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The retinal pigmented epithelium – from basic developmental biology research to translational approaches

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ABSTRACT The development of the eye has been a topic of extensive investigation, from the early studies on tissue induction to more recent breakthroughs in resolving the mechanism regulating progenitor patterning and their gradual and coordinated differentiation into diverse tissue types that function together throughout life. Among the ocular tissue types, the retinal pigmented epithelium (RPE) is at the forefront of developmental biology and stem cell research. The growing interest in this lineage stems from its importance for photoreceptor function as well as from its requirement during embryogenesis for the development of the photoreceptors and the choroid. Indeed mutations in RPE genes and epigenetic changes that occur during aging are the cause of monogenic as well as multifactorial retinal diseases. Importantly, the RPE is readily generated from stem cells, and these stem cell-derived RPE cells are currently being tested in clinical trials for transplantation in cases of retinal dystrophies; they also constitute an important model to study developmental processes *in vitro*. This review summarizes recent advances in our understanding of RPE development and its requirement for the development of photoreceptors and choroidal vasculature. We discuss the contribution of basic findings to therapeutic applications and the future challenges in uncovering developmental processes and mimicking them *ex vivo* to further advance research and therapy of retinal disorders.

KEY WORDS: *RPE, photoreceptor, choroidal vasculature, retina*

Introduction

Photoreceptors (PRs) and pigment cells form a functional unit which is found in various types of eyes in the different animal phyla (Arendt, 2003, Charlton-Perkins and Cook, 2010, Gehring, 2014). In vertebrates, the ocular pigmented cells form a single layer of polarized epithelium termed retinal pigmented epithelium (RPE), which is located between the PRs and choroidal vasculature. The RPE has multiple and complex functions required for PR development, homeostasis and physiology (Strauss, 2005).

RPE functions are executed by distinct cellular compartments. The apical side of the RPE extends microvilli, which are long and thin actin processes that engulf the outer segments of the PRs and are required for their daily renewal by phagocytosis (Bonilha, 2014; reviewed in Kevany and Palczewski, 2010). The basal side the RPE is attached to the anterior layer of Bruch's membrane, a connective tissue that separates the RPE from the choriocapilaris and is an important component of the blood–retina barrier (BRB; Bhutto and Lutty, 2012, Rahner *et al*., 2004). The interaction of the RPE with Bruch's membrane consists of basal infoldings, short invaginations which increase the surface area of the RPE (Bonilha, 2014). The polarized distribution of ion channels and transporters in the RPE further enable it to control the composition of the subretinal space and to support survival and function of the PRs and other retinal cell types (Lehmann *et al*., 2014).

Considering the importance of the RPE to PR survival and activ-

Abbreviations used in this paper: AMD, age-related macular degeneration; BRB, blood–retina barrier; EMT, epithelial to mesenchyme transition; FGF, fibroblast growth factor; HD, homeodomain; IHH, Indian hedgehog; iPSC, induced pluripotent stem cell; miRNA, microRNAs; NR, neuroretina; OC, optic cup; OV, optic vesicle; PEDF, pigmented epithelium-derived factor; PR, photoreceptor; pSMAD, phosophorylated smad; RA, retinoic acid; RPE, retinal pigmented epithelium; SE, surface ectoderm; SHH, Sonic hedgehog; TF, transcription factor.

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ity, it is not surprising that RPE dysfunctions are associated with various retinal dystrophies, including the common cause of aging-related blindness in humans—age-related macular degeneration (AMD, Jackson *et al.*, 2002; Wright *et al.*, 2010). Stem-cell-based therapies for RPE dysfunctions are currently being evaluated in clinical trials (Carr *et al.*, 2013, Song and Bharti, 2015). Thus, resolving the gene regulatory networks functioning in the course of RPE development and regulating its multiple activities is of interest for the field of regenerative medicine. In this review, we summarize the findings on RPE differentiation and maintenance, and the progress—as well as the challenges—in stem cell technologies aimed at replacing damaged RPE with healthy tissue for the purpose of preserving and regenerating retinal function.

Gene regulatory networks involved in establishing the progenitor domains of the optic cup

In humans and other vertebrates, the sensory neural retina and pigmented lineages of the eye originate during embryonic development from an outgrowth of the ventral forebrain termed optic vesicle (OV). The oval OV rapidly undergoes morphogenesis to become the hemispheric optic cup (OC, Kwan *et al.*, 2012; Picker *et al.*, 2009). The dorsal outer layer of each OC will form the RPE, the ventral outer layer will give rise to the optic stalk, and the inner layer of the OC contains the pseudostratified retinal progenitor cells that proliferate and gradually differentiate into retinal lineages (Shaham *et al.*, 2012). The borders between inner and outer layers of the OC will give rise to the non-neuronal, pigmented epithelia of the ciliary body and iris (Davis-Silberman and Ashery-Padan, 2008).

The OV originates from a group of cells at the anterior neural plate that express a combination of transcription factors (TFs) important for forebrain and eye development (reviewed in Fuhrmann *et al.*, 2014; Sinn and Wittbrodt, 2013). These TFs include the homeodomain (HD) proteins Six3, Otx2 and Rx, the paired and HD protein Pax6, and the LIM-HD domain protein Lhx2. In the OV, these TFs play multiple roles. Rx and Lhx2 are required for OV morphogenesis and later, together with Pax6, Otx2 and Six3, function to maintain ocular fates and proliferation of the early neuroepithelial progenitors (Bovolenta *et al.*, 1997; Farhy *et al.*, 2013; Oron-Karni *et al.*, 2008; Philips *et al.*, 2005; Porter *et al.*, 1997; Roy *et al.*, 2013; Gueta *et al.,* 2016). At later stages, these factors are further involved in the differentiation of specific retinal cell types (Beby and Lamonerie, 2013, de Melo *et al.*, 2016, Emerson *et al.*, 2013, Gordon *et al.*, 2013, Koike *et al.*, 2007, Muranishi *et al.*, 2011, Samuel *et al.*, 2016).

In the RPE, Mitf is considered to be the earliest indication of a pigmented epithelium fate. Mitf is a basic helix-loop-helix leucine zipper protein and a key regulator of pigment cell development from both the neural crest and neural epithelium as it transactivates crucial genes for pigment biogenesis (e.g. *Dct*, *Tyrp1* and *Tyr*) (Tachibana, 2000, Tachibana *et al.*, 1994). Mutations in Mitf result in the generation of neuroretina (NR) instead of RPE, whereas ectopic expression of Mitf in the NR prevents neurogenesis and triggers expression of genes involved in pigment biogenesis (Horsford *et al.*, 2005, Nguyen and Arnheiter, 2000). Mitf is detected in the OV of mouse embryos prior to division of the pigmented and neural progenitor domains and by the OC stage, its expression is restricted to the pigmented lineages (Baumer *et al.*, 2003, Nguyen and Arnheiter, 2000). Mitf regulation has therefore been extensively investigated to resolve the mechanisms involved in early patterning of the OC. *Mitf* contains multiple promoters (Bharti *et al.*, 2008). The variants expressed in the RPE are Mitf A, J, H and D, in contrast to Mitf M, which is predominant in melanocytes (Bharti *et al.*, 2008). Studies of mutants in specific Mitf variants have revealed a compensatory mechanism among these variants, as downregulation of one leads to upregulation of others, as well as of another Mitf family member, TFEC (Bharti *et al.*, 2012). These intricate regulatory feedback loops among the Mitf variants probably evolved to ensure robust expression of Mitf, which is required for normal differentiation of an essential tissue for PR development and function (Bharti *et al.*, 2012, Raviv *et al.*, 2014).

Based on analyses of mouse mutants, the onset of Mitf expression is regulated by TFs expressed in the OV. Six3 seems to inhibit Mitf, possibly indirectly, through inhibition of Wnt8b (Liu *et al.*, 2010), whereas Otx2 was found to directly regulate Mitf as well as to transactivate the expression of pigment genes in cooperation with Mitf (Martinez-Morales *et al.*, 2003, Martinez-Morales *et al.*, 2004). Similarly, Pax6 together with Pax2 are required for the onset of Mitf expression (Baumer *et al.*, 2003). Pax6 also seems to regulate TFEC, which is upregulated upon Mitf loss in embryos with an intact Pax6 gene but not in double mutants of both Mitf and Pax6 (Bharti *et al.*, 2012). Lhx2 is required for the expression of both Mitf and Chx10/Vsx2, the initiating factors for RPE and retina specification, respectively (Gordon *et al.*, 2013, Yun *et al.*, 2009). It is not clear whether Lhx2 directly regulates Mitf expression, as the consequences of Lhx2 loss include upregulation of hypothalamic and thalamic eminence genes, which may interfere with the acquisition of eye fates (Roy *et al.*, 2013).

Restriction of Mitf expression to the prospective pigmented progenitors occurs in mice during the transition from OV to OC and is dependent on exogenous cues emanating from the surface ectoderm (SE) and the ocular mesenchyme (reviewed in Fuhrmann, 2010). Early studies using explants in chick embryos suggested that fibroblast growth factors (FGFs) emanating from the SE inhibit Mitf expression in the distal OC and thus enable upregulation of the NR TF, Vsx2 (Nguyen and Arnheiter, 2000). Vsx2 is important for NR progenitor proliferation and for inhibition of Mitf. In fish, Vsx2 also regulates the expression of proteins involved in OC morphogenesis (Gago-Rodrigues *et al.*, 2015, Green *et al.*, 2003, Nguyen and Arnheiter, 2000). As FGF-induced reprogramming of the RPE lineage does not occur in Vsx2-null mutant mice, it has been suggested that Vsx2 is an essential downstream node in the MAPK–FGF pathway responsible for NR fate determination (Horsford *et al.*, 2005, Nguyen and Arnheiter, 2000).

Explant studies in chick embryos expose complex roles for TGFβ/BMP proteins in patterning and morphogenesis of neural plate and the OC. During early neurolation BMP activity in the anterior neural plate prevents the acquisition of eye-field identify from the prospective telencephalic cells, whereas, at the early neural tube stage BMP from the SE promote neural retina identity in adjacent OV (Pandit *et al.*, 2015; Huang *et al.*, 2015). Finally, TGFβ/BMP proteins, together with Wnt ligands emanating from the extraocular mesenchyme and SE around the lens are implicated in triggering RPE fate (Carpenter *et al.*, 2015; Fuhrmann, 2010; Fuhrmann *et al.*, 2000; Muller *et al.*, 2007; Steinfeld *et al.*, 2013; see below). A recent study using live imaging in zebrafish embryos indicated roles for BMPs in regulating the cellular move-

ments required for OC morphogenesis, in addition to triggering RPE-differentiation genes. In this model, BMP controls the flow of cells from outside toward the inside of the developing OC, and BMP-mediated inhibition of the flow results in ectopic NR in the RPE domain (Heermann *et al.*, 2015). Whether cell migration contributes to OC morphogenesis and patterning in mammals, where proliferation plays a major role in expansion of the retinal progenitors, has yet to be determined.

Wnt signaling is another important pathway for RPE differentiation. Conditional inactivation of β-catenin, the mediator of the canonical Wnt pathway in the OV, using Lhx2-Cre (Hagglund *et al.*, 2013), or in the presumptive RPE using Tryp1-Cre (Westenskow *et al.*, 2009), prevents RPE differentiation and results in the formation of NR instead. Using chromatin immunoprecipitation and reporter assays, this phenotype is suspected to be a result of β-catenin's direct regulation of both Otx2 and Mitf (Fujimura *et al.*, 2009, Westenskow *et al.*, 2009). The regulation of Mitf by β-catenin has also been reported in melanocytes, where β-catenin functionally interacts with Mitf and is redirected by Mitf to activation of Mitf-specific target promoters (Lang *et al.*, 2005, Schepsky *et al.*, 2006); as such, this may represent a conserved pathway in pigmented cell differentiation.

In a recent study that explored the role of secreted Wnt ligands during mouse eye development, it was established that the autocrine secretion of Wnts by RPE cells is not sufficient for normal RPE differentiation. In contrast, Wnt ligands originating from the SE elicited specification of RPE progenitors near the OC rim. This suggestion was based on the observation that inhibition of Wnt ligands secretion from the SE reduces RPE cell number and results in a saucer-shaped OC (Carpenter *et al.*, 2015). This abnormal OC morphology is consistent with the suggestion that RPE stiffness is an important driver for cup morphogenesis (Eiraku *et al.*, 2011).

The Wnt ligands seem to affect both BMP and retinoic acid (RA) signaling pathways. Tissue ablation and transplantations in chick embryos suggest that Wnt ligands emanating from the dorsal SE direct dorsal OV cells to develop into RPE through a stabilizing effect of BMP signaling. BMPs and Wnts cooperate via a GSK3β-dependent pathway at the level of pSmad to ensure RPE specification in dorsal OV cells (Steinfeld *et al.*, 2013). Accordingly, upon loss of Lrp6, a Wnt receptor mediating canonical Wnt signaling, expression of both BMP and RA is reduced in the dorsal OC of mice (Zhou *et al.*, 2008). Interestingly, RA seems to contribute to proliferation of the RPE as well as to affect the morphogenetic movements of the mesenchyme that surrounds the OC (Carpenter *et al.*, 2015, Molotkov *et al.*, 2006). Thus, it seems that Wnt is required for the generation of sufficient quantities of RPE cells through β-catenin and by regulating BMP, as well as for the RA-mediated morphogenetic movements of ocular mesenchyme.

Autonomous formation of OV and OC structures can be achieved from a 3D culture of mouse and human embryonic stem cells and induced pluripotent stem cell (iPSC) aggregates (Eiraku *et al.*, 2011, Kuwahara *et al.*, 2015, Meyer *et al.*, 2011, Nakano *et al.*, 2012). The isolated OC structures suggest an autonomous program for OC morphogenesis in the neural progenitors. Nevertheless, as these OC cultures are supplemented with Wnt ligands and RA ligands (Eiraku *et al.*, 2011, Kuwahara *et al.*, 2015, Nakano *et al.*, 2012), it remains likely that in vivo, the SE is an important source of ligands which are required for RPE differentiation and normal OC morphogenesis.

Sonic hedgehog (SHH) molecules play critical roles in establishing the bilateral eye fields and in determining the proximal–distal axis of the eye primordium (Chiang *et al.*, 1996, Li *et al.*, 1997, Macdonald *et al.*, 1995). SHH also influences the expression patterns of BMP4 and Otx2 and thus may play a role in RPE development (Zhang and Yang, 2001). Recently, two antagonists of HH signaling in the OC were identified: Lrp2, a cell-surface receptor, mediates endocytic clearance of SHH and antagonizes its morphogenetic action (Christ *et al.*, 2015) and Cdon, a cell-adhesion molecule that interacts with the SHH receptor Patched 1 (Ptc1) (Cardozo *et al.*, 2014) and seems to antagonize HH by trapping the HH protein in the neuroepithelial basal end-foot. Cdon and LRP2 play a role in generation of the retina and adjacent ciliary body and iris, although their roles in RPE differentiation are unknown. Indian hedgehog (IHH), secreted from endothelial cells adjacent to the RPE, is required for RPE differentiation. In mice mutated in IHH, RPE differentiation is abnormal; the nuclei are elongated and deformed, the basal and apical microvilli are abrogated and pigmentation is reduced (Dakubo *et al.*, 2008). Also the sclera and the retina are affected in the IHH mutant eyes and therefore the specific roles of IHH on RPE differentiation remain to be determined.

The Hippo signaling pathway was recently recognized to mediate the initial specification of RPE cells in fish embryos (Miesfeld *et al.*, 2015). The Hippo kinase signaling cascade regulates cell-fate decisions during development by controlling the localization and stability of the transcriptional coactivators Yes-associated protein 1 (Yap) and WW domain containing transcription regulator 1 (Taz) (Varelas, 2014). The main nuclear binding partners for Yap and Taz are the Tea domain (Tead) TFs. Yap/Taz–Tead activity is necessary and sufficient for OV progenitors to adopt RPE identity in zebrafish. Yap mutants lack a subset of RPE cells and exhibit coloboma; the phenotype is exacerbated when combined with Taz mutant alleles, such that when Yap and Taz are both absent, OV progenitor cells completely lose their ability to form RPE. Consistent with the fact that mutation within the Yap-binding domain of Tead1 causes Sveinsson chorioretinal atrophy (SCRA), autosomal dominant loss of RPE and choroid, and altered choroid fissure closure (Fossdal *et al.*, 2004, Jonasson *et al.*, 2007), loss of function of Yap and Taz affects choroid fissure closure as well (Miesfeld *et al.*, 2015). Current efforts are being aimed at determining the role of this pathway in mammalian RPE and deciphering the mechanisms upstream and downstream of the Yap/Taz–Tead activity in RPE specification and differentiation.

Growth and differentiation of the retinal pigmented epithelium

Growth and differentiation of the specified RPE occurs gradually, similar to the pattern of proliferation and differentiation in the adjacent NR (Defoe and Levine, 2003). In mice, on embryonic day 15, there are approximately 14,000 RPE cells and by postnatal day 15, the total number of RPE cells reaches 54,000, a fourfold increase (Bodenstein and Sidman, 1987). At the molecular level, it was shown in rats that the cyclin-dependent kinase inhibitor p27 (Kip1) is gradually upregulated, starting from the central OC and progressing toward the peripheral OC, similar to the differentiation wave observed in the adjacent retina (Defoe and Levine, 2003). Expression of Kip1 decreases gradually during postnatal stages

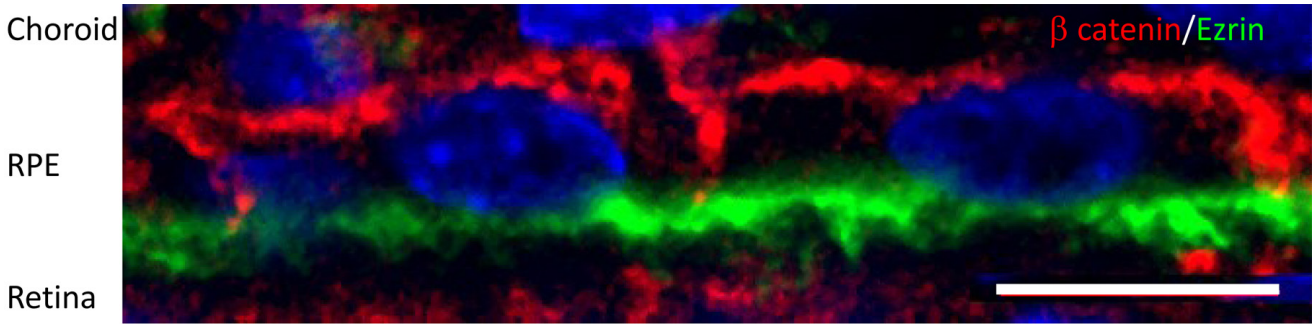

Fig. 1. Retinal pigmented epithelium (RPE) polarity at postnatal day 5 (P5) is evident with the accumulation of beta-catenin (red) in the basolateral membrane and phosphor-Ezrin (green) in the developing apical microvilli of the RPE. *Nuclei are labeled with DAPI (blue). Scale bar, 10 μm.*

(Defoe and Levine, 2003). In addition to the increase in cell number due to proliferation, the RPE cells grow in size, so that the total area of the RPE increases 10-fold (Bodenstein and Sidman, 1987).

In contrast to the tight coupling of differentiation and cell-cycle exit observed in neural differentiation, differentiation is evident early on in RPE progenitors, with the accumulation of pigment granules (melanosomes). During embryogenesis as well, features of apical–basal polarity are evident with the apical localization in the developing microvilli of the scaffold protein phospho-ezrin, the basolateral distribution of beta-catenin and the basal deposition of components of Bruch's membrane, the basement membrane for both RPE and choriocapillary endothelium and an important component of the BRB (Bonilha *et al.*, 2006; Hirabayashi *et al.*, 2003; Fig. 1). RPE completes its differentiation during postnatal stages, in conjunction with choroid maturation and PR outer segment formation. These late events include the onset of expression of proteins involved in recycling and the continuous supply of 11-cis-retinal to PRs, growth of the apical microvilli, and establishment of the BRB, which is dependent on the formation of adherent tight junctions between the cells and on the polar distribution of ion channels and transporters that are required for selective transport across the barrier (reviewed in Rahner *et al.*, 2004; Rizzolo *et al.*, 2011; Lehmann *et al.*, 2014).

Gene regulatory networks in the late stages of retinal pigmented epithelium differentiation

The gradual differentiation of RPE cells is mediated by the hierarchical expression of key TFs (Fig. 2). Pax6, which is important at early stages of RPE specification, is also required later on for the pigmentation of RPE cells. During this process, Pax6 regulates the RPE-specific D-isoform of Mitf (Bharti *et al.*, 2008) and interacts in a feed-forward regulatory loop with Mitf proteins to activate the pigment-biogenesis genes (Cavodeassi and Bovolenta, 2014, Raviv *et al.*, 2014). Around midgestation (Fig. 2B), expression of the TF Sox9 is upregulated and probably promotes, based on bovine cell-based assays, the expression of genes related to the visual cycle through a synergistic cooperation with Lhx2 and Otx2 (Masuda *et al.*, 2014). Sox9 has been further shown to regulate the expression of Best1, a Ca-dependent Cl channel, through interaction with Mitf and Otx2 (Esumi *et al.*, 2007, Esumi *et al.*, 2009, Masuda and Esumi, 2010). Moreover, conditional mutation of Otx2 in the adult mouse RPE provided further support for its requirement for RPE physiology (Housset *et al.*, 2013). Together, these findings support key roles for Sox9 and Otx2 in regulating genes that are important for the diversity of RPE functions in adults.

In addition to TFs, microRNAs (miRNAs) have been shown to have an important role in RPE maturation and function (Sundermeier and Palczewski, 2016). Ohana *et al.,* (2015) reported that RPE cells, in which miRNAs were diminished due to conditional mutation in Dicer1 or DgcR8, preserve the expression of RPE TFs Sox9 and Otx2, but the cells are smaller than normal, show reduced pigmentation and fail to express enzymes required for the recycling of retinal (Ohana *et al.*, 2015). miR204 which is essential for normal lens and retinal development in fish is highly enriched, together with miR211, its closely related paralog in mammals, in the developing and differentiated RPE (Conte *et al.*, 2010, Ohana *et al.*, 2015). A dominant gain-of-function mutation in miR204 resulted in a severe ocular phenotype, supporting an important role for this miRNA in multiple eye lineages in humans (Conte *et al.*, 2015). Further support for miR204's role in the RPE was obtained through functional studies in primary human fetal RPE cultures, which revealed TGFBR2 and Snail2 to be direct targets of miR204/211, indicating the importance of this miRNA family in maintaining cell-adhesion properties and in RPE physiology (Wang *et al.*, 2010). Interestingly, in the mammalian genome, miR211 and miR204 are encoded by two genes which are located within introns of the transient receptor potential genes *Trpm1* and *Trpm3*, and these miRNAs seem to be co-regulated with their host genes. Trpm1/miR211 were found to be direct transcriptional targets of Mitf in primary cultures of human fetal RPE and melanocytes (Adijanto *et al.*, 2012, Levy *et al.*, 2010), while Pax6 was found to regulate Trpm3/miR204 in the lens, retina, ciliary body progenitors and iris (Shaham *et al.*, 2013). Thus miR211 and miR204 are important mediators of key TFs required for differentiation of diverse eye lineages.

The levels of additional miRNAs are elevated during differentiation of RPE from pluripotent stem cells (Greene *et al.*, 2014, Hu *et al.*, 2012, Wang *et al.*, 2014, Yuan *et al.*, 2015), but very little is known about their expression profiles or functions *in vivo* at early developmental stages. Recent in-vitro functional studies using RPE generated from iPSCs have implicated roles for miR184 in promoting RPE differentiation by inhibiting the AKT2/mTOR signaling pathway (Jiang *et al.*, 2016). Additional in-vitro studies on miRNAs in mature RPE have suggested that the visual cycle genes may be regulated by miR137 (Masuda *et al.*, 2014), while catalase, an important component of the cell's antioxidant defense mechanism, seems to be regulated by miR30b (Haque *et al.*, 2012). These studies support important roles for miRNAs in the RPE. However,

considering possible redundancy between miRNAs and other compensatory mechanisms, further in-vivo functional studies are critical to substantiate the miRNAs' contributions to RPE development and function.

RPE functions in the development of photoreceptors and choroidal vasculature

The importance of the RPE for retinal development was documented early on by ablation of the embryonic RPE using targeted expression of toxins or misexpression of Fgf9, which resulted in the development of NR instead of RPE (Raymond and Jackson, 1995, Zhao and Overbeek, 2001). More recently, conditional mutations of Dicer1, DGCR8 or DNMT1 in the RPE were shown to result in failure to differentiate the PR outer segment (Nasonkin *et al.*, 2013, Ohana *et al.*, 2015). These findings demonstrate the importance of a functional RPE for PR outer segment formation and implicate the requirement of factors from the RPE for PR maturation.

Further support for the importance of RPE-secreted factors for PR differentiation comes from the observation that PRs grown in culture require supplementation of conditioned medium from RPE culture (Sheedlo *et al.*, 2007). Moreover, it has been shown that different concentrations of RPE-conditioned medium drive retinal stem cells to different cell fates (Dutt *et al.*, 2010). One candidate molecule for the mediation of RPE–PR communication is pigmented epithelium-derived factor (PEDF). In vitro, PEDF was able to rescue PR development (Jablonski *et al.*, 2000). Nevertheless, a determination of the role of PEDF in PR differentiation awaits analysis of the retinal phenotype of PEDF mutant mice (Doll *et al.*, 2003). Another suggested candidate is SHH, as it is able to support PR development *in vitro* (Levine *et al.*, 1997). However, while HH signalling is required for normal differentiation of the RPE (Dakubo *et al.,* 2008), the HH ligand expressed in this tissue is not known. Thus, although there is ample evidence for the importance of RPE-secreted factors in PR differentiation, identification of these factors constitutes an important challenge for future studies. Understanding the interaction between the RPE and PRs is critical for the use of RPE-based therapies for retinal degenerative pathologies, as it can lead to better-directed therapies. In addition, it may provide a more indicative tool for the evaluation of RPE function and maturity.

The impact of RPE on PR differentiation can be also indirect, through its role in regulating development of the choroidal vascu-

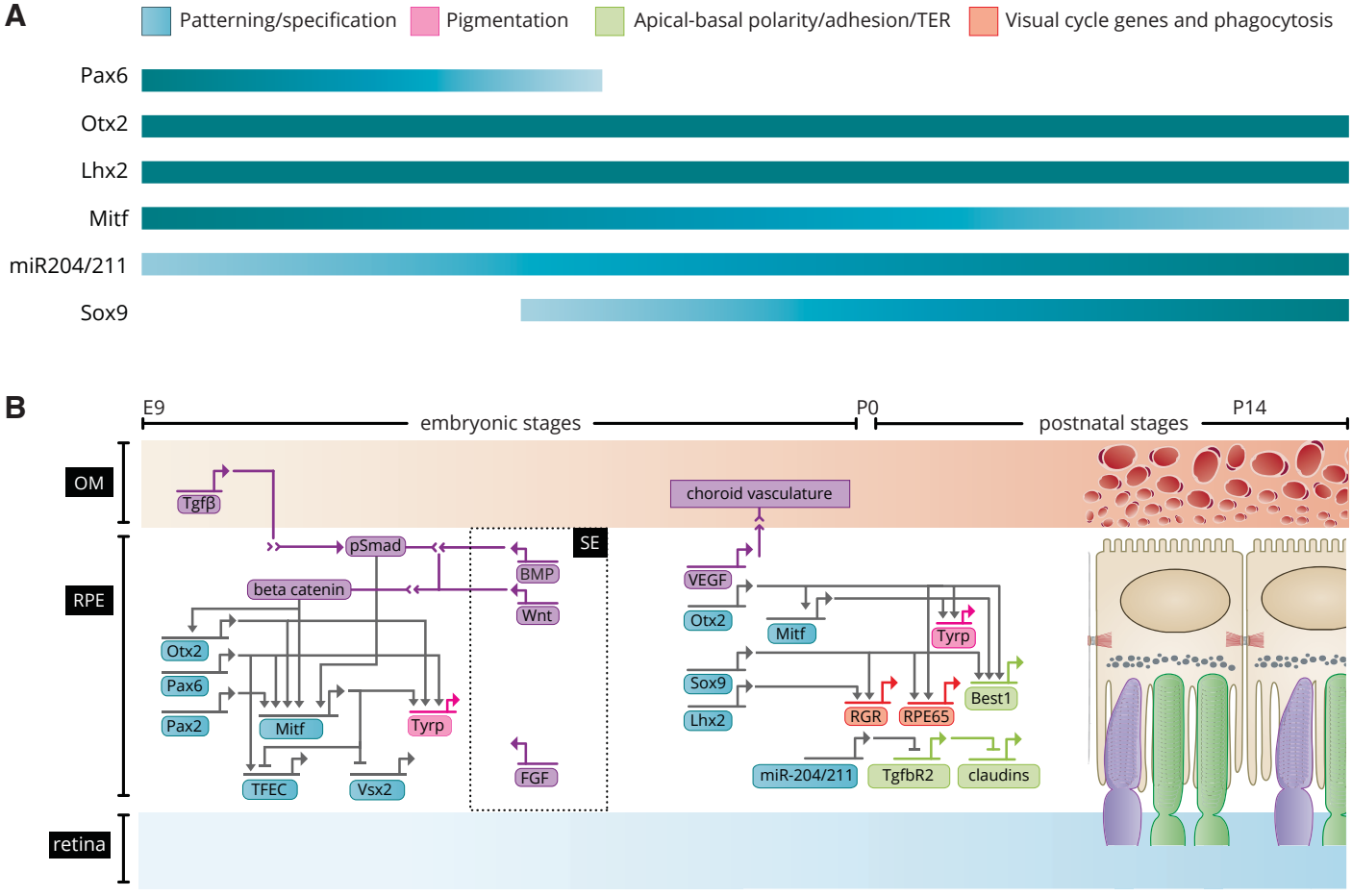

Fig. 2. Gene regulatory networks in retinal pigmented epithelium (RPE) differentiation. (A) *The level of expression of transcription factors and miR204/211 family during RPE differentiation, demonstrated by color intensity and respectively to mouse embryonic stages mentioned in B.* **(B)** *The early and late regulatory networks of the RPE expressed genes and the signaling pathways mediating interaction with adjacent surface ectoderm (SE), retina and ocular mesenchyme (OM) from which the choroid vasculature evolves. The transcription factors or miRNAs are in blue, while their targets are labeled according to color code mentioned at top based on the biological function. The mature RPE cells and their adjacent tissues are illustrated on the lower right panel.*

lature and mediating the selective transport, and thus the microenvironment, required for PR differentiation, survival and physiology. This vascular system starts to form early on during vertebrate eye development (Gage *et al.*, 2005), yet the molecular mechanisms regulating its formation are only starting to be uncovered. The RPE seems to be essential for choroid blood vessels' formation, as well as maintenance as the chemical destruction or transdifferentiation of the RPE resulted in abnormal choroid development (Korte *et al.*, 1984, Zhao and Overbeek, 2001). Accordingly, co-culture of RPE cells with bovine choroidal endothelial cells stimulated the formation of the typical mesh network of the choriocapillaris. Interestingly, this stimulatory angiogenesis was attenuated by inhibition of VEGF and FGF, but was not affected by TGFβ neutralization (Sakamoto *et al.*, 1995).

Further examination of the molecular signals from the RPE which may regulate choroid development and maintenance focused on proteins that are secreted to its basal side (Blaauwgeers *et al.*, 1999). VEGF polar secretion (Blaauwgeers *et al.*, 1999) was established to be essential for choroid development, since mice with conditional mutagenesis of VEGF in the RPE failed to develop choriocapillaris and demonstrated a microphthalmic phenotype. This was not observed following Hif1a mutagenesis and was thus considered independent of the Hif1a pathway (Marneros *et al.*, 2005). In addition to VEGF expression by the RPE during choroid development, its expression is maintained in the adult, suggesting a continuous role for VEGF in maintenance of the choroidal vasculature (Saint-Geniez *et al.*, 2006, Zhao and Overbeek, 2001). Different isoforms of VEGF are generated by alternative exon splicing and contain 0 to 2 heparan sulfate sites, which affect their solubility (Tischer *et al.*, 1991). The main isoforms expressed by the adult RPE are the soluble isoforms VEFG164 and VEFG120 (Saint-Geniez *et al.*, 2006). Examination of mice expressing only the insoluble isoform, VEFG188, emphasized the need for soluble isoforms since their absence resulted in VEGF's inability to diffuse through Bruch's membrane and led to decreased phosphorylation of the VEGF receptor VEFGR2 in the choroid (Saint-Geniez *et al.*, 2009).

FGF was shown to be a more potent angiogenic factor than VEGF (Cao *et al.*, 2003). Nevertheless, the role of FGF2 in choroid vascularization is currently under debate; FGF2 administration induces choroidal angiogenesis (Soubrane *et al.*, 1994), while Fgf2 null mice demonstrate normal development of choroid vessels (Ozaki *et al.*, 1998). This contradiction may be settled by the ability of other FGF proteins, such as FGF5, to compensate for the loss of FGF2. This hypothesis was supported when transgenic mice expressing a dominant negative FGFR1 demonstrated delayed and decreased vascularization of the choroid (Rousseau *et al.*, 2000). However, it is still unclear whether FGF affects choroidal angiogenesis directly or via other signaling pathways of the RPE. One suggestion for such a pathway is the coupling of FGFR2 upon activation with Ca^{2+} L-type channels (Rosenthal *et al.*, 2001), which have been shown to promote VEGF secretion from RPE cells (Strauss *et al.*, 2003).

Generating RPE from pluripotent stem cells for the modeling of RPE development and diseases, and for cell-based therapy for retinal degeneration

The 3D OC presents an excellent model to study early stages of retina and RPE development, while late stages of retinogenesis, as well as complete differentiation, are limited under long-term culture conditions. However, there have been great advances in the generation of differentiated RPE from human pluripotent stem-cells using 2D cultures, which are currently being tested in clinical settings as well as employed to model RPE diseases (extensively reviewed in Carr *et al.*, 2013; Wahlin *et al.*, 2014; Zhang *et al.*, 2013). Initially, the differentiation protocols for RPE in 2D culture were generated by spontaneously differentiating colonies (Buchholz *et al.*, 2009, Kawasaki *et al.*, 2002, Klimanskaya *et al.*, 2004). Later, enhanced differentiation efficiency was achieved by stepwise protocols that took into account knowledge of RPE-differentiation mechanisms in vivo, as well as factors that inhibit epithelial to mesenchyme transition (EMT) and support cell survival in culture (Buchholz *et al.*, 2009; Buchholz *et al.*, 2013; Idelson *et al.*, 2009; Lamba *et al.*, 2006; Maruotti *et al.*, 2013; Osakada *et al.*, 2009; Zahabi *et al.*, 2012; Zhu *et al.*, 2013; reviewed in Parvini *et al.*, 2014). Important advances were made with the introduction of reporters for RPE differentiation, which are triggered by promoters of RPE genes such as *RPE65*, *Mitf D*, *Otx2* and *Bestrophin* (Leach *et al.*, 2015, Zhang *et al.*, 2014). These reporters further enhanced the ability to monitor differentiation following manipulation of the cells with small molecules, ligands of signaling pathways or expression of TFs to enhance effective differentiation, and even direct conversion to RPE from fibroblasts (Maruotti *et al.*, 2015, Zhang *et al.*, 2014).

The successful generation of RPE from pluripotent cells allows researchers to model diseases ex vivo by employing patient-derived iPSCs. Cellular models for RPE dystrophies have been generated for Best disease (Singh *et al.*, 2013), and mutations in frizzled-related proteins (Li *et al.*, 2014) and Mer tyrosine kinase receptor (MERTK; Lukovic *et al.*, 2015). The findings obtained from these cellular models have contributed to our understanding of disease pathologies in humans and are expected to provide useful tools to test therapies (Lukovic *et al.*, 2015).

The progress in generating RPE from stem cells has led to recent clinical trials in which these cells are used for therapy of AMD (Lu *et al.*, 2016, Song *et al.*, 2015). However, variability in differentiation efficiency among donors has to be considered, as well as the possibility that growth factors and chemicals that stimulate differentiation or expression of stem cell genes are not completely inactivated and that unexpected gene activities may appear, at least in some of the genetic backgrounds. These concerns have indeed had a major impact on the ongoing clinical trial with the detection of oncogene expression in RPE generated from iPSCs; a major challenge is to determine the level of differentiation of these cells in culture and following transplantation (Miyagishima *et al.*, 2016). Another major challenge is to determine the capacity of the stem cell-derived RPE to functionally integrate the choroid and PRs for long-term tissue replacement. Future studies should therefore aim to provide a deeper understanding of the crosstalk between RPE and PRs and between RPE and choroid during development, differentiation and maintenance. Such findings will contribute to our understanding of the mechanism of organ formation, provide insights into the etiology of congenital retinal diseases and hopefully, lead to improved approaches for cell-replacement therapies.

To conclude, although great advances have been made in developing stem cell-based therapies for RPE diseases, there remains a need for a more comprehensive basic science understanding of the intrinsic program, as well as of the signaling to and from the RPE during eye development. Resolving TFs, miRNAs and epigenetic components that regulate RPE differentiation and

the generation of adjacent lineages is critical for trials aimed at reducing heterogeneity between iPSCs, to improve differentiation and safety and to ensure proper integration and function within the host tissue to assure effective and safe cell-replacement therapies.

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