Published in final edited form as:

Exp Eye Res. 2014 September ; 126: 5–15. doi:10.1016/j.exer.2014.04.021.

Plasma membrane protein polarity and trafficking in RPE cells: Past, present and future

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Abstract

The retinal pigment epithelium (RPE) comprises a monolayer of polarized pigmented epithelial cells that is strategically interposed between the neural retina and the fenestrated choroid capillaries. The RPE performs a variety of vectorial transport functions (water, ions, metabolites, nutrients and waste products) that regulate the composition of the subretinal space and support the functions of photoreceptors (PRs) and other cells in the neural retina. To this end, RPE cells display a polarized distribution of channels, transporters and receptors in their plasma membrane (PM) that is remarkably different from that found in conventional extra-ocular epithelia, e.g. intestine, kidney, and gall bladder. This characteristic PM protein polarity of RPE cells depends on the interplay of sorting signals in the RPE PM proteins and sorting mechanisms and biosynthetic/recycling trafficking routes in the RPE cell. Although considerable progress has been made in our understanding of the RPE trafficking machinery, most available data have been obtained from immortalized RPE cell lines that only partially maintain the RPE phenotype and by extrapolation of data obtained in the prototype Madin–Darby Canine Kidney (MDCK) cell line. The increasing availability of RPE cell cultures that more closely resemble the RPE *in vivo* together with the advent of advanced live imaging microscopy techniques provides a platform and an opportunity to rapidly expand our understanding of how polarized protein trafficking contributes to RPE PM polarity.

Keywords

retinal pigment epithelium; reversed polarity; RPE culture; vectorial transport; clathrin adaptors; sorting signals; stem cells

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1. Structural and functional polarity of RPE cells

1.1. The specialized polarity of RPE cells is key for retinal homeostasis

The retinal pigment epithelium (RPE) comprises a monolayer of polarized pigmented epithelial cells interposed between the photoreceptors (PR) and the fenestrated choroid capillaries (Fig. 1). One RPE cell provides support for 30–50 adjacent PR. The RPE performs an amazing variety of support functions for the neural retina (Sparrow et al., 2010; Strauss, 2005).

(i) **Selective blood-retinal barrier**, which depends on the possession of functional tight junctions (see review by Rizzolo 2014); (ii) **Absorption of stray light**, essential for vision, by the abundant melanin granules; (iii) **Retinoid processing and recycling**, key for the visual cycle; (iv) Vectorial transport of nutrients and metabolites, essential for generating the appropriate ionic environment for PR's light-sensing function; and (v) Receptor-mediated engulfment of shed outer segments (see Finnemann's review in this issue), essential for the regeneration of PR, that compensates for the highly oxidative environment of the retina. All of these RPE functions are essential for retinal homeostasis.

To perform these multiple functions, RPE cells display a characteristic structural and biochemical polarity, which differs in different regions of the retina and depending on the adjacent PR type. For example, RPE is a high cuboidal epithelium in the fovea, but transitions to a lower cuboidal type at the equatorial regions of the human retina (Feeney-Burns et al., 1984). RPE cells display extremely long microvilli (20–30 μm) that surround the rod outer segments; in contrast, RPE cells surround the cone outer segments with large apical folds (Spitznas and Hogan, 1970; Steinberg et al., 1977). The basal PM of RPE cells displays highly convoluted microinfolds that increase drastically the surface area of this domain. The formation and maintenance of both microvilli and basal infolds depends on the presence of active ezrin and the ezrin-associated PDZ-containing proteins EBP50 and SAP-97, respectively (Bonilha and Rodriguez-Boulton, 2001; Bonilha et al., 1999). RPE cells and the underlying choroid capillaries participate in the synthesis of Bruch's membrane (BM) (Takei and Ozanics, 1975), formed by several distinct layers. Maintenance of a permeable BM is key for the movement of nutrients, metabolites and oxygen between the choriocapillaris and the outer retina, and depends on a fine-tuned balance between synthesis of BM components and their degradation by metalloproteinases secreted by the RPE (Booij et al., 2010).

Like other epithelia, RPE display one primary cilium (PC) at the apical domain. The PC is an antenna-like organelle involved in the organization of signaling pathways (e.g. Hedgehog) and the transduction of environmental stimuli (mechano, chemo, and osmosensory functions) (Gerdes, 2009; Goetz, 2010). Early studies reported that adult RPE display a PC that is spatially correlated with the presence of cones in the neural retina (Fisher and Steinberg, 1982). More recent immunofluorescence analysis on mouse RPE flatmounts using antibodies against acetylated tubulin concluded that RPE PC is present in developing RPE but disappears in the mature retina (Nishiyama et al., 2002). However, our preliminary studies (Lehmann-Mantaras et al., 2013) suggest that the reported absence of PC in mature RPE is largely an artefact resulting from mechanical peeling after neural retinal

removal. Indeed, recent experiments suggest that the PC may have important functions in retinal development, as previously shown for skin (Ezratty et al., 2011). Nasonkin et al. (2013), reported that RPE-specific knock-out of DNA methyltransferase 1 (DNMT1) disrupts RPE polarity and prevent secondarily the formation of PR outer segments (Nasonkin et al., 2013). Interestingly, RNA levels of Indian Hedgehog (IHH) in RPE/choroid (which were not analysed separately) were concomitantly altered. As IHH is believed to be produced by the choroid endothelium (CE) (Dakubo et al., 2008) and RPE cells express the HH receptor machinery (GL, IB and ERB, preliminary results), these studies suggest that IHH, secreted by CE cells interacts with specific receptors in RPE's PC, to promote RPE and PR differentiation. Hence, understanding the role of PC in RPE development and physiology is a very important future goal in retinal research.

In addition to their characteristic structural polarity, RPE cells display a highly polarized distribution of ion and nutrient transporters, channels and receptors (Strauss, 2005) that significantly differs from the configurations observed in most extraocular epithelia. The trafficking mechanisms underlying this discrepancy have only partially been unravelled and are discussed below.

1.2. Functional polarity of RPE

A major function of the RPE is to transport a net amount of fluid out of the subretinal space, while keeping tightly controlled the K^+ and lactate levels in this compartment. Fluid transport out of the subretinal space maintains a negative hydrostatic pressure within this compartment, essential for the adhesion between RPE and PR; failure of this transport system leads to retinal edema and retinal detachment (Hamann, 2002; Strauss, 2005). The strategy used by the RPE to transport fluid between the apical and the basal domain is different from that employed by other fluid transporting epithelia, i.e. kidney proximal tubule or gall bladder. In the latter, passive diffusion of Na^+ into the cell via apical channels in tandem with active extrusion of Na^+ via a basally located Na,K-ATPase provides the driving force for secondary fluid transport (Diamond and Bossert, 1967). In RPE, chloride transport, proposed to occur via a tandem of apically located Na, K, Cl cotransporter and basal chloride transporters (CLC2, CFTR), is thought to provide the driving force for secondary fluid transport (Hamann, 2002; Strauss, 2005) (Fig. 2). The basal localization of CFTR (Blaug et al., 2003) is opposite to that found in other epithelia, e.g. intestine and lung, where CFTR is apically localized (Levin and Verkman, 2006). Water transport is mediated by Aquaporin-1 water channels, which have been found to be apically expressed both in hfRPE primary cultures (Stamer et al., 2003), and more recently in stem cell-derived RPE cells (Juuti-Uusitalo et al., 2013). The localization of the Na,K-ATPase in RPE cells is also unconventional, as it is localized to the apical surface, rather than to the basolateral PM, the characteristic localization of the sodium pump in most epithelia (Bok, 1982; Gundersen et al., 1991; Ostwald and Steinberg, 1980; Rizzolo, 1990). The apical localization of Na,K-ATPase in RPE cells is believed to be associated to the process of phototransduction. When photoreceptors are dark-adapted, there is an intense and continuous intracellular flux of Na^+ from the OS to the inner segments via the connecting cilium that is balanced by the efflux of K^+ at the inner segments, which reduces the polarization of the PR plasma membrane. A high Na^+ photoreceptor environment is required to support this process (Ames et al., 1992).

A second major function of the RPE is to tightly regulate the pH of the subretinal space. This function requires the removal of large amounts of lactate produced by the neural retina. Normally, lactate levels in the subretinal space are 3–10-fold higher than in the blood (Strauss, 2005). Lactate transport is carried out by proton-coupled monocarboxylate transporters (MCT) distributed in a polarized fashion, MCT1 at the apical PM and MCT3 at the basolateral PM (Philp et al., 1998; Yoon et al., 1997). Interestingly, the apical localization of MCT1 is opposite to that found in other epithelial cells, where it is found on the basolateral PM (Deora et al., 2005; Garcia et al., 1995; Koho et al., 2005).

RPE cells transport a net amount of glucose in the apical direction, which is mediated by the glucose transporter Glut-1 localized on both apical and basolateral PM domains (Sugasawa et al., 1994; Takata et al., 1992; Tserentsoodol et al., 1998). RPE cells transport a net amount of bicarbonate in the apical to basolateral direction, a result of the polarized distribution of bicarbonate transporters NBC1 (SLC4A4) to apical and NBCn1 (SLC4A7) to basolateral PM domains (Bok et al., 2001; Adijanto et al., 2009). Surprisingly little is known about the amino acid transporters expressed by RPE cells.

RPE cells secrete key growth factors in a polarized fashion, e.g. PEDF in the apical direction, necessary for the maintenance of differentiated PR (Becerra et al., 2004) and VEGF in the basolateral direction, necessary for the maintenance of functional choroid capillaries (Blaauwgeers et al., 1999; Kurihara et al., 2012; Sonoda et al., 2010).

In addition to the examples mentioned above, RPE cells display a non-conventional distribution of several other PM proteins (Table 1). A number of PM proteins that are sorted basolaterally in most epithelia have an apical or non-polar distribution in RPE cells. These include the Transferrin Receptor (TfR) (Perez Bay et al., 2013), the Coxsackie-Adenovirus Receptor (CAR) (Diaz et al., 2009), JAM C (Daniele et al., 2007) and the Neural Cell Adhesion Molecule (NCAM) (Gundersen et al., 1993). The apical localization of CAR and TfR results from the transcytosis of these receptors to the apical PM from common recycling endosomes (CRE) (Diaz et al., 2009; Perez Bay et al., 2013).

1.3. Compartments and mechanisms that determine the surface polarity of PM proteins in RPE cells

Studies carried out in the prototype kidney epithelial cell line MDCK have identified subcellular compartments and mechanisms involved in the sorting and distribution of apical and basolateral PM proteins (Rodriguez-Boulán et al., 2005) (Fig. 3). PM proteins are produced in the Endoplasmic Reticulum (ER) and are transported by COP-2 coated carrier vesicles to the Golgi complex. At a distal Golgi compartment, the *trans* Golgi Network (TGN), apical and basolateral PM proteins are sorted into different routes to their respective PM domain. Most of these routes involve intermediate endosomal compartments (Apodaca et al., 2012; Rodriguez-Boulán and Macara, 2014). Basolateral proteins are targeted either directly from the TGN to the basolateral PM or indirectly via common recycling endosomes (CRE) (Gonzalez and Rodriguez-Boulán, 2009). Apical PM proteins are targeted either directly to the apical PM or via intermediate apical recycling endosomes (ARE) or apical sorting endosomes (ASE) (Weisz and Rodriguez-Boulán, 2009).

In addition to these polarized biosynthetic routes, epithelial cells display polarized endocytic routes (Apodaca et al., 2012; Rodriguez-Boulán and Macara, 2014). An example of a fast recycling apical endocytic receptor is megalin, which, as a dimer with cubilin, conforms a “Velcro”-like mechanism to recover proteins from the kidney ultrafiltrate (Christensen et al., 2012). Fast recycling receptors are found more frequently on the basolateral PM, where they function to deliver a variety of blood nutrients to the cell, e.g. transferrin and low density lipoprotein (LDL) receptors (Apodaca et al., 2012; Rodriguez-Boulán and Macara, 2014). Apical and basolateral PM receptors are internalized into spatially segregated apical sorting endosomes (ASE) and basal sorting endosomes (BSE), respectively, and are mixed in perinuclear common recycling endosomes (CRE), where they are sorted by sorting signals similar to those employed in the biosynthetic pathways (see below) into different recycling routes to the PM (Fig. 3). The apical route includes an additional trafficking step through apical recycling endosomes (ARE), which have a more neutral luminal pH than CRE and sorting endosomes. Recently, we have shown that the biosynthetic route of rhodopsin, an apical PM protein when expressed in epithelial cells (MDCK), involves an intermediate stop at ARE, from where it is transported in vesicles that require dynamin 2 for fission from ARE and Rab11a for docking at the apical PM (Thuenauer et al., 2014).

Epithelial cells vary considerably in terms of how they traffic apical PM proteins. Whereas some epithelial cells use predominantly vectorial routes (direct or transendosomal) to the apical membrane (e.g. MDCK cells), other epithelial cells use transcytotic routes, i.e., the apical protein is first delivered to the basolateral membrane and is then transcytosed to the apical domain (e.g. liver, intestine epithelial cells) (Weisz and Rodriguez-Boulán, 2009). The RPE is known to use the transcytotic route for the model apical PM protein influenza hemagglutinin (Bonilha et al., 1997), but how widespread is the use of this route by RPE cells is still unknown. Also unknown to a large extent, is the mechanisms underlying the preference for transcytotic or direct vectorial routes in different epithelia.

Many of the signals and mechanisms that direct PM proteins along their biosynthetic and recycling routes have been elucidated (Apodaca et al., 2012; Mellman and Nelson, 2008; Mostov et al., 2003; Rodriguez-Boulán and Macara, 2014; Rodriguez-Boulán et al., 2005). Apical sorting signals are usually recessive to baso-lateral sorting signals and may be present in the ectodomain, membrane-associated domain or cytoplasmic domain of the PM protein (Weisz and Rodriguez-Boulán, 2009). In the ectodomain, they usually consist of N-glycans or O-glycans (Scheiffele et al., 1995; Yeaman et al., 1997). Glycans are recognized by lectins such as galectin 3 (Delacour et al., 2008, 2006), galectin 4 (Delacour et al., 2005; Stechly et al., 2009) and galectin 9 (Mishra et al., 2010). Specialized transmembrane domains or lipid anchors such as glycosyl-phosphatidylinositol (GPI) may also act as apical sorting signals (Lisanti et al., 1989; Scheiffele et al., 1997), and are sorted through interaction with cholesterol-sphingolipid domains called “rafts” (Simons and Ikonen, 1997). Finally, some apical sorting signals are found in the C-terminal domain; this is the case for rhodopsin (Sung et al., 1994; Tai et al., 1999), which is sorted apically by the MT motor dynein (Tai et al., 2001) and Megalin (Marzolo et al., 2003; Takeda et al., 2003) for which the apical signal decoding mechanism has not been identified yet.

Basolateral signals are short, simple peptide motifs that often resemble endocytic signals such as those used by PM receptors for internalization via clathrin coated pits (Gonzalez and Rodriguez-Boulan, 2009; Rodriguez-Boulan et al., 2005). Like clathrin-mediated endocytosis, TGN sorting of basolateral proteins requires clathrin and clathrin adaptors, such as AP-1A and AP-1B (Carvajal-Gonzalez et al., 2012; Deborde et al., 2008; Fölsch et al., 1999; Gravotta et al., 2012; Ohno et al., 1999; Rodriguez-Boulan et al., 2013). These twin adaptors are heterotetramers that share three subunits (β 1, σ 1 and γ) and differ in the possession of different medium subunits (μ 1A and μ 1B). AP-1A is ubiquitously expressed across tissues and organs whereas AP-1B is expressed only in epithelial cells (Ohno et al., 1999). Their functions are partly overlapping, albeit the nature of the overlap is under discussion. Evidence accumulated over the past two decades suggests that the two adaptors function in different trafficking routes and compartments, AP-1A in biosynthetic trafficking at the TGN and AP-1B in post endocytic recycling and transendosomal biosynthetic trafficking at CRE (Ang et al., 2004; Fölsch et al., 2001, 2003; Gan et al., 2002; Fölsch et al., 2003; Gravotta et al., 2007, 2012). However, recent work by Bonifacino and coworkers suggests that both adaptors function at TGN and CRE and differ only in their ability to bind different basolateral cargo proteins (Bonifacino et al., 2014; Guo et al., 2013; Rodriguez-Boulan et al., 2013). Future work should address this controversy.

Interestingly, RPE lacks AP-1B (Diaz et al., 2009); two other epithelia have been shown to lack this adaptor, the kidney proximal tubule (Schreiner et al., 2010) and hepatocytes (Ohno et al., 1999). Since AP-1A cannot compensate fully for the absence of AP-1B (Carvajal-Gonzalez et al., 2012; Gravotta et al., 2012), several basolateral PM proteins are missorted to the apical PM (see Table 1). One of these proteins is transferrin receptor, which utilizes a transcytotic route mediated by MT and the plus end kinesin KIF16B, as well as by the small GTPase rab 11a (Perez Bay et al., 2013).

The mechanisms that determine the reversed polarity of other PM proteins in RPE are only starting to be elucidated. Our laboratories (NP and ERB) described the signals that mediate polarized sorting of monocarboxylate transporters in MDCK and RPE cells. Like Na,K-ATPase, these transporters possess a multispan transporting subunit (MCT1,3, or 4) and a single span heavily glycosylated chaperone subunit (CD147). Our studies identified a novel leucine-based basolateral sorting signal in the MCT chaperone CD147, that is recognized by MDCK cells but not by RPE cells (Deora et al., 2004). CD147 determines the final polarity of MCT1, which lacks sorting signals and thus depends on CD147 for its surface polarity (Deora et al., 2005). These studies also identified strong basolateral sorting signals in MCT3 and MCT4 that determine the surface localization of the complex in both MDCK and RPE cells (Castorino et al., 2011). In spite of many years of research, the sorting signals and mechanisms that mediate polarized sorting of Na,K-ATPase are still poorly understood.

2. Models to study RPE polarity

The ability of epithelial cells to form polarized monolayers in culture, either on polycarbonate filters or within 3D Collagen or Matrigel matrices, has allowed the study of their polarized properties (Bryant and Mostov, 2008; Rodriguez-Boulan et al., 2005). Ideally, the culture conditions must reproduce the properties of the original epithelium *in*

situ as close as possible. For instance, the culture of epithelial cells on plastic or glass surfaces is usually not suitable because when the epithelial cells form fully differentiated tight junctions, the basolateral membrane does not have access to the media. This may restrict the uptake of key nutrients via transporters polarized to the basolateral PM. To circumvent this problem, epithelial cells are usually seeded over permeable supports (e.g. polycarbonate filter chambers or Transwells), which allow culturing of the cells in media designed to resemble the apical and basal environments of the epithelium *in situ* (Rodriguez-Boulan et al., 2005). A number of functional, biochemical and optical assays can be performed on filter-grown monolayers to evaluate their polarized epithelial properties, e.g. the polarity of their protein secretion, the steady state localization of their PM proteins, the polarized delivery of PM proteins to apical and basolateral domains and the integrity and maturity of their tight junction tightness (Deora et al., 2006; Kreitzer et al., 2006) (Table 2). These include biochemical assays such as domain selective biotinylation and surface immune-precipitation assays, morphological assays and the transepithelial electrical resistance (TER) assay.

Polarized RPE culture systems have been developed that recapitulate to a variable extent the properties of polarized RPE cells *in situ*. The closest to RPE *in situ* is to place the RPE/choroid membrane in a Ussing chamber to study RPE transport properties (Joseph and Miller, 1991). This approach has been used with RPE obtained from several species, i.e. frog, pig, human, chicken, monkey, etc (Gallemore and Steinberg, 1993; Immel and Steinberg, 1986; Treumer et al., 2012; Tsuboi and Pederson, 1988). However, to obtain a monolayer of pure RPE cells *in vitro*, RPE cells must be extracted from the underlying choroid avoiding cell contaminants and maintaining their tissue-specific properties. A common feature to all diploid cell cultures is their tendency to lose tissue-specific properties after isolation and expansion. This is also true for RPE cells, as the disruption of cell-cell contact during cell passage triggers a process of epithelial to mesenchymal transition (EMT) resulting in loss of RPE identity that can become irreversible (Adijanto et al., 2012; Gallagher-Colombo et al., 2010; Grisanti and Guidry, 1995; Tamiya et al., 2010). Pioneering research by Pfeffer demonstrated that RPE cells retain their phenotype when the cell-cell interaction is maintained during tissue harvesting (Pfeffer et al., 1986). Once the primary culture is established, RPE cells can be passaged by changing to a low-calcium media, a condition that induces the release of clusters of proliferating RPE cells into the culture medium that can be collected for subculturing without the requirement of proteases (Flannery et al., 1990; Pfeffer et al., 1986). More recently, Blenkinsop et al. (2013) reported that mild treatment with dispase generates fragments of RPE sheets from the primary culture that can be expanded by plating at ~50% subcon-fluency without significant loss in RPE identity. Contrary to human adult RPE cells, RPE obtained from human fetal eyes are more likely to recover the RPE-phenotype upon expansion after full cell dissociation (e.g. by trypsin) (Maminishkis et al., 2006). Some immortalized RPE cell lines have been produced, but all those currently available have a transcriptome very different from that of RPE *in situ* (Strunnikova et al., 2010) and some (e.g. hTERT-RPE-1, D407) lack even basic epithelial polarity features (Davis et al., 1995). None of these primary cell culture or immortalized cell models is therefore ideal; hence, there is considerable room for improvement in the future. Some features are common to all models. First, the development of polarity takes about 1

month post-confluence to be completed in both ARPE-19 (Dunn et al., 1996; Luo et al., 2006) and RPE primary cultures (Ablonczy et al., 2011; Burke, 2008; Hu and Bok, 2001; Maminishkis et al., 2006; Sonoda et al., 2009), comparable with the polarization time of Caco-2 intestinal cells (Sambuy et al., 2005) but considerably longer than for MDCK cells (3–4 days). This likely reflects the longer time needed to upregulate the expression of genes associated to fully differentiated cells and to develop the molecular machinery involved in membrane protein localization in RPE and intestinal cells. Understanding the molecular basis of RPE maturation will greatly help to elucidate the relative slow maturation of the polarized trafficking machinery in these cells. Second, both primary cultures and cell lines have a tendency to lose RPE-specific properties with successive passages as outlined above. For this reason, the source of cells is not unlimited as with happens with commonly used cell lines. Finally, like other epithelial cells, once fully polarized, RPE cells are difficult to transfect with conventional methods (liposomes/electroporation). Hence polarized trafficking studies in RPE would benefit from flexible and efficient vectors to transfect RPE cells; adenoviruses and lentiviruses hold promise in this regard. Interestingly, RPE cells are easily infected with adenoviruses as they express the corresponding receptor, coxsackie-adenovirus receptor (CAR) at the apical surface as a consequence of the lack of expression of the basolateral sorting adaptor AP-1B (Table 1) (Diaz et al., 2009).

For all of these reasons, and because analysis of trafficking by biochemical assays requires a large number of epithelial cells plated on filters, most data available on RPE protein trafficking to date has been obtained by extrapolation of data obtained with the Madin–Darby canine kidney (MDCK). MDCK cells have been engineered to lack AP-1B, thus resembling RPE cells in this regard (Diaz et al., 2009; Gravotta et al., 2007). The studies on MDCK cells, when properly complemented with key experiments in RPE cells, have provided an important window to explore the RPE trafficking machinery.

2.1. RPE cell lines

A number of rodent and human RPE-immortalized cell lines are available. These include the rat-derived cell line RPE-J (Nabi et al., 1993) and the human RPE-derived cell lines D407 (Davis et al., 1995), hTERT-RPE1 (Bodnar et al., 1998), and ARPE-19 (Dunn et al., 1996). Among these, only RPE-J and ARPE-19 show sufficient preservation of polarity and barrier function for studies of protein trafficking.

2.1.1. RPE-J—The RPE-J cell line was derived from primary cultures of rat RPE transformed with a temperature-sensitive SV40 virus (Nabi et al., 1993). The cell line exhibits a transformed phenotype very convenient for cell expansion when cultured at 33 °C. Upon switching to 40 °C, the oncogenes are inactivated and thus the cells differentiate into polarized RPE. RPE-J cells exhibits several RPE-specific properties such as phagocytosis, expression of some RPE-specific genes and a TEER of over 200 Ω cm². Studies performed in this cell line were critical to determine the basolateral localization of bestrophin-1 (Marmorstein et al., 2000). Additionally, the RPE-J cell line appears to express most of the RPE machinery for OS phagocytosis (see Mazzoni, 2014). A clear disadvantage is that RPE-J cells fail to reproduce some structural and functional RPE polarity features. Their microvilli are short and sparse, the basal infoldings are almost absent (Bonilha et al., 1999;

Nabi et al., 1993; Marmorstein et al., 1998), and the localization of N-CAM, CD147 (Marmorstein et al., 1998) and Na, K-ATPase is non-polar (Nabi et al., 1993). Although a systematic study to compare the transcriptome of RPE-J cells with that of RPE *in situ* was not carried out, it is clear that there are substantial differences as these cells completely lack E-cadherin (Marrs et al., 1995). E-cadherin, characteristically expressed by most body epithelia, is highly expressed by human fetal RPE (Strunnikova et al., 2010) but is variously expressed by RPE cells of different origins both *in vitro* and *in situ* (Burke and Hong, 2006; Burke, 2008; Burke et al., 1999).

2.1.2. ARPE-19—The ARPE-19 cell line was obtained from a spontaneously transformed human RPE primary culture (Dunn et al., 1996). Among the advantages of this cell line are their normal karyotype, fast proliferation rates and preservation of several RPE-specific features (Dunn et al., 1998). ARPE-19 cells require 4–6 weeks in confluent culture on filters to acquire a ‘cobblestone’ epithelial appearance, to develop functional tight junctions with a TER~50 Ω cm² (Dunn et al., 1996) lower than the estimated TER of native RPE (Quinn and Miller, 1992). However, ARPE-19 cells have significant deficiencies as a bona fide RPE model. 74 out of 154 RPE-signature genes are under expressed when compared with primary cultures or human RPE *in situ* (Strunnikova et al., 2010). Some examples of the downregulated genes are the basolateral transporter bestrophin-1, the melanogenesis proteins pMel17 and dopachrome tautomerase, and the cell adhesion molecule E-cadherin (Strunnikova et al., 2010). The trafficking machinery is likely different from RPE *in situ*, since the Na, K-ATPase was reported to be basolateral in ARPE-19 cells with the exception of a small subset of pigmented (possibly more differentiated) cells, in which it was found to be apical (Ahmado et al., 2011).

2.2. Primary cultures of RPE cells

Primary hFRPE cells are considered the most representative culture model of native RPE cells. The culture system was first characterized by Bok and coworkers (Flannery et al., 1990; Hu and Bok, 2001) and later modified by others (Maminishkis et al., 2006; Sonoda et al., 2009). When fetal RPE cells are cultured at confluency they form polarized monolayers of heavily pigmented epithelial cells, with cobblestone appearance and abundant apical microvilli (Hu and Bok, 2001; Maminishkis et al., 2006; Sonoda et al., 2009). In contrast with ARPE-19, the gene expression profile of hFRPE resembles more closely the native tissue, with only a set of 34 out of 154 ‘signature genes’ under represented (Strunnikova et al., 2010). When seeded at high density (>150,000 cells/cm²) hFRPE develop functional tight junctions evidenced by a progressive increment of the TER to about ~800 Ω cm². For comparative purposes, the TER of hFRPE measured *in situ* can reach 486 Ω cm² (Quinn and Miller, 1992). Analysis of the distribution of polarized membrane proteins has shown that hFRPE correctly localize the proteins Na,K-ATPase (Hu and Bok, 2001; Maminishkis et al., 2006; Sonoda et al., 2009), CAR (Diaz et al., 2009) and MCT1 (Deora et al., 2005; Maminishkis et al., 2006) to the apical domain and bestrophin-1 (Gamm et al., 2008) and MCT3 (Adijanto et al., 2012; Deora et al., 2005) to the basolateral domain. Additionally they secrete asymmetrically α B crystalline (Gangalum et al., 2011; Sreekumar et al., 2010) and PEDF (Maminishkis et al., 2006) to the apical medium; and VEGF (Ablonczy et al., 2011; Maminishkis et al., 2006; Sonoda et al., 2010) to the basolateral medium (for a recent

review see (Kay et al., 2013)). We utilize RPE primary cultures provided in T-25 flasks by Miller's laboratory (Maminishkis et al., 2006). We consistently generate first passage cultures from the initial primary culture by trypsin dissociation and high density cell seeding (on filters, 200,000/cm²) to prevent epithelial–mesenchymal transition (EMT). After 4 weeks in culture the RPE monolayers become fully polarized and are ready to be used. While the original protocols recommend using filters coated with recombinant laminin (Hu and Bok, 2001) we have found that RPE cells attach readily to the bare filter with no impact on the TER development, polarity or expression of RPE “signature” genes, defined as genes highly expressed in RPE cells in comparison with to most human tissues (Strunnikova et al., 2010).

As discussed above, monolayers of well polarized RPE primary cultures or ARPE-19 cells can be obtained after 4 weeks in culture (Ablonczy et al., 2011; Hu and Bok, 2001; Maminishkis et al., 2006; Sonoda et al., 2009). An important consideration is that when fully differentiated, RPE cells become particularly resistant to gene transfection via commonly used methods such as liposome gene delivery and electroporation. For this reason we use a lentiviral system to deliver exogenous proteins and specific shRNA, with a transduction efficiency close to 100%. Another limitation of hRPE primary cultures is that they must be used at low passages (P1 preferentially, P2 at most) to avoid EMT development with further passaging (Adijanto et al., 2012; Gallagher-Colombo et al., 2010; Tamiya et al., 2010). In addition, because the RPE cells originate from anonymous donors that are not usually genotyped, experiments should be repeated on different cultures or performed on populations of mixed cells to obtain representative results (as discussed by Hu and Bok, 2013). Lastly, the heavy pigmentation can be a problem for immunofluorescence microscopy of intracellular or basolateral membranes, although there are protocols available that allow melanosome quenching (Mishima et al., 1999). Overall, hRPE primary cultures have greatly impacted the study of RPE cells, and constitute a very powerful tool to explore the polarity and trafficking mechanisms in RPE cells.

2.3. RPE from human pluripotent stem cells

RPE cells have been derived from human pluripotent stem cells (HPS), either from embryonic (hES) or induced pluripotent cells (iPS). When the beta FGF that is normally required to maintain the pluripotency of the stem cells is removed from the culture medium, a small population of HPS will spontaneously differentiate into pigmented RPE-like cells after several weeks (Klimanskaya et al., 2004). The efficiency of this protocol has been recently improved by a number of laboratories by first using a variety of specific Wnt and BMP inhibitors to induce a pool of neuroectodermal progenitors (Meyer et al., 2009), followed by treatment with Activin, a TGF beta family signaling factor, to promote RPE differentiation (Buchholz et al., 2013; Idelson et al., 2009). Although with some differences among the protocols and lines used, the HPS-RPE cells are similar to the native RPE, as they form cobblestone monolayers of fully pigmented epithelial cells, and express multiple genes characteristic of native RPE markers such as CRALBP, RPE65, tyrosinase and the transcription factors MITF and OTX2 (for a review see (Bharti et al., 2011)). Furthermore, a number of functional studies revealed that HPS-RPE cells can phagocytose rod outer segments, develop functional tight junctions and rescue visual function in rodent models of

retinal degeneration (Carr et al., 2009; Li et al., 2012; Lu et al., 2009). The suitability of HPS-RPE cells to analyse polarity and trafficking pathways has not been assessed yet. However, preliminary evidence is promising as they develop functional tight junctions and localize Na,K-ATPase, integrin $\alpha v \beta 5$ and CD147 to the apical domain (Buchholz et al., 2009; Kokkinaki et al., 2011) and bestrophin-1 to the basolateral domain (Maruotti et al., 2013; Singh et al., 2013). There are methodological issues that remain to be solved, such as the low yield and extended time required for RPE differentiation (Bharti et al., 2011). However, HPS-RPE cells constitute a promising experimental platform to explore trafficking and polarity mechanisms as they offer an unlimited source of cells for RPE cultures and provide a unique *in vitro* system to model patient-specific RPE diseases.

3. Future directions

Clearly, a task for the future is the direct analysis of trafficking routes in cultured RPE cells with a phenotype as close as possible to RPE cells *in situ*. This is now possible thanks to the availability of RPE culture cell models that resemble closely the phenotype of native RPE and advanced microscopy techniques that allow for the recording of the subcellular localization of a given cargo protein, tagged with a fluorescent protein, in multiple regions of interest in the RPE monolayer. It is now possible to synchronize the trafficking of a cargo protein using reversible aggregation via either FM domains (Rivera et al., 2000; Thuenauer et al., 2014) or biotin trapping systems (Boncompain et al., 2012). The passage of the cargo protein through a given subcellular compartment, e.g. TGN, CRE, ARE, can be studied using markers for these compartments tagged with a fluorescent protein with a spectrum different from that utilized for the cargo protein. The technology for this type of studies is quickly developing, so we can foresee a fast increase in our knowledge of how RPE trafficking contributes to RPE polarity.

Acknowledgements

This work was supported by NIH grants GM34107, EY08538 and EY022165 and by funding from the Research to Prevent Blindness Foundation and from the the Dyson Foundation to ERB, by NIH grant EY012042 to Nancy Philp, and by a Pew Latin American Fellowship to Guillermo L. Lehmann.

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Fig. 1.
Schematic representation of the outer retina and the support functions of RPE.

Fig. 2.

Polarized ion channels and transporters in RPE. The asymmetrical distribution of transporters allows a vectorial flux of water and lactate from the subretinal space (top) to the choroid (bottom). Modified from (Adijanto et al., 2009).

Fig. 3.
Trafficking routes, sorting signals and mechanisms in polarized epithelial cells.

Table 1

Membrane proteins with reversed polarity in RPE cells.

Protein	RPE topology	Topology IN conventional epithelia	System	Assay	Reference
MCT1	Apical	Basolateral	Cultured hfRPE	Immunofluorescence	Deora et al., 2005; Maminishkis et al., 2006
			Ex-vivo adult rat eyes	Immunofluorescence	Philp et al., 1998
Na,K-ATPase	Apical	Basolateral	Cultured hfRPE	Immunofluorescence	Hu and Bok, 2001; Maminishkis et al., 2006; Sonoda et al., 2009
			Native adult rat eyes	Immunocytochemistry	Gundersen et al., 1991; Okami et al., 1990
NCAM	Apical	Basolateral	Native developing and adult rat eyes	Immunofluorescence	Gundersen et al., 1993; Marmorstein et al., 1998
EMMPRIN (CD147; RET-PE2)	Apical	Basolateral	Cultured hfRPE	Immunofluorescence	Deora et al., 2004
			Native and ex-vivo adult rat eyes	Immunofluorescence, <i>in situ</i> biotinylation	Marmorstein et al., 1998, 1996
JAM-C	Apical/ tight-junctions	Basolateral/ tight-junctions	Native adult mouse eyes	Immunofluorescence	Daniele et al., 2007
			Cultured hfRPE	Immunofluorescence	Economopoulou et al., 2009
CAR	Non polar	Basolateral	Cultured hfRPE; ARPE-19 cells	Immunofluorescence	Diaz et al., 2009
TfR	Non polar	Basolateral	ARPE-19 cells	Transferrin recycling	Perez Bay et al., 2013
CFTR	Basolateral/ non polar	Apical	Native hfRPE	Immunofluorescence, electrophysiology	Blaug et al., 2003

Table 2

Experimental approaches to study epithelial polarity.

Technique	Methodology	Aspect of trafficking and polarity analyzed				Output	References
		Steady state distribution	Biosynthetic pathway	Recycling pathway	Transcytotic pathway		
Biochemical analysis	Cell surface biotinylation	Selective labelling of surface proteins with biotin analogues followed by purification and Western blot analysis.	x			Relative percentage of the protein localized in the apical vs. the basolateral domain.	Deora et al., 2006; Gottardi and Caplan, 1992; Sargiacomo et al., 1989
	Biotin targeting assay	Metabolic labelling combined with domain-selective biotinylation and recovery.		x	x	Targeting of proteins from biosynthetic pools to the plasma membrane domain.	Le Bivic et al., 1990, 1989
	Surface immunolabeling	Surface proteins are labelled with specific antibodies. Serves as an alternative method when biotin permeates the TJs (leaky monolayers).	x			Relative percentage of the protein localized in the apical vs. the basolateral domain.	Gan et al., 2002
	Biotin assay for endocytosis	Selective labelling of surface proteins with a cleavable biotin analogue. Only internalized proteins remain protected upon exposure to a reducing media..		x		Rate of protein internalization calculated from the amount of protein protected of cleavage.	Graeve, 1989
	SulfoTag-labelling recycling assay	The cargo protein is labelled with biotin bound to a luminophore (SulfoTag). After incubation and thorough washing, the media is collected and quantitated in streptavidin coated wells.		x	x	Rate of endocytosis and/or transcytosis monitored by	
Confocal microscopy	electrochemiluminescence analysis of the culture media.						
	Surface immunolabeling	Selective immunofluorescence labelling in non permeabilized cells using primary antibodies.	x			Distribution of membrane proteins by colocalization with domain-specific surface markers.	Deora et al., 2006
	Short-term reporter expression	Intranuclear injection of reporters	x	x		Spatial and temporal distribution of proteins by colocalization with surface and endosomal markers.	Kreitzer et al., 2000, 2003, 2006
	Cargo synchronization	iDimerize	x	x	x		Rivera et al., 2000; Thuenauer et al., 2014
		RUSH system	x	x	x		Boncompain et al., 2012

Technique	Methodology	Aspect of trafficking and polarity analyzed				Output	References
		Steady state distribution	Biosynthetic pathway	Recycling pathway	Transcytotic pathway		
Fluorescent labelling uptake assay	interaction is mediated by streptavidin, so the addition of biotin releases the cargo to the secretory pathway.						
	Cells are incubated with labelled cargoes or antibodies against extracellular epitopes.			x	x		Blot and McGraw, 2008

Biochemical assays and confocal analysis of intranuclear microinjections can be combined with the following strategies to synchronize the movement of protein/cargo: *Temperature switch*: Newly synthesized proteins are blocked in the ER at 15 °C, but released to the TGN when switching to 20 °C (Malin and Simons., 1983). *Thermoreversible VSVG*: At 39.5 °C, the misfolded version tsO45 is retained in the ER but trimerizes and traffics to the plasma membrane through the Golgi at 32 °C (Lafay, 1974). *Chemical block*: Brefeldin A blocks the traffic from the ER to the Golgi by reversibly disassembling the Golgi complex (Lippincott-Schwartz et al., 1989). Monensin is an ionophore that blocks transport through the Golgi complex (Tartakoff, 1983).