, which is initially specified as a patch of cells, the “eye field”, in the anterior neuroectoderm. Some progress has been made on resolving the mechanistic underpinnings of these early developmental steps, in large part because they provide insight into how the forebrain is initially organized. In the following subsections, we describe the

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recent findings describing the mechanisms and steps leading to the specification and early differentiation of the RPE.

1.1. Regulation of anterior neuroectodermal fate

Eye development is initiated by the formation of a single eye field in the anterior neural plate, comprised of neuroepithelial cells that give rise to the optic stalk, retina and RPE. The eye field arises within the future forebrain region that develops into telencephalon and diencephalon but it is unclear how its formation is initiated. FGF, BMP, Wnt and retinoic acid signaling exert multiple roles during gastrulation and early neural development; however, the complex morphogenetic movements make it challenging to examine the exact role of these pathways in specification of the anterior neural plate, including the eye field.

Observations from different model systems suggest that anterior neuroectodermal fate is a ubiquitous state, and that specification of posterior neuroectoderm requires additional signals to prevent spreading of anterior fate across the neural plate. Wnt/ β -catenin signaling is one such signal, acting at different threshold requirements to elicit distinct morphogenetic responses in different brain regions (for reviews, see (Andoniadou and Martinez-Barbera, 2013; Beccari et al., 2013; Fuhrmann, 2008; Wilson and Houart, 2004). In rostral regions, several mechanisms exist to maintain anterior fate, such as the presence of antagonists of the Wnt/ β -catenin pathway (e.g. Dkk1, sFRP) or direct suppressors of Wnt ligand transcription (e.g. the homeodomain transcription factor Six3 in the eye field). Consequently, loss of Wnt/ β -catenin inhibition leads to suppression of forebrain development, resulting in eyeless or headless embryos in the most severe cases (Kim et al., 2000; Lagutin et al., 2003). Studies in sea urchins suggest a potential mechanism by which non-canonical Wnt signaling through the receptors Frizzled-1/-2/-7 and JNK induces the Wnt/ β -catenin antagonist Dkk1 and is required for expression of Six3 in the anterior neural plate (Range et al., 2013). Consistent with this, non-canonical Wnt signaling is required for repression of Wnt/ β -catenin signaling and for promoting expression of eye-specific genes during eye field formation in frog and zebrafish (Fig.1; (Cavodeassi et al., 2005; Kibardin et al., 2006; Maurus et al., 2005; Rasmussen et al., 2001); for review, see (Fuhrmann, 2008). Thus, it is possible that the anterior neural fate is specified as a “default state” with an initially more widespread expression of Six3 and the paired transcription factor Rx in the rostral forebrain (Mathers et al., 1997; Muranishi et al., 2012; Oliver et al., 1995).

Further fine-tuning of the forebrain into different territories requires additional signaling centers or organizers. During establishment of the eye field in zebrafish, BMP acts as an instructive signal to pattern the anterior-most part of the neural plate into telencephalon and represses Rx3 inhibiting eye field formation (Fig.1; (Bielen and Houart, 2012). BMP may be also provided from the paraxial rostral mesoderm to restrict the eye field; experimental and functional manipulations in chick embryos demonstrate that BMP signaling mediates the inhibitory effect of the paraxial rostral mesoderm on optic vesicle formation, potentially through differential modulation of distinct Wnt pathways (Teraoka et al., 2009).

Another potential mechanism for regionalization of the forebrain could be that different target genes in distinct brain regions are regulated by specific stoichiometric ratios of transcriptional activators such as Six3, the orthodenticle-related transcription factor Otx2

and the high mobility group transcription factor Sox2 (Beccari et al., 2012; Danno et al., 2008). Recent studies in frog indicate that both Otx2 and Sox2 proteins can directly and synergistically interact to transactivate a conserved, non-coding sequence upstream of the Rx promoter (Danno et al., 2008). Rx may not be upregulated in the brain outside of the eye field because the ratio of Sox2 and Otx2 is too high or too low for transcriptional activation of Rx; for example, excess Sox2 can inhibit transcription cooperatively induced by Sox2 and Otx2 (Danno et al., 2008). Therefore, these and other mechanisms may tightly control gene expression levels, resulting in distinct target gene activation in different brain regions.

1.2. Formation of the eye field

The eye field is characterized by the restricted expression of a specific combination of key transcription factors, the eye field transcription factors (EFTFs) that include Rx, Pax6, Six3, Six6, and Lhx2 (Fig.1; (Bailey et al., 2004; Zuber et al., 2003). Fate mapping studies in *Xenopus* and zebrafish reveal that the cells in the eye field move in a complex and highly coordinated pattern to generate retina, RPE and lens (England et al., 2006; Kwan et al., 2012; Lee et al., 2006; Moore et al., 2004). These morphogenetic movements are guided by interactions with surrounding tissues and lead to evagination of the optic vesicles; for example, the underlying mesendoderm acts through sonic hedgehog to guide eye field progenitor cells laterally, resulting in separation of the eye field into two domains and to suppress Pax6 expression in the midline (Li et al., 1997; Macdonald et al., 1995). Ephrin and PCP/JNK pathways regulate movement of progenitors cells into the eye field (Lee et al., 2006). The EFTFs exert several important functions; for example, Rx (RAX in humans, Rx3 in zebrafish), is required for changes in cell shape and segregative behavior of eye field progenitor cells through Nlcam and Cxcr4, it controls proliferation, and it maintains non-canonical Wnt signaling and expression of eye-specific genes (Bielen and Houart, 2012; Brown et al., 2010; Cavodeassi et al., 2005; Chuang et al., 1999; Deschet et al., 1999; Mathers et al., 1997; Ohuchi et al., 1999; Svoboda and O'Shea, 1987; Voronina et al., 2004); for review, see (Fuhrmann, 2010; Muranishi et al., 2012).

Another important EFTF is Six3, which, as mentioned above, directly suppresses expression of Wnt1 and Wnt8b (Fig.1; (Lagutin et al., 2003; Liu et al., 2010). Loss of Six3 at the late eye field stage results in defective patterning of the optic vesicle by ectopic activation of the Wnt/ β -catenin pathway and, consequently, the whole optic vesicle develops into RPE (Liu et al., 2010). In contrast, active Wnt/ β -catenin signaling is required later in the optic cup for RPE differentiation in mouse and chick (see below), or specifically in frog to promote proneural fate and retinal progenitor proliferation (Agathocleous et al., 2009).

A distinct mechanism for promoting formation of the EFTFs was discovered recently in frog. A physiological screen for bioelectric patterns revealed the existence of bilateral hyperpolarized cell clusters in the anterior neural plate in *Xenopus* (Pai et al., 2012). Depolarization of the transmembrane voltage potential in eye progenitors interferes with expression of Pax6 and Rx1, while forced hyperpolarization in areas outside of the neural plate results in ectopic eye induction. It needs to be shown, however, how hyperpolarization

translates into induction of gene expression and whether this mechanism is conserved across species.

1.3. RPE specification

Following eye field evagination and optic vesicle formation, complex tissue interactions guide specification and differentiation of retina, RPE, optic stalk, and lens. The distal portion of the optic vesicle invaginates upon contact with the overlying surface ectoderm, leading to formation of the optic cup. The overlying ectoderm invaginates, pinches off as a vesicle and differentiates into the lens. Proper specification of the optic stalk is dependent on interaction with the adjacent ventral neuroectodermal and surrounding mesenchymal tissue. The inner layer of the optic cup receives signals from the overlying surface ectoderm to differentiate into the neural retina. The outer layer interacts with the surrounding extraocular mesenchyme and differentiates into the RPE. Two morphological features that distinguish the RPE domain from the remainder of the optic cup is the switch from a pseudo-stratified to a monolayer epithelial arrangement and the appearance of pigment granules. Both properties are acquired by E11.5 in mice and are the first overt features of ocular differentiation that are maintained in the mature eye. A failure of the presumptive RPE domain to initiate or retain these properties is interpreted as a failure of RPE specification, which is oftentimes accompanied by a transdifferentiation into neural retina (discussed in more detail below). The RPE is essential for ocular growth and morphogenesis of the eye as well as for proper retinal differentiation in many aspects (Bumsted and Barnstable, 2000; Martinez-Morales et al., 2001; Nasonkin et al., 2013; Nguyen and Arnheiter, 2000; Pearson et al., 2005; Raymond and Jackson, 1995; Scholtz and Chan, 1987; Tibber et al., 2007) for reviews, see (Bharti et al., 2006; Fuhrmann, 2010; Martinez-Morales et al., 2004; Strauss, 2005).

Among the earliest molecular indicators of RPE specification is the expression of the bHLH-leucine zipper transcription factor microphthalmia associated transcription factor (Mitf; (Hodgkinson et al., 1993). Mitf directly transactivates genes for terminal pigment differentiation and RPE functions such as tyrosinase, tyrosinase-related protein, dopachrome tautomerase, melanosomal proteins and the Ca-dependent chloride channel protein bestrophin. Loss of Mitf function in the embryonic mouse RPE leads to pigmentation defects throughout the RPE, while a restricted region in the dorsal RPE hyperproliferates and transdifferentiates into retina (Bumsted and Barnstable, 2000; Nguyen and Arnheiter, 2000). Mitf also transactivates expression of miRNAs, and the dynamic regulation of miRNA expression is strongly associated with RPE differentiation; e.g. miR-204 and miR-211 (Adijanto et al., 2012; Hu et al., 2012; Li et al., 2012; Wang et al., 2010a).

Onset of Mitf expression occurs during the early optic vesicle stage, initially throughout the vesicle (~E9.0 in mouse; (Liu et al., 2010; Yun et al., 2009). Mitf downregulation in the presumptive neural retinal domain depends on the expression of the retinal homeodomain transcription factor Vsx2, which comes on at the late optic vesicle stage, just prior optic cup formation (~E9.5 in mouse; (Liu et al., 1994; Yun et al., 2009). The current evidence supports a mechanism in which Vsx2 regulates Mitf through direct transcriptional repression (Bharti et al., 2008; Zou and Levine, 2012). The Mitf locus is complex, however, with 9 distinct promoters identified, and it is unlikely that Vsx2 controls the transcriptional activity

of all promoters. In a recent analysis of Vsx2 function, we found that Vsx2 and Mitf proteins interact, leading us to suggest that in addition to transcriptional repression, Vsx2 may regulate Mitf activity through direct protein interaction, such as by preventing access to Mitf target genes (Zou and Levine, 2012). Further work is needed, however, to determine if this is the case *in vivo*.

The mechanisms for initiating Mitf expression in the optic neuroepithelium are only beginning to be understood (Fig.1). Rx is expressed prior to onset of Mitf in different vertebrates (Mathers et al., 1997), and in zebrafish, the Rx family member Rx3 is cell-autonomously required for RPE development (Rojas-Munoz et al., 2005). In other vertebrates, however, further evidence is needed to determine whether Rx is required to initiate RPE-specific gene expression.

In mice, the earliest specific requirement for Mitf expression is the LIM homeodomain transcription factor Lhx2 (Yun et al., 2009). Lhx2 is an integral component of the eye field transcription factor network and its absence causes anophthalmia due to a failure of the optic vesicle to transition into an optic cup (Porter et al., 1997; Tetreault et al., 2009; Yun et al., 2009; Zuber et al., 2003). Lhx2 does not appear to be sufficient for initiating Mitf expression, rather, Lhx2 is likely to act in a permissive manner to allow Mitf expression in the optic neuroepithelium, thus, additional factors are responsible for Mitf activation (Yun et al., 2009).

Otx2 is initially expressed throughout the whole optic vesicle and becomes restricted to the RPE in the optic cup, similar to Mitf (Bovolenta et al., 1997) (Martinez-Morales et al., 2001). Proper levels of Otx transcription factors are required for RPE development; reduction of Otx levels results in a decrease of Mitf expression in the optic vesicle and transdifferentiation of RPE into retina (Martinez-Morales et al., 2001; Nishihara et al., 2012).

Experiments in chick revealed that loss of Otx2 leads to ectopic upregulation of FGF8 and Sox2, which can induce transdifferentiation into retina (Ishii et al., 2009; Nishihara et al., 2012; Vogel-Hopker et al., 2000). Since Otx2 is widely expressed in the forebrain before the onset of Mitf expression and can promote Mitf expression (Martinez-Morales et al., 2003; Westenskow et al., 2010), it may act as a competence factor working with additional signals to induce Mitf. Studies in several species suggest that such a signal originates in the surrounding mesenchyme but the identity of extrinsic factor(s) that initiates Mitf is still unclear (Buse and de Groot, 1991; Fuhrmann et al., 2000; Kagiya et al., 2005). Culture experiments and *in vivo* manipulations suggest that TGF β family members mediate the effect of extraocular tissues, however, more work is needed to define precisely which ligand(s), receptors, and intracellular pathway(s) are involved ((Adler and Belecky-Adams, 2002; Buse and de Groot, 1991; Fuhrmann et al., 2000; Hyer et al., 2003; Lopashov, 1963; Muller et al., 2007; Stroeva, 1960), for review, see (Fuhrmann, 2010)).

1.4 Maintenance of RPE fate in the optic cup

Ocular progenitor cells in the optic vesicle exhibit bi-potential competence, they can adopt either the neural retina or the RPE fate and it is the signal from the extraocular tissues that

promote either retinal or RPE fate (Araki and Okada, 1977; Coulombre and Coulombre, 1965; Horsford et al., 2005; Itoh, 1976; Opas et al., 2001; Pritchard et al., 1978; Reh and Pittack, 1995; Rowan et al., 2004; Stroeve, 1960; Stroeve and Mitashov, 1983; Westenskow et al., 2010). Ectopic activation of these signals/factors can expand one tissue at the expense of the other. For example, the competence of the RPE to transdifferentiate into retina is retained in chick and mouse during a restricted developmental window but can occur throughout life in frog and certain amphibians (see below).

FGFs from the overlying surface ectoderm promote retina differentiation by upregulating *Vsx2* expression in the distal optic vesicle, which restricts *Mitf* expression, promotes retinal progenitor cell proliferation, and controls the timing of onset of retinal neurogenesis (Fig.1); (Cai et al., 2010; Green et al., 2003; Nguyen and Arnheiter, 2000; Pittack et al., 1997; Sigulinsky et al., 2008). On the other hand, RPE differentiation requires Wnt/ β -catenin signaling, among other signals (Fujimura et al., 2009; Westenskow et al., 2009). Early, ectopic activation of the Wnt/ β -catenin pathway is sufficient to convert retinal progenitor cells into RPE, with absence of retina specification, and this is dependent on proper levels of *Otx2* (Liu et al., 2010; Westenskow et al., 2010).

Further differentiation and maintenance of the RPE in the optic cup requires the participation of several transcription factors and signaling pathways, such as *Pax2*, *Gas1*, *CoupTF1/2*, hedgehog, BMP and retinoid signaling, as well as *Dnmt1*-mediated DNA methylation (Fig.1) ; (Adler and Belecky-Adams, 2002; Baumer et al., 2003; Dakubo et al., 2008; Grondona et al., 1996; Huh et al., 1999; Lee et al., 2001; Lohnes et al., 1994; Matt et al., 2008; Muller et al., 2007; Nasonkin et al., 2013; Stenkamp et al., 2000; Tang et al., 2010; Zhang and Yang, 2001) for reviews, see (Bharti et al., 2006; Fuhrmann, 2010; Martinez-Morales et al., 2004). Interestingly, Bharti et al. (Bharti et al., 2012) showed recently in mouse that the transcription factor *Pax6* is critical for maintaining RPE fate; *Pax6* acts in concert with *Mitf* to promote transcription of RPE-specific genes, and depletion of *Pax6* in *Mitf* mutants greatly increases a transdifferentiation phenotype, while misexpression of *Pax6* has the opposite effect. Importantly, in *Pax6*;*Mitf* compound mutants, expression of *FGF15* and *Dkk3* is upregulated, which can also cooperate to induce RPE transdifferentiation in explant culture of wildtype embryos (Bharti et al., 2012). Together these observations demonstrate that *Pax6* has a bifunctional role depending on the tissue-specific context; it acts “pro-retinogenic” in the developing retina, while in the developing RPE it cooperates with *Mitf*/TFEC in an “anti-retinogenic” manner.

Interestingly, different regions of the RPE in the optic cup are distinct with respect to their propensity to transdifferentiate into retina. For example, in *Mitf* mutant mice, pigmentation is lost throughout the RPE, but only a small portion in the dorsal RPE transdifferentiates into retina (Bumsted and Barnstable, 2000; Nguyen and Arnheiter, 2000). A recent study revealed that *Vax* transcription factors (*Vax1*, *Vax2*) can counteract the loss of *Mitf* function in the ventral optic cup (Mui et al., 2005; Ou et al., 2013); in the absence of *Mitf*, *Vax* gene expression extends from the optic stalk and into the region of the RPE where transdifferentiation into retina is not observed. When *Vax* activity is genetically reduced in *Mitf* mutant mice, transdifferentiation is much more extensive, an outcome that may be mediated by ectopic FGF signaling (Ou et al., 2013). In this context, the *Vax* genes appear

to be acting as anti-retinogenic factors, but it is not clear whether the non-transdifferentiated RPE cells in the *Mitf* mutant still retain the identity of RPE, have acquired the identity of optic stalk, where *Vax* genes are normally expressed, or if the cells are of mixed identity, expressing features of both RPE and optic stalk.

2.1 Classical studies of RPE transdifferentiation

The capacity of the RPE to transdifferentiate into retina is well established, and this knowledge initially emerged, not from genetic studies, but from experiments in which various vertebrate species were evaluated for their ability to regenerate retinal tissue following ablation. As we now know, the origins and identities of the regenerative cell sources vary widely across species (Barbosa-Sabanero et al., 2012; Lamba et al., 2008; Locker et al., 2010; Nelson and Hyde, 2012). With respect to RPE transdifferentiation into retina, urodele amphibians, anuran amphibians, and embryonic chick stand out because they show bona-fide metaplasia *in vivo* without the addition of exogenous factors and they generate all major retinal cells classes through dedifferentiation, proliferation, and activation of retinal neurogenic programs (for reviews, see (Araki, 2007; Barbosa-Sabanero et al., 2012; Lamba et al., 2008; Reh and Levine, 1998); refer to Chiba/El-Hodiri in this issue). RPE to retina transdifferentiation during adult stages appears to be specific to amphibians with it best documented in urodeles (i.e. newts), animals that display among the most regenerative capacities of all vertebrates. In anurans such as *Xenopus*, transdifferentiation potential was thought to be lost at metamorphosis, but is now known to extend into adulthood if the retinal vascular membrane is not removed (Yoshii et al., 2007). The period of transdifferentiation potential of the chick RPE is restricted to a short window during embryonic development, and is thus quite limited, as is the case for rat (Coulombre and Coulombre, 1965; Zhao et al., 1997; Zhao et al., 1995). In teleost fish, RPE transdifferentiation has been suggested to not occur at all (Knight and Raymond, 1995).

Although the morphological features of RPE transdifferentiation differ between amphibians and chick, with amphibians producing a new retina with the correct tissue polarity as the ablated retina, and chick producing a new retina with the opposite tissue polarity and at a loss of RPE tissue in the transdifferentiated region, there are common features with respect to the extrinsic and intrinsic factors associated with the transdifferentiation process.

One common theme is that RPE transdifferentiation is dependent on interactions with the extracellular matrix and soluble factors. First described in frog, it was discovered that association of the RPE with the vascular membrane is required for transdifferentiation (Reh and Nagy, 1987; Reh et al., 1987). The avian mutant *Silver* quail exhibits transdifferentiation of RPE in a limited portion, which indicates that some specific tissue interactions between RPE and other neighboring tissues must take place in that area. When the RPE layer from *Silver* mutant embryos was isolated from surrounding tissues and placed into culture, transdifferentiation was only observed when recombined with choroid (Araki et al., 2002; Araki et al., 1998). In frog, the choroid contains high concentrations of the basement membrane component laminin and in high levels of laminin, dissociated RPE cells migrate, loose their pigment, start to proliferate extensively and assume a neuroepithelial phenotype (Reh and Nagy, 1987). In an organotypic co-culture system of newt RPE and choroid, the

choroid-derived activity that stimulates RPE transdifferentiation was identified as an activator of the FGF/MAP kinase pathway, and, importantly, transdifferentiation was suppressed with MEK/ERK and FGF receptor inhibitors (Mitsuda et al., 2005). Work in multiple labs has further revealed the importance of the FGF family in RPE transdifferentiation in amphibians, chick and mammals (Galy et al., 2002; Guillemot and Cepko, 1992; Ikegami et al., 2002; Mitsuda et al., 2005; Park and Hollenberg, 1989, 1991; Pittack et al., 1991; Sakaguchi et al., 1997; Spence et al., 2007; Zhao et al., 1995) for reviews, see: (Araki, 2007; Barbosa-Sabanero et al., 2012; Zhao et al., 1997). Interestingly, IGF-1 was shown to further enhance FGF's effects on promoting transdifferentiation by increasing the number of transdifferentiating cells, while hedgehog and activin signaling act in an antagonistic manner (Mitsuda et al., 2005; Sakami et al., 2008; Spence et al., 2004). In newt, notch signaling has been suggested to promote retinal neurogenesis during the later stages of RPE transdifferentiation by maintaining progenitor cells through the neurogenic interval (Nakamura and Chiba, 2007).

As described in the previous sections, these extracellular factors and signaling pathways are important for development of the retina and RPE. Similarly, this also appears to be the case for the intrinsic factors linked to transdifferentiation activity. Sox2, essential for maintenance of embryonic and neural progenitors, is normally downregulated in the developing chick RPE, however, exogenous FGF treatment sustains its expression resulting in transdifferentiation of RPE (Ishii et al., 2009). On the other hand, Sox2 overexpression directly induces RPE depigmentation, decreases expression of RPE genes such as Mitf and Otx2, and promotes proliferation and differentiation into ganglion cell, amacrine cells and/or photoreceptors (Ishii et al., 2009; Ma et al., 2009). Rx and Pax6 are upregulated and required at the stage of transdifferentiation when RPE cells begin to acquire retinal progenitor characteristics (Arresta et al., 2005; Azuma et al., 2005; Ishii et al., 2009; Kuriyama et al., 2009; Martinez-De Luna et al., 2011; Nabeshima et al., 2013). Since Pax6 exerts also RPE-promoting functions under certain conditions (see above (Bharti et al., 2012)), it is possible that a high expression level of ectopic Pax6 expression in the RPE is critical for the transdifferentiation effect. Downstream effectors of Pax6 can also promote RPE transdifferentiation; the homeobox transcription factor Six6 induces retina-like structures in the RPE of embryonic and mature chicks, and misexpression of Mash1 in the embryonic mouse RPE results in transdifferentiation into retina (Lanning et al., 2005; Toy et al., 1998).

2.2 Limitations in RPE transdifferentiation activity

An important, but unresolved, issue is what temporally limits the transdifferentiation capacity of the RPE except in otherwise highly regenerative animals such as the newt. This is a difficult issue to address for a couple of reasons. One is that the constraints placed on RPE transdifferentiation activity may be species-specific, so a general or unified explanation for this phenomenon may not be forthcoming. It may also be that the window of transdifferentiation activity is limited by multiple factors. Regardless, it is important to address this issue because understanding these limitations could provide important insights into how transdifferentiation properties can be activated or inhibited, depending on the

circumstances. In this section, we discuss possible reasons for the limitations on RPE transdifferentiation capacity.

One possibility is that transdifferentiation is only possible before the RPE differentiation program is completed (Fig. 2; step #1). Although the RPE acquires pigmentation at a relatively early step in eye organogenesis, it remains that embryonic RPE cells have not achieved their final differentiated state nor have they completed the phase of tissue expansion. The RPE follows a pattern of tissue growth and cell addition that is in close register with the neural retina. Cell proliferation is completed postnatally, and the RPE epithelium matures in a central to peripheral manner, as indicated by patterns of proliferation and the progressive adoption of a monolayer epithelial morphology from a pseudostratified epithelial arrangement along this axis (Bodenstein and Sidman, 1987; Kong and Nagata, 1995; Kong et al., 1992; Rapaport et al., 1995; Stroeve and Panova, 1983). Additionally, refinements in the features associated with the apical-basal polarity of RPE cells are not completed until postnatal stages (Marmorstein et al., 1998; Rizzolo, 2007). Thus, it appears that embryonic RPE cells exist in a “quasi-progenitor state”, performing functions associated with progenitor and stem cells (i.e. tissue expansion), but also exhibiting features of the differentiated state (i.e. pigmentation, initiation of cell polarity, morphogenesis into a monolayer epithelium).

We still know very little about the mechanisms controlling RPE tissue expansion, but some factors and pathways have been implicated including the cyclin-dependent kinase inhibitor protein p27Kip1, the Adenomatous Polyposis Coli (APC) gene, the tetraspanin protein CD81 and as in many developing tissues, Notch signaling (Defoe et al., 2007; Liou et al., 2004; Marcus et al., 1997; Marcus et al., 2000; Pan et al., 2011; Yoshida et al., 2004) (Schouwey et al., 2011). Genetic inactivation of the Notch effector gene *Rbpj* in mice causes RPE hypoplasticity and the eyes are microphthalmic, and overactivation of the Notch pathway causes RPE hyperplasia and interferes with differentiation (Schouwey et al., 2011). However, sustained Notch pathway activation does not lead to transdifferentiation into retina. Thus, the quasi-progenitor state of developing RPE cells is not the only property that underlies transdifferentiation potential.

Another consideration is that transdifferentiation potential is a transient vestige of the bipotential nature of the early optic neuroepithelium (Fig. 2; step #2), and as RPE cells become committed to the RPE fate, the specification pathways are no longer needed and are shut down. For example, *Mitf* expression is downregulated early postnatally (Nakayama et al., 1998), and yet the RPE fate is stable. Additionally, postnatal, RPE-specific inactivation of *Otx2* causes defects in metabolic and homeostatic functions in the RPE but does not cause transdifferentiation into retina (Bey et al., 2010; Housset et al., 2013). Furthermore, the ability of the RPE to reactivate the retinal program may be actively repressed over time, potentially through epigenetic remodeling of chromatin. However, misexpression of individual neurogenic bHLH genes in chick or mouse RPE cells beyond the period of transdifferentiation is sufficient to activate retinal cell type-specific gene expression (Wang et al., 2010b). Although this is not transdifferentiation in the classical sense because a complete retinal tissue is not produced by these manipulations, these studies suggest that retinal gene expression is not irreversibly repressed in mature RPE.

Another possibility is that the RPE retains transdifferentiation potential longer than the activity is observed, but is blocked by extrinsic influences (Fig. 2; step #3). For example, the retina may prevent RPE transdifferentiation by providing an inhibitory factor. If true, then other tissues must also produce inhibitory signals since retinal removal only promotes transdifferentiation for a brief period. Alternatively, the retina may prevent exposure to a factor that promotes transdifferentiation, such as from the retinal vasculature in anurans (Reh and Nagy, 1987; Yoshii et al., 2007). We note, however, that this last possibility has not been reported in mammals, and chickens lack a retinal vascular membrane; but if the retina restricts access to a transdifferentiation promoting signal, the signal must only exist during the window when transdifferentiation is observed in chick and mammals. The idea that the retina acts as barrier that limits the exposure of the RPE to transdifferentiation promoting signals may not be so far-fetched. In rhegmatogenous retinal detachment, in which a hole or tear in the retina exposes the RPE to vitreous, RPE cells undergo transdifferentiation into mesenchymal-like fates, which contribute to the formation of epiretinal membranes, a type of scar associated with retinal detachment and proliferative vitreoretinopathy (Hiscott et al., 1999; Umazume et al., 2012). While this type of transdifferentiation is not related to the ability of RPE cells to adopt retinal fates, it does support the idea that RPE cells may have a high intrinsic capacity for transdifferentiation, but are normally held in check by limited exposure to stimulatory signals.

3.1 Tissue homeostasis in the mature RPE

The RPE exhibits many properties of a classic barrier epithelium and a common feature of these types of tissues is that they undergo cell turnover throughout life. To ensure tissue homeostasis is maintained, the rate of cell removal is matched by new cell production. This is achieved primarily through the retention of stem cells in the mature tissue, typically in a compartmentalized manner. Well studied examples of high turnover tissues include the intestinal epithelium, epidermis, hair follicle, and corneal epithelium (Barker et al., 2010; Mort et al., 2012). In each case, there is a continuous rate of turnover, and as a consequence, the stem cell activities of these tissues are robust. They are also responsive to sudden perturbations, such as injury or inflammation. In other cases, such as the mammary gland epithelium, the rate of turnover may be low relative to the life of the organism, but growth and differentiation can be initiated rapidly in response to hormonal signals (Visvader and Smith, 2011).

In contrast, the mature RPE maintains tissue homeostasis through long-term cell survival, with little evidence of cell turnover and essentially no evidence of a stem cell compartment for de novo cell production. This is consistent with a sharp diminishment of proliferation not long after birth (Bodenstein and Sidman, 1987; Kong and Nagata, 1995; Kong et al., 1992; Rapaport et al., 1995; Robb, 1985; Stroeva and Panova, 1983). It is well established that the density of RPE cells across the epithelium decreases over time, most notably in the peripheral RPE. The decrease in cell density is thought to be due to two primary processes; continued growth of the eye, especially during the juvenile period, and a gradual loss of cells, especially as related to aging (Del Priore et al., 2002; Gao and Hollyfield, 1992; Harman et al., 1997; Robb, 1985; Ts'o and Friedman, 1968). To maintain the epithelium then, RPE cells generally undergo hypertrophy without loss of contact with their

neighboring cells. A serious consequence of this type of static homeostasis is that as RPE cells age, the tissue undergoes significant changes that reduce its function and compromise its structural integrity, problems that are considered to be primary factors in the etiology of age-related macular degeneration (Boulton, 2012; Kozlowski, 2012; Swaroop et al., 2009). It should be noted, however, that the ability of the RPE to cope with cell loss through hypertrophy may be greater than previously thought. In an experiment where RPE cells were killed by genetic ablation using an inducible Cre recombinase-mediated expression of Diphtheria toxin, the RPE was able to maintain itself as a continuous sheet through hypertrophy without proliferation, even in the presence of greater than 60% cell loss (Longbottom et al., 2009). Nevertheless, significant deficits occurred within the retina as evidenced by photoreceptor rosetting and degeneration, which suggests that maintenance of the epithelial sheet is not enough to maintain retinal integrity if other aspects of RPE cell function are compromised.

Recent studies are beginning to challenge the idea that the mature RPE is a static, homogeneous epithelium. Immunohistochemical studies in mice have revealed a subpopulation of RPE cells in the peripheral domain that proliferate throughout life (Al-Hussaini et al., 2008; Kokkinopoulos et al., 2011). Differential expression of cell cycle and adhesion related genes were also observed between central (posterior) and peripheral RPE with a bias toward proliferation promoting genes in the peripheral RPE, although it should be noted that the analysis was focused on a select set of genes (Kokkinopoulos et al., 2011). Cell to cell differences have also been documented for melanosome content and for the expression of genes and proteins associated with RPE cell function, suggesting that cell heterogeneity may be a feature that exists throughout the epithelium (Burke and Hjelmeland, 2005). Heterogeneity may also exist with respect to latent stem cell-like characteristics within the mature RPE. Clonal cultures of RPE cells isolated from humans at multiple ages revealed that approximately 10% of the cells had self-renewal capacity and the ability to activate RPE, neural, and several mesenchymal programs of differentiation (Salero et al., 2012). The sum of these studies are pointing to the idea that RPE cells as a population are not homogeneous and that certain subsets may contain distinct properties that could contribute not only to enhanced repair and regeneration, but also to pathology.

It is also becoming more apparent that the mature RPE has the capacity to undergo limited regeneration. Proliferation, self-repair, and restoration of the epithelium has been observed in paradigms of RPE damage in which small lesions were induced by low energy laser exposure or low doses of sodium iodate (Machalinska et al., 2013; von Leithner et al., 2010). These studies indicate that the RPE possesses proliferative potential and determining ways to enhance proliferation could further stimulate regenerative capacity. However, as with hypertrophy as a mechanism to maintain the epithelial sheet, simply stimulating RPE proliferation will have its limitations. For example, overexpression of the Y-box transcription factor ZONAB or knockdown of its negative regulator, the tight junction protein ZO-1 enhanced RPE proliferation, but also caused RPE de-differentiation, expression of genes associated with epithelial to mesenchymal transition (EMT), and retinal degeneration (Georgiadis et al., 2010), not unlike the phenotypes observed in RPE-specific

inactivation of the phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*), or by pharmacological activation of the mTOR pathway (Kim et al., 2008; Zhao et al., 2011).

Interestingly, the regenerative response of the RPE to sodium iodate injury is enhanced in the Murphy Roths Large mouse strain (MRL) or “healer mouse”, and is correlated with elevated proliferation in the damaged region (Xia et al., 2011). Improved regenerative responses have been observed in several different tissues in the MRL mouse (Heber-Katz et al., 2004), but it is not clear whether the enhanced regenerative potential is due to alterations in systemic responses to injury or to a cell-intrinsic advantage within the regenerative cells themselves. Genetic mapping studies have linked over 20 loci to the regenerative phenotype on multiple chromosomes, but specific genes have yet to be identified (Blankenhorn et al., 2009; McBrearty et al., 1998). Intriguingly, expression of the cyclin-dependent kinase inhibitor p21 is lower in the regenerating tissue in an earpunch closure assay in MRL mice and genetic inactivation of p21 in non-healer strain conferred a regenerative response that was comparable to the MRL mouse (Bedelbaeva et al., 2010). Whether reducing p21 levels in damaged RPE similarly enhances a regenerative response has yet to be determined.

4. Conclusions

An increasing understanding of the developmental processes governing specification and differentiation of the RPE is providing more insight into the mechanisms that underlie plasticity of the RPE before it fully matures. For example, progress has been made in identifying several of the factors important for transdifferentiation of the RPE into retina, among other cell types, and to some extent their interactions with each other. We still lack, however, a comprehensive understanding of the molecular networks that drive transdifferentiation. Thus, future studies should be aimed at resolving these networks.

Furthermore, the adult RPE is remarkably non-regenerative; even though it exhibits some degree of cell addition and self-repair. Ultimately, the limitations on RPE self-repair and regenerative capacity may boil down to two fundamental issues: the lack of a bona-fide stem cell compartment, and an extracellular environment that is not supportive for controlled regenerative repair, especially in aged or injured eyes, the latter topic not discussed in this review. Much of the properties discussed above appear to arise from mature RPE cells that revert to a proliferative state. If the differentiation characteristics were maintained, this mode of self-repair could be successful. But, an emerging theme is that proliferation is oftentimes accompanied by dedifferentiation and activation of pathological differentiation programs. Thus, the challenges ahead should include an examination of how acquisition of re-entry into a proliferative state can be uncoupled from dedifferentiation.

Acknowledgments

Supported by NIH/NEI (EY014954 to S.F., EY013760 to E.M.L.) and by an unrestricted grant from Research to Prevent Blindness, Inc., to the Department of Ophthalmology, University of Utah. Our apologies to those authors whose work is not cited due to space limitations.

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Highlights

- Update of early eye development and RPE specification
- Limitations in RPE transdifferentiation activity
- Regeneration and self-repair in the mature RPE

Fig. 1.

Regulation of early eye and RPE development by extracellular signals and transcription factors. The eye field is initiated in the anterior neural plate by induction of eye field transcription factors: Pax6, Tll, Rx, T/Tbx3, Six3/6. Rx may be upregulated cooperatively by Otx2 and Sox2 that are broadly expressed in the anterior neural plate. Additional interaction with extracellular pathways (BMP, Wnt, sonic hedgehog) separate the eye field from other brain regions. Eye field transcription factors promote Lhx2 expression that acts as a competence factor to allow patterning of the optic vesicle. Otx2, Mitf (possibly induced by TGFβ-like signals), and Pax6 are initially present throughout the optic vesicle, however Mitf is then downregulated by Vsx2 in the presumptive retina domain, which is initiated by FGF signals. Otx2 may further suppress expression of FGF and Sox2 to support RPE formation. In the optic cup, additional factors such as Wnt, hedgehog, retinoids, Gas1, CoupTFs, BMP4, Notch and Vax stabilize the RPE fate and promote differentiation into RPE subdomains. For more details, see text. A: anterior, P: posterior.

Fig. 2. Limitations in RPE transdifferentiation activity

The temporally restricted transdifferentiation capacity of the RPE (#1) may be determined by cell-intrinsic factors including the stage of development in which RPE progenitors still retain the competence to activate a retinal program (RPE/retina bipotentiality, #2), or the stage of development in which RPE cells are undergoing cellular differentiation and tissue growth (RPE differentiation/expansion). Cell-extrinsic influences (#3) may further limit the period of transdifferentiation potential by exposing RPE cells to inhibitory signals or blocking access to transdifferentiation-promoting signals. Although not shown, cell-extrinsic influences may also play a prominent role in limiting proliferative potential and fate plasticity in the mature RPE.