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Cell culture models to study retinal pigment epithelium-related pathogenesis in age-related macular degeneration

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Abstract

Age-related macular degeneration (AMD) is a disease that affects the macula – the central part of the retina. It is a leading cause of irreversible vision loss in the elderly. AMD onset is marked by the presence of lipid- and protein-rich extracellular deposits around the retinal pigment epithelium (RPE), a monolayer of polarized, pigmented epithelial cells located between the photoreceptors and the choroidal blood supply. Progression of AMD to the late nonexudative "dry" stage

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of AMD, also called geographic atrophy, is linked to progressive loss of areas of the RPE, photoreceptors, and underlying choriocapillaris leading to a severe decline in patients' vision. Differential susceptibility of macular RPE in AMD and the lack of an anatomical macula in most lab animal models has promoted the use of *in vitro* models of the RPE. In addition, the need for high throughput platforms to test potential therapies has driven the creation and characterization of *in vitro* model systems that recapitulate morphologic and functional abnormalities associated with human AMD. These models range from spontaneously formed cell line ARPE19, immortalized cell lines such as hTERT-RPE1, RPE-J, and D407, to primary human (fetal or adult) or animal (mouse and pig) RPE cells, and embryonic and induced pluripotent stem cell (iPSC) derived RPE. Hallmark RPE phenotypes, such as cobblestone morphology, pigmentation, and polarization, vary significantly between different models limiting their usability for investigating different aspects of AMD biology. Here the AMD Disease Models task group of the Ryan Initiative for Macular Research (RIMR) provides a summary of several currently used *in vitro* RPE models, historical aspects of their development, RPE phenotypes that are attainable in these models, their ability to model different aspects of AMD pathophysiology, and pros/cons for their use in the RPE and AMD fields. In addition, due to the burgeoning use of iPSC derived RPE cells, the critical need for developing standards for differentiating and rigorously characterizing RPE cell appearance, morphology, and function are discussed.

Keywords

retinal pigment epithelium; ARPE19; primary cells; iPSC-RPE; Organ-on-a-Chip; complement system

INTRODUCTION

Age-related macular degeneration (AMD) affects over 30 million individuals world-wide, and these numbers are expected to double by 2050 (Rein et al., 2009). AMD affects the macula, the central part of the retina that is responsible for central and sharp vision (Swaroop et al., 2009). Clinically, AMD is classified into two advanced stages, nonexudative 'dry' AMD or geographic atrophy, characterized by degeneration of the retinal pigment epithelium (RPE) and photoreceptors in the macula, and exudative 'wet' AMD or choroidal neovascularization (CNV), characterized by hyperproliferation and migration of choroidal capillaries into the sub-RPE, subretinal, or the intraretinal space (Swaroop et al., 2009). AMD onset and progression have been linked to genetic and environmental risk-factors including age and lifestyle (e.g. diet and smoking); and genetic factors, which include risk alleles in genes (Swaroop et al., 2009) identified through genome-wide association studies (GWAS) (Fritsche et al., 2016). Several features of AMD pathophysiology make this one of the hardest diseases to model *in vitro* and *in vivo*. For instance, most routinely used lab animals do not have a macula; animal cells cannot replicate human genetic risk alleles associated with AMD; diet and aging are not easy to mimic in animals or cell culture; and specifically macular cells have not been successfully cultured *in vitro*. However, a growing body of evidence suggests that reliable models can be developed/utilized to study certain aspects of AMD pathogenesis.

There has been significant interest in the biology of the RPE, a pigmented monolayer of post-mitotic cells located between the photoreceptors and the choroidal blood supply (Bharti et al., 2006). Multiple studies have suggested the RPE monolayer as the primary site of disease initiation (Wang et al., 2018). Accordingly, a majority of *in vitro* models focus on RPE cells, fidelity of the cell systems to the *in vivo* biology of RPE, and how it relates to AMD pathogenesis.

The AMD Disease Models task group of the Ryan Initiative for Macular Research (RIMR) aimed to provide direction on currently available and upcoming *in vitro* models used for better understanding of RPE cell biology and AMD pathogenesis related to RPE dysfunction. Between February and April 2021, the task group convened in four virtual sessions. The task group discussed the following models: immortalized RPE cell lines, primary RPE cells obtained from animals (mice and pigs) and donor human eyes (adult and fetal), as well as RPE cells differentiated from induced pluripotent stem cells (iPSCs). This review provides a summary of the discussions held during the RIMR AMD Disease Models task group meetings about usability, strengths, and limitations of these models for AMD research. This review also provides guidance on assays to compare RPE models across labs and quantitative readouts of RPE cell health under physiological stressors related to AMD pathogenesis.

RPE CELL LINES

Cell lines spontaneously formed from primary cultures (e.g. the ARPE19 cell line, Dunn et al., 1996) or by introduction of one of a number of immortalizing genes (hTERT-RPE1, Bodnar et al., 1998; Jiang et al., 1999) provide a readily available source and represent the most tractable models. However, several of the immortalized cell lines have abnormal karyotypes, and often lack some physiological characteristics of RPE *in vivo*, so that their use comes with a potential trade-off. In many cases, though, a cultured cell line may possess the characteristics required for the particular study at hand. Importantly, these characteristics depend, not just on the origin of the cell line, but also on the conditions under which it has been cultured. Unfortunately, numerous published studies have used an RPE cell line where the cells do not have a basic epithelial organization, such as a cobblestone-like appearance and apical-basal polarity. That said, it is possible in some cases that the lack of RPE characteristics is less of a concern. For example, use of hTERT-RPE1 cells (see below) as a model for the study of the pocket cilium (Molla-Herman et al., 2010) does not require RPE-like functions. On the other hand, an RPE cell culture that is not well polarized is inappropriate for studying many key RPE functions. For example, manifestation of the apical-basal localization and motility of RPE organelles, such as phagosomes, lysosomes, and mitochondria, requires cells with apical-basal polarity that is determined by a well-differentiated cytoskeleton. The maturation and degradation of phagosomes from the tips of the photoreceptor outer segments involve an apical to basal migration of the phagosomes. Indeed, defects in phagosome migration inhibit degradation and lead to AMD-like pathology (Pfeffer and Philp, 2014; Jiang et al., 2015; Lakkaraju et al., 2020).

The RPE-J line was generated by simian virus 40 (SV40) transformation of primary rat RPE cells and was the first immortalized RPE cell line to be described (Nabi et al., 1993). In early

studies, RPE-J cultures were shown to possess numerous characteristics of differentiated RPE. They formed a cobblestone-like appearance based on circumferential localization of the tight-junction protein, ZO-1, exhibited extensive apical microvilli, and displayed significant trans-epithelial resistance (TER) (350 $\Omega.cm^2$) (Nabi et al., 1993). However, the cells lacked some characteristic features dependent on polarity, such as the specific apical localization of the Na^+/K^+-ATPase and neural cell adhesion molecule (NCAM) (Nabi et al., 1993), which are critical for RPE and photoreceptor function. Nearly three decades later, the RPE-J cell line continues to be used as an RPE model; however, the true passage number of these cells is often not known, and their ability to differentiate seems limited, at least by common culturing methods. Growing currently available RPE-J cell cultures in the presence of 10 nM retinoic acid has been reported to induce a differentiated RPE phenotype, however, resulting cultures typically lack a cobblestone-like appearance and demonstration of apical-basal differentiation (Kim et al., 2013). On the other hand, RPE-J cells use the same apical phagocytic receptors and signaling pathways as primary rat RPE to phagocytose shed outer segment fragments (Finnemann 2003). Overall, there has been minimal utility of RPE-J cells in modeling of AMD pathobiology *in vitro*; the focus of most of RPE-J studies has been on RPE cell biology.

The human telomerase (hTERT)-RPE1 cell line was derived from a young donor's RPE cells, using human telomerase reverse transcriptase activity. These cells have been reported to become pigmented in the absence of serum after four to eight weeks in culture and to express some RPE-associated proteins (e.g. cellular retinaldehyde-binding protein [CRALBP]) (Rambhatla et al., 2002). However, they do not form a differentiated epithelium with a cobblestone-like organization and have been used mainly for studies on the primary cilium (Molla-Herman et al., 2010). After a few days of serum starvation, ciliogenesis is robustly initiated, although the cilium typically extends between the cell and the substrate, rather than from an apical surface as in normal RPE cells. For vision scientists, the most applicable use of hTERT-RPE1 cells appears to be as a model for the photoreceptor cilium (Trivedi et al., 2012), since its cilium arises from a pocket (Molla-Herman et al., 2010; Chadha et al., 2021), like that of the photoreceptor cilium (Liu et al., 2007).

The first descriptions of the adult RPE-19 (ARPE-19) and D407 lines (Davis et al., 1995; Dunn et al., 1996) showed that they differentiate readily into polarized RPE cells. However, these cell lines have abnormal karyotypes (Davis et al., 1995; Fasler-Kan et al., 2018; Hazim et al., 2019), and having been passaged over many years have diminished ability to demonstrate characteristics of differentiated RPE (Strunnikova et al., 2010; Lehmann et al., 2014). Nevertheless, the ARPE-19 cell line remain widely used, and mimics many characteristics of RPE *in vivo – provided they are cultured under conditions that promote differentiation*.

Improved culture conditions that promote differentiation of ARPE-19 cells have been reported. They involve culture on a porous Transwell® filter and media additives that affect metabolism. One method involves the addition of pyruvate in high-glucose DMEM. Under these conditions, cells recapitulate key features of RPE cells, including RPE-specific differentiation markers, a cobblestone-like organization, polar expression of proteins, extensive microvilli, the ability to phagocytose, and secretion of VEGF (Ahmado et al.,

2011). The expression of the premelanosome marker Pmel17 was observed, although, as with the original study (Dunn et al.,1996), cylindrical melanosomes characteristic of the RPE were not evident. In addition, the cells did not exhibit a TER greater than 51 Ω.cm^2. However, the same protocol employed by another group reported a TER of 126 Ω.cm^2 (Samuel et al., 2017), but it required a three-to-four-month period for RPE differentiation. A more recent method incorporates the addition of nicotinamide instead of pyruvate, resulting in rapid differentiation of ARPE-19 cells. In the presence of nicotinamide, cells form a tightly-packed, cobblestone-like appearance after one week, and a well-polarized epithelium within four weeks (Hazim et al., 2019). While the epithelium still possesses a relatively low TER, this method of culturing ARPE-19 cells in media supplemented with nicotinamide preserves the ease of use inherent in a cell line.

ARPE-19 cells have already been used to study many characteristics of AMD, including EMT (Yang et al., 2021), inflammation (Tseng et al., 2013), phagocytosis defects (Xu et al., 2012, and complement activation (Chung et al., 2017; Fernandez-Godino et al., 2018). Unfortunately, most studies appear to have been performed on undifferentiated, or, at best, poorly differentiated cells. Nevertheless, the studies suggest that differentiated ARPE-19 cells may be amenable to such studies. Importantly, gene editing has been used successfully with ARPE-19 cells; for example, a mutant line was obtained by editing the *EFEMP1* gene to generate a mutation that underlies dominant macular degeneration (Fernandez-Godino et al., 2018). Thus, while there are limitations in the use of RPE cell lines, the use of appropriate culture conditions, in order to promote a differentiated epithelium, make the ARPE-19 cell line useful for various types of RPE studies, including those related to AMD (Table 1). A recent editorial by Pfeffer and Fliesler provides additional guidance for using ARPE19 cells with appropriate culture conditions (Pfeffer and Fliesler 2022).

PRIMARY RPE CELLS (MOUSE, PORCINE, BOVINE, MONKEY, HUMAN)

Primary RPE cultures have been established from freshly harvested retinas of mouse, porcine, or human donors. These cells are plated at confluence on collagen- or laminin or other extracellular matrices-coated semipermeable membrane inserts (Transwells®), culture conditions leading to cells that recapitulate several features of RPE *in situ*. The time to full maturation depends on the species: about two weeks for porcine RPE and more than six weeks for cultures established from human donors. After this period, the cultures have formed well-differentiated monolayers with tight junctions and TERs >300 Ω.cm^2. The cells exhibit a polarized RPE phenotype, with precisely organized microtubules that are essential for the appropriate localization of apical (e.g., Na^+,K^+-ATPase, αvβ5 integrin, MCT1) and basolateral (e.g., MCT3, bestrophin-1 , Stra6) proteins (Lakkaraju et al., 2020). The cells of these cultures degrade photoreceptor outer segments with kinetics comparable to RPE *in vivo*. Detailed protocols that have been validated in multiple publications are available (Blenkinsop et al., 2013; Gibbs and Williams, 2003; Maminishkis et al., 2006; Toops et al., 2014).

PRIMARY MOUSE RPE

Mouse RPE cultures are usually established from trypsinized RPE cells isolated from eyes of newborn pups and survive about 10 days in culture, which is sufficient for experiments using transfection, transduction, or live imaging approaches (Gibbs et al., 2003; Gibbs and Williams, 2003; Fernandez-Godino et al., 2016). These cultures can be generated from transgenic mice, which allows investigation of subcellular mechanisms that can contribute to RPE dysfunction and retinal degenerations (Nandrot et al., 2004; Esteve-Rudd et al., 2018; Jiang et al., 2015). Advantages of mouse RPE cultures include short post-mortem times and consistency of cultures due to inbred mouse lines. Some caveats are that cultures are easier to establish from younger mice than adult mice, which could limit the ability to model age-dependent disease pathology. On the other hand, younger mice have smaller eyes, so more eyes might be needed to generate a sufficient number of RPE cells. Another caveat, especially with regards to studying AMD, is that mice do not express key AMD-associated genes such as *ARMS2* and *CETP* and express only one isoform of APOE.

Recently, a new promising method of culturing an intact mouse RPE layer without the loss of its properties and function has been described (Shang et al. 2021). Briefly, the eye is dissected under sterile conditions to remove the anterior segment and the retina, followed by flattening the RPE-choroid-sclera explant on a polyvinylidene difluoride (PVDF) membrane with the sclera attached. Such RPE flat mounts can then be kept in culture for at least seven days without the loss of RPE viability and properties or significant alterations in the expression of RPE-specific genes. The main benefit of this method is that it is the gentlest technique to date for maintaining intact RPE cultures, where the procedure-induced stress to the RPE is minimal. An additional benefit is that the RPE can be obtained from any genetic background, including known mouse models for dry AMD (briefly discussed below), making it a good model for studying various aspects of AMD development and treatment *in vitro*.

One of the strongest genetic associations with the development of AMD has been shown to be a common polymorphism in the complement factor H (*CFH*) gene (Edwards et al. 2005; Hageman et al. 2005; Haines et al. 2005; Klein et al., 2005). Aged transgenic mice expressing the Y402H variant of the *CFH* gene on the $Cfh^{-/-}$ background (*CFH-H/H*) developed an AMD-like phenotype only after being fed a high fat, cholesterol-enriched diet. Moreover, this phenotype was not associated with complement activation but rather with lipoprotein dysregulation, supporting a noncanonical role for CFH in AMD pathogenesis (Landowski et al. 2019). We speculate that cultured RPE flat mounts (Shang et al. 2021) from the aged Y402H transgenic mice may be an interesting and highly relevant *in vitro* model to study AMD pathogenesis. The applications may include gene therapy studies (silencing the existing H402 variant and replacing it with the low-risk Y402 variant of the gene) as well as investigations aimed at elucidating the interplay between the complement system and lipid homeostasis in AMD.

Another notable genetic risk factor for AMD is determined by the variants of the apolipoprotein E (*APOE*) gene (Klaver et al. 1998). While *APOE3* represents the wild-type variant, the presence of the *APOE2* allele increases the risk of developing AMD in humans

(reviewed in Hu et al. 2021). APOE2 is associated with a slightly increased risk of AMD (odds ratio [OR] 1.124–1.83) while APOE4 is associated with a slightly reduced risk (OR 0.43–0.81) (McKay et al. 2011; Hu et al. 2021). This is in stark contrast to the risk association of these alleles in Alzheimer's disease where APOE4 is a major genetic risk factor (OR 4.86), while APOE2 is protective (OR 0.60) (Ang et al. 2008). Potentially contributing to this paradox, the frequency distribution of the APOE4 allele halves between the ages of 60 and 85 years (McKay et al. 2011; Hu et al. 2021) which could diminish the effect of the APOE4 allele in late AMD in this age range. ApoE is known to be an abundant component of drusen in both healthy individuals and in AMD patients. Being secreted from both the apical and basal sides of the RPE, ApoE plays a role in lipid trafficking. Importantly, aged mice overexpressing human *APOE2*, *APOE3*, or *APOE4*, developed isoform-dependent pathologies when maintained on a high fat, cholesterol-rich (HFC) diet in which *APOE4* mice developed an AMD-like phenotype (Malek et al. 2005). In this original characterization of these *APOE* transgenic mice, all of the animals expressing the human ApoE were *APOE* homozygotes whereas, in the human association studies, the subjects were predominantly, if not exclusively, heterozygous for the *APOE2* or *APOE4* alleles (Malek et al. 2005). In addition, in the human studies, the effect of diet as a modifier of the *APOE* allele effect on AMD risk was not investigated. It should also be noted that the homozygote *APOE2* mice are 100% hyperlipoproteinemic unlike humans in which there is a 30% incidence (Sullivan et al. 1998), and this is not a feature of AMD. The AMD-like phenotype observed in old *APOE4* fed an HFC diet could be blocked with systemic anti-amyloid immunotherapy which resulted in structural preservation of the RPE, reduction of activated complement components in the sub-RPE deposits and protection of visual function (Ding et al. 2011). Another clear driver of AMD development is the dysregulation of lysosomal function in the RPE. To date, several animal models exist that develop an AMD-like phenotype due to defective lysosomes and deregulated autophagy, including the *Mcd/mcd* mice (enzymatically inactive cathepsin D) (Zhang et al. 2002), *Lamp 2* KO mice (Notomi et al., 2019), and *Cryba1* cKO mice (RPE-specific conditional knockout of gene encoding βA3/A1-crystallin) (Valapala et al. 2014; Zigler and Sinha 2015). Several genes involved in the lysosomes and autophagy have been shown to be critical in RPE cells (Frost et al., 2014; Gomez et al., 2018). It may be promising to use RPE cultures obtained from these animal models to further examine this relationship.

Despite several limitations, primary mouse RPE cultures – both from trypsinized cells and intact RPE monolayers, provide an excellent source to connect RPE biology to genetics, and to phenotypic data in animals, and its use continues to grow in the AMD field. Furthermore, mouse RPE cells can be expanded by one-two passages making them amenable to moderate drug screens. (Table 1).

PRIMARY PORCINE RPE

An increasing number of studies have used RPE from freshly harvested porcine eyes to establish well-polarized monolayers (Toops et al., 2014; Georgiannakis et al., 2015; Klingeborn et al., 2017). After approximately two weeks in anti-mycoplasma antibiotic (e.g. ciprofloxacin) on collagen-coated Transwell® filters, these monolayers express RPE polarity markers mentioned above, establish functional tight junctions, and have a TER >400

Ohms.cm^2 (Toops et al., 2014). An advantage of using porcine RPE cultures is the similarity between human and porcine immune systems (Henderson and Hicks 2002; Sanchez et al., 2011; Mair et al., 2014), which is important for investigating a disease with a strong immune component like AMD. In support of this, studies using porcine RPE have increased our insight into complement regulation in the RPE (Georgiannakis et al., 2015 Tan et al., 2016) and led to the discovery that intracellular complement activation is a novel modulator of RPE physiology (Kaur et al., 2018). Live imaging and bioinformatics analyses of the impact of *APOE* isoforms on porcine RPE cultures have identified that mitochondrial redox status regulates drusen nucleation in the RPE. Complement-mediated mitochondrial injury induces oxidation of proteins with reactive cysteines, such as APOE2 (the AMD risk isoform), which drives the formation of APOE biomolecular condensates as potential drusen precursors (La Cunza et al., 2021). Caveats for using porcine RPE cultures include the facts that: **i)** eyes are obtained from local abattoirs, meaning that tissue quality and therefore, ability to generate viable cultures, depends on the harvesting procedures used at different facilities; **ii)** pooling eyes from animals of different ages and sexes increases variability; and **iii)** RPE from some pig breeds are better able to generate healthy cultures compared to others, and provide sufficient quantity of cells for moderate size drug screens (Table 1).

PRIMARY BOVINE and MONKEY RPE

Previously primary cultures of bovine and monkey RPE cells have also been attempted (Oka et al., 1984; Pfeffer 1991; Hartnett et al., 2003; Becerra et al., 2004; Fronk and Vargis 2016). Bovine RPE cells required bovine serum for attachment, but serum free conditions led to better epithelial morphology in passaged cells (Oka et 1984). Hartnett et al determined that although the TER of primary bovine cultures increased for the first two weeks of cultures, it barely reached 100 Ohms.cm^2 and stayed stable only for additional two weeks. In co-culture with endothelial cells the TER of these cells dropped significantly (Hartnett et al., 2003). Pfeffer developed a protocol to derive confluent monolayers of monkey RPE cells and studied the effect of media components on cell proliferation and attachment to the substrate. These cells were found to secrete PEDF mainly apically, similar to cultures of fetal human RPE and iPSC-derived RPE (Becerra et al., 2004; Maminishkis et al., 2006; Sharma et al., 2019). Bovine globes are rather easier to obtain but monkey globes are relatively hard to obtain because of continually reduced usage of monkeys in research. Overall, neither of cultures were used extensively in AMD research and their current use in this field is negligible.

PRIMARY HUMAN RPE

Primary cultures from human donors have been generated from fetal RPE (Hu and Bok 2001; Maminishkis et al., 2006; Sonoda et al., 2009). These cultures are highly pigmented, exhibit a polarized epithelial morphology with tight junctions, and express RPE-specific proteins. Fully polarized monolayers develop after six to eight weeks in culture and display a very high TER (500–800 ohm.cm^2). After one to three months of culture on semi-permeable membrane supports, human fetal RPE monolayers constitutively secrete deposits containing APOE into the basal matrix, which can be increased by exposing the cultures to active complement components (Johnson et al., 2011).

More recently a method for obtaining RPE cells from adult human RPE (ahRPE) monolayers has been described (Ferrington et al., 2017). Here, the cells are derived from cadaver donor eyes. RPE cultures established from adult donors have effectively been used to identify autophagic defects, mitochondrial dysfunction, secreted proteins, and decreased expression of nuclear hormone receptors in AMD donors compared with cultures established from donors without AMD (An et al., 2006; Hu et al., 2013; Ferrington et al., 2016; Ferrington et al., 2017; Choudhary et al., 2020; Zhang et al., 2020).

A significant obstacle to using human RPE cultures is the scarcity of donor tissue, ideally collected within short post-mortem times (Table 1). Moreover, RPE cells dedifferentiate with passaging and lose pigmentation, and acquire a fibroblastic morphology with loss of their cobblestone morphology. Some of these characteristics can be restored by long term culture (>8 weeks) on porous substrates, which facilitate the induction of polarity and related phenotypes (Hazim et al., 2019). That said, establishing primary RPE cultures would be especially useful when studying the epigenetic aspects of AMD induction. An increasing body of evidence suggests that epigenetics plays a role in AMD in the form of abnormal DNA and RNA methylations and histone modifications (reviewed in Li et al. 2020). We speculate that working with primary RPE cultures obtained directly from AMD donors, with their “naturally occurring” and unique patterns of epigenetic modifications, would allow researchers to identify important modifications and to potentially develop effective treatments to reverse them and to rescue RPE physiology and function. Despite the technical difficulties, donor-derived primary RPE seems to be ideal for this application, considering the complexity of the epigenetic modifications that may be hard, if not impossible, to recreate in other models (Table 1).

INDUCED PLURIPOTENT STEM CELL-DERIVED RPE

Over the last two decades, embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have been developed as additional sources of differentiated human RPE cells (Sharma et al., 2020). Unlike the other sources of RPE cells discussed above, RPE differentiation from ESCs/iPSCs is a laborious and resource-consuming process. However, key advantages of this technology are: **(1)** the ability to obtain an unlimited supply of fully mature and functional RPE cells, making it feasible to perform disease modeling and high throughput drug screens that require large amounts of cells (Sharma et al., 2020; Miyagishima et al., 2021; Ferrer et al., 2014) and **(2)** derivation of patient-specific iPSC-derived RPE cells allowing the possibility of performing comparative analysis between diseased and healthy cells and correlating cellular endophenotypes with patient symptoms and genotypes (Sharma et al., 2020; Miyagishima et al., 2021).

iPSC-RPE cell cultures are both diverse and accessible, as iPSCs can be obtained by reprogramming of fibroblasts collected through skin biopsies or reprogramming of peripheral blood mononuclear cells through blood withdrawal. Their utility can be seen as three-fold: **(1)** iPSC-RPE may provide material for restoration/transplantation due to the very large numbers of high-quality cells available, a direction taken by several academic and commercial research groups globally. We will not elaborate on these efforts here; suffice it to say that numerous materials and methodologies are under investigation and being tested

in clinical trials (see review by Sharma et al 2020); **(2)** iPSC-RPE may provide high quality RPE cell culture models for mechanistic RPE research in general and specifically for AMD "disease-in-a-dish" research (May-Simera et al., 2018; Sharma et al., 2020) and; **(3)** as an extension from exploring AMD disease mechanisms and identification of differences at the cellular level, iPSC-RPE may be interrogated for responsiveness to interventions and therapies such that AMD patients or individuals with high risk of developing AMD may receive personalized therapy or preventive treatment (Sharma et al., 2019).

The initial observation of RPE differentiation from human ESCs dates to 2004, when ESCs were spontaneously differentiated into RPE cells (Klimanskaya et al., 2004). Over the last two decades multiple iterations of RPE differentiation protocols have been developed (see review by Sharma et al 2020). One key advancement in RPE differentiation methods is the use of what is called directed or guided differentiation methods (Idelson et al., 2009; Osakada et al., 2009; Sharma et al., 2019), using specific growth factors at defined timepoints to developmentally alter the fate of ESCs/iPSCs into RPE cells. Although the use of growth factors significantly increases the cost of RPE differentiation, there are multiple advantages to this approach: **(1)** it generates fully mature and functional RPE (May-Simera et al., 2018); **(2)** it improves the efficiency of differentiation from less than 10% in spontaneous differentiation to 60–80% with directed differentiation (Sharma et al., 2019); **(3)** it improves reproducibility of the protocol across multiple iPSC lines (Sharma et al., 2019); and, **(4)** it shortens the differentiation time from 30 weeks in spontaneous differentiation to 10 weeks in directed differentiation (Sharma et al., 2019).

iPSC-DERIVED RPE DIFFERENTIATION AND CHARACTERIZATION STANDARDS

One major concern with iPSC-RPE differentiation is that currently there are no standard protocols to differentiate and propagate iPSC-derived RPE. In addition to donor tissue source and individual donor characteristics, parameters that are likely to affect iPSC-RPE phenotype and function include substrate surface, plating density, feeding medium and frequency, and duration of culture. Depending upon the kind of substrate-coating, RPE cell change their gene expression profile especially of ECM-related genes (Sorkio et al., 2014). Unlike, immortalized cell lines, iPSC-derived RPE cells cannot be propagated forever. However, it may be possible to propagate iPSC-RPE cultures while maintaining their epithelial character, at least for a few passages, by using milder dissociation methods like low calcium containing medium, as suggested by Ramachandran Rao et al. 2018. There is nevertheless a need to develop standardized methods that are universally utilized for differentiation of iPSCs. In addition, there is a demand for commercial sources of AMD iPSC lines and derived RPE cells. The National Eye Institute recently started a repository of 65 AREDS2 iPSC lines at the New York Stem Cell Foundation (Wright et al., 2020). Some of the widely utilized iPSC-RPE commercial sources are iCell RPE from Fujifilm (catalog #R1102), Lagenlabs (RPE-catalog number not available), and Tempo Bioscience iRPE (Tempo-iRPE™).

However, there continues to be a need to functionally validate iPSC-RPE across labs so that results derived in different labs, using a given patient cell line, can be directly compared. A visual inspection of RPE monolayer confluency, hexagonality, and pigmentation along with expression analysis of a subset of genes is frequently used to validate RPE cells (Bharti et al., 2011). These assays do support a RPE phenotype but are not sensitive enough to compare and validate cells across multiple labs and from different AMD patients. Structural assessment of the RPE monolayer has been performed using scanning and transmission electron microscopy (Sharma et al., 2019). It has been shown that iPSC-derived RPE cells contain robust apically located pigment granules, basally located nuclei, tight junctions between neighboring cells, and confluent apical processes supporting fully polarized and mature feature of these cells (Miyagishima et al., 2016; Sharma et al., 2019). Functional assessment of the tight junctions is confirmed by measuring the TER of cells grown on semi-permeable Transwell® filters. As cells continue to mature, tight junctions seal the paracellular space increasing the ability of RPE cells to resist the flow of current passed via an electrode on the top and another on the bottom of RPE monolayer. TER measurement is one of the easiest assays to validate the quality of RPE cells derived from different patients or in different labs. TER values slowly increasing in the initial weeks of culture reaching a plateau of several hundred $\Omega.cm^2$ have been demonstrated in iPSC-RPE cells derived from different donors (Miyagishima et al., 2016; Sharma et al., 2019). It is worth noting that TER measurements are extremely sensitive to outside variables like the composition, temperature, and pH of the cell culture medium and placement of electrodes (Srinivisan et al., 2015). Care must be taken to control these outside variables when comparing TER across labs or across cultures and additional assays of RPE barrier function must be performed. For instance, TER measurements in conjunction with quantification of apical and basal secretion of VEGF and PEDF are accepted assessments of barrier functions and RPE specific epithelial polarity. These tests have the benefit of being non-invasive and thus can be performed longitudinally to monitor cell differentiation over time of culture and under specific conditions of manipulation (e.g. stress challenge) or pharmacological treatment. Other common assessments include phagocytosis of photoreceptor outer segment fragments (POS), a key task of RPE cells in the eye. Phagocytosis by RPE cells in culture involves two well characterized surface receptors, $\alpha v\beta 5$ integrin and the TAM receptor MERTK, that function in POS recognition and internalization, respectively (Finnemann et al., 1997; D'Cruz et al., 2000). These receptors engage purified POS indirectly via extracellular bridge ligand proteins, e.g. MFG-E8 for $\alpha v\beta 5$ integrin (Nandrot et al., 2007), and Protein S for MERTK (Burstyn-Cohen et al., 2012). Phagocytosis assays where challenging RPE cells in culture with and without bridge ligand supplementation can therefore specify recognition and engulfment capacity of RPE cells (Mazzoni et al., 2019). These assessments require the addition of POS and ligand proteins at concentrations that match the levels of available cell surface phagocytic molecules, which may vary depending on cell origin or culture conditions. The kinetics of POS uptake vary significantly between iPSC-RPE cell populations. Both POS binding and engulfment are saturable processes. Thus, POS phagocytosis assays are informative and might be used to compare RPE functionality across cell sources and laboratories if they are optimized to yield maximal POS engulfment. Reporting of stringent assessment of phagocytic capacity of any RPE population should include quantification of two distinct characteristics, namely average numbers of particles

engulfed per cell and fraction of phagocytic cells in the population. With regard to AMD, the most critical stage of POS phagocytosis concerns the degradation of the engulfed POS membranes, with inefficient degradation likely contributing to lipid deposition. This degradation phase can be most readily studied by removing the POSs from the RPE cells after a short interval, and then following the subsequent degradation of the POS membranes that were engulfed during the “pulse” interval. The rate of POS degradation in iPSC-RPE cells have been found to compare well with *in vivo* rates, although variation among RPE cultures from different iPSC lines was observed further highlighting the need for standardized protocols for iPSC-RPE differentiation (Miyagishima et al., 2016; Sharma et al., 2019 Hazim et al., 2017).

Functional validation of iPSC-RPE not only helps with establishing high-quality cultures and improve rigor, but it also sets the stage of translational work when using such cells for disease modeling or drug discovery. For instance, assessment of RPE polarity, marker expression, and phagocytosis are sensitive and quantitative, thus allowing identification of modest differences between non-AMD/low-risk genotype and AMD/high-risk genotype RPE cells. Indeed, there is some evidence that reduced polarized growth factor secretion and phagocytic activity are associated with AMD/high-risk iPSC-RPE (Sharma et al., 2021). However, given the somewhat limited number of experimental parameters routinely tested for iPSC-RPE to date, additional insight into disease genotype or disease associated functional differences will likely be uncovered in future studies with more expansive testing. Interestingly donor dependent differences in purinergic receptor-based calcium signaling in iPSC-RPE cells has been reported, supporting the need for more nuanced assays to discern donor-specific differences in RPE cells (Miyagishima et al., 2016 & 2017). Observations from non iPSC adult human RPE studies suggest that quantifying F-actin stress fiber content of differentiated RPE may provide insight (Muller et al., 2018).

Unlike bona fide iPSC-RPE obtained through directing RPE differentiation from PSC, adult donor RPE-derived cultures may be maintained under conditions that promote proliferation and re-dedifferentiation into large numbers of polarized, functional RPE cells, known as RPE stem cell-derived RPE (RPESC-RPE) (Salero et al., 2012; Blenkinsop et al., 2013). Compared to iPSC-RPE, RPESC-RPE generation and maintenance is economical and practical if donor globes, or posterior shells are available. Side-by-side comparison of RPESC-RPE from different donors has revealed that RPESC-RPE (derived from non-AMD donors only) falls into two distinct categories with respect to F-actin morphology and phagocytic function (Muller et al., 2018). Genotyping and AMD risk assessment of donors will be needed to determine if they underlie these or other line-to-line differences in RPESC-RPE functionality. Altogether, testing additional RPE properties should advance our insight into functional deficits of AMD RPE and may suggest new avenues for therapy or prevention. One exciting question that remains unanswered to date is whether we will be able to utilize functional assessment of specific parameters of iPSC-RPE cells in culture to predict AMD risk of donor individuals regardless of known low-risk/high-risk genotypes as this would be an enormous clinical advance.

Naturally, some of the early “disease in a dish” studies in the iPSC-RPE field focused on discovering the specific cellular phenotypes associated with the major risk-alleles for AMD

discovered in the *CFH* and *ARMS2/HTRA1* loci. Strikingly, these studies agree on the intriguing principal insight that AMD risk genotypes and/or AMD patient donor origin result in measurable functional differences among cell populations even if these are extensively propagated in cell culture. Moreover, the functional differences detected may be mitigated by manipulation of cell culture conditions. For instance, it was demonstrated that iPSC-RPE from AMD donors, some of whom carried the *ARMS2/HTRA1* high-risk alleles, normalized their gene and protein expression of pro-inflammatory markers including complement protein C3 following exposure to the metabolite precursor nicotinamide (Saini et al., 2017). A separate study comparing *ARMS2/HTRA1* high-risk versus low-risk iPSC-RPE shows association of decreased superoxide dismutase 2 and decreased antioxidant defense capacity with the high-risk genotype (Yang et al., 2014). Whether or not nicotinamide supplementation restores antioxidant capacity to high-risk AMD has not yet been reported. An important implication of these findings is that genotyping of known risk alleles of donor cells is essential to be able to distinguish variability among cell cultures due to technical issues and from differences in disease relevant genotypes.

Recently it was demonstrated that RPE derived from AMD iPSCs with the *CFH* (rs1061170, Y402H) high-risk allele show reduced mitochondrial function and increased expression of inflammatory markers as compared to cells with low-risk for the *CFH* allele (Ebeling et al., 2021). Importantly, AMD risk genotype or disease differences were readily detectable in iPSC-RPE only if these were subjected to moderate, sub-lethal levels of oxidative stress but not under normal cell culture conditions (Ebeling et al., 2021). Whether nicotinamide supplementation would ameliorate the mitochondrial deficiencies/stress responses was not tested. Others have pursued a similar strategy of comparing phenotypic differences between *CFH* low- and high-risk expressing RPE cells and also tested the effect of locally activated complement proteins C3a and C5a on iPSC-derived RPE cells (Sharma et al., 2021). Activation of local complement led to sub-RPE lipid deposits, loss of epithelial tight junctions, activation of the NF-kB pathway, and downregulation of autophagy (Sharma et al., 2021). Cells with the high-risk allele showed predisposition to disease similar to previous observations (Sharma et al., 2021). A proof-of-concept drug screen identified two drugs, a dopamine receptor antagonist and a serine protease inhibitor, that could suppress the effect of activated complement on RPE cells (Sharma et al., 2021). While these studies will benefit from follow-up studies using iPSC-RPE generated through different protocols and with different types of starting donor tissue, they suggest that altered cell metabolism may contribute to or even underlie diverse AMD iPSC-RPE defects described earlier. Altogether, metabolic profiling of donor iPSC-RPE should be included in RPE validation assays to preclude studying secondary effects of metabolic deficiency (Table 1).

ORGAN-ON-A-CHIP

RPE cells have been utilized most intensively as a cellular model system for AMD, since many of the disease processes associated with AMD are related to RPE function. However, recent single cell sequencing studies of the retina, RPE and choroid demonstrate that AMD-associated genes are expressed in many different cell types beyond the RPE, including glial cells, vascular cells, fibroblasts, monocytes, photoreceptors, bipolar cells and horizontal cells (Menon et al., 2019; Cowan et al., 2020; Orozco et al., 2020). Furthermore, multiple RPE

subtypes that are differentially sensitive to AMD have been recently discovered (Ortolan et al., 2019). These finding suggests that modeling AMD in a 'generic' RPE cell and RPE alone may be too simplistic, and more complex model systems may be needed that include other retinal cell types. In early attempts to develop complex culture systems, RPE and endothelial cells were grown in two separate compartments of a transwell® culture system - RPE on top of the transwell insert membrane and endothelial cells at the bottom of the plastic dish well that holds the transwell insert. Despite cell culture medium separating the two cell types, this co-culture system showed improved TER of the RPE monolayer likely induced by cytokines secreted by endothelial cells in the cell culture medium (Benedicto et al, 2019). This work underscores that co-culture of different ocular cell types does affect their physiology. Bioengineering can aid in the development of *in vitro* models with higher complexity.

In recent years, organ-on-a-chip technology has demonstrated potential to establish *in vitro* models that can more closely mimic human tissue anatomy and functionality. Organ-on-chips are microfluidic cell culture systems that contain continuously perfused chambers with controlled, dynamic conditions for co-culturing multiple cell types, and simulating tissue- and organ-level physiology (Bhatia and Ingbar 2014). Organ-on-a-chip technology enables high-resolution, real-time imaging and *in vitro* analysis of biochemical, genetic and metabolic activities of living cells in the context of a functional tissue or organ.

Several organs-on-a-chip have been developed to model tissues relevant to AMD pathogenesis, in particular the RPE-choroid interface (Jong et al., 2021). The first organ-on-a-chip model of the RPE-choroid interactions consisted of ARPE-19 and HUVEC monolayers grown on opposite sides of a synthetic porous membrane, mimicking the *in vivo* anatomy where RPE cells are separated from the choroid by Bruch's membrane. The ARPE-19 and HUVEC monolayers were cultured under perfusion in separate microfluidic channels (Chen et al., 2017). The co-culture platform was used to study the effect of culture conditions on ARPE-19 cells and the subsequent response of HUVEC cells. ARPE-19 cells responded to lowered glucose and hypoxic microenvironments by increasing VEGF secretion, and HUVEC cells responded to increased concentrations of VEGF by moving through the pores towards the source of VEGF inside the microfluidic device. While this organ-on-a-chip model does not mimic the entire angiogenesis process, endothelial cell migration was used as a surrogate for the initial stage in angiogenic tube formation.

A second organ-on-a-chip device aimed to obtain a vascular network consisting of capillary-like structures. Endothelial cells (ECs) were allowed to self-assemble by embedding HUVECs in a fibrin scaffold together with lung fibroblasts to stabilize the vascular network (Chung et al., 2018). The microfluidic platform was designed to form a perfusable 3D blood vessel network adjacent to an ARPE-19 monolayer, close enough to interact. Instead of a membrane, fibrin channels were introduced among the ARPE-19 cells and the blood vessel network. The model was used to mimic the pathogenesis of CNV. In response to excessive VEGF concentrations, penetrating angiogenic sprouts from the blood vessel network resulted in breakdown of the RPE monolayer. The sprouting neovessels regressed when VEGF was added along with bevacizumab, an anti-VEGF agent used clinically for CNV treatment.

A similar vascular network-based organ-on-a-chip was developed using more physiologically relevant cells; human retinal microvascular ECs and primary human ocular choroidal fibroblasts were used to form a three-dimensional vascular network, and iPSC-derived RPE cells were co-cultured in direct contact with the vascular network (Paek et al., 2019). RPE maturation markers, such as melanosome number, were observed to increase in the co-culture.

Self-assembled vascular networks may lead to low reproducibility and consistency between different devices. To avoid this problem, an organ-on-a-chip device has been developed that consists of a monolayer of ARPE-19 cells in an open-top culture chamber, and a monolayer of HUVEC cells in a capillary-like channel, separated by a semipermeable membrane (Arik et al., 2021). The three-dimensional vascular structures within the chip were imaged by optical coherence tomography (OCT), a medical imaging technique that is routinely applied in ophthalmology. Upon inducing oxidative stress by treatment with hydrogen peroxide, a dose dependent increase in barrier permeability was observed using a dynamic assay for fluorescence tracing, analogous to the clinically used fluorescence angiography.

Another organ-on-a-chip concept, designed to reproduce the interface between the neural retina and the RPE has also recently been reported (Achberger et al., 2019). This device allows the co-culture of retinal organoids in advanced stages of maturation, containing photoreceptors, with apical exposure to an RPE monolayer. Phagocytosis of POS by the RPE was detected on-chip. Although the device is perfusable through a channel below the RPE compartment, there is no vascular system in this model.

For accurate disease modeling, it would be valuable to develop an organ-on-a-chip device that combines organoids, RPE and choroidal vasculature, thereby containing the wide variety of cell types that have been implicated in AMD pathology by single cell sequencing studies (Menon et al., 2019; Cowan et al., 2020; Orozco et al., 2020). In addition, it is necessary to extend the cellular lifespan of the cells on the chip, to allow increased maturation and observation of prolonged molecular processes (Jong et al., 2019). Further, it would be very useful to generate a multiplexed organ-on-a-chip device that allows high throughput screens for testing the effect of various disease stimuli and for drug screening purposes.

ASSAYS USING CULTURED RPE TO STUDY THE PATHOGENESIS OF AMD

As described in the previous sections, various cellular and molecular assays have been developed and used to not only validate the fidelity of *in vitro* cultures relative to the *in vivo* state, but also to characterize disease processes in the context of AMD pathology. Below we provide a brief review of culture models that present with AMD-associated morphological features and highlight a few assays relevant when studying AMD.

Drusen-like extracellular deposit formation

In vitro modeling of sub-RPE deposit formation has been achieved, to some degree, in a number of studies to date. A seminal study using ARPE-19 cells cultured over time observed an increase in the amount of condensed deposition of banded fibrillar material with a

periodicity similar to fibrous long spacing collagen, typical of basal laminar deposits seen in human AMD samples (Amin et al., 2004). Challenge with albumin and retinal homogenates led to a significant increase in the total amount of both membranous and fibrillar containing deposits. With the availability of human fetal RPE cells, the accumulation of basal laminar-like deposits containing fibrillar material, resembling fibrous long spacing collagen, has also been reported when cells were cultured for two to four months (Maminishkis et al., 2006). Exposure of early passage human fetal RPE cultures to human serum was shown to result in deposition of drusen-associated proteins including APOE, vitronectin, clusterin, serum amyloid P, as well as the terminal complement complex C5b-9 indicating activation of the complement system (Johnson et al., 2011). These studies collectively demonstrate that drusen originate from the RPE. A plausible mechanism for drusen biogenesis was recently identified using polarized porcine RPE cultures: complement-induced mitochondrial injury leads to aggregation of proteins with reactive cysteines such as APOE, thereby 'nucleating' drusen within the RPE (La Cunza et al., 2021).

Studies using stem cells as platforms for generating *in vitro* RPE model systems have shown that stressing a sub-population of self-renewing multipotent stem cells isolated from adult human RPE cells with the oxidant tert-butylhydroperoxide (TBHP) stimulates upregulation of drusen-associated crystallins, apolipoproteins, and extracellular matrix molecules intracellularly (Rabin et al., 2013). Notably, cultures of RPE cells derived from human iPSCs derived from AMD patients carrying the *ARMS/HTRA1* homozygous high-risk genotype secrete several AMD relevant proteins including complement proteins. Lipid droplet accumulation, visualized using Oil Red O histochemistry, has been reported in iPSC-derived RPE cells from patients homozygous for the low- and high-risk *CFH* genotypes (Hallam et al., 2017; Saini et al., 2017; Cerniauskas et al., 2020; Sharma et al., 2021). Finally, as touched on earlier, complement competent human serum treated iPSC-derived RPE cells from donors carrying the low- and high-risk *CFH* genotypes have been shown to induce extracellular APOE and lipid deposition (Sharma et al., 2021).

Extracellular deposits have also been reported in cultures of RPE cells isolated from non-human species, including mice and pigs. RPE harvested from mice with a dominant mutation in the EGF-containing fibulin-like ECM protein 1 (EFEMP1) produce basal deposits (Fernandez-Godina et al., 2015), whereas sub-RPE deposits containing drusen components have been observed in cultures harvested from porcine eyes, cultured for up to six months on polyester membrane inserts. The composition of the deposits was well-characterized and included neutral lipids as illustrated with Oil Red O staining, APOE, and mineralization as seen with Von Kossa staining and hydroxyapatite fluorescence labeling and mass spectrometry, adjacent to highly pigmented RPE cells, after as little as 12 weeks of culture (Pilgrim et al., 2017). Collectively, these model systems should be useful for studying the genesis of basal deposits and/or testing the ability of drugs to target and clear/block drusen-associated proteins.

It should be noted that *in vitro* models able to induce sub-retinal drusenoid deposits/reticular pseudodrusen, that accumulate on the apical side of RPE cells, are sparse (Rudolf et al., 2008; Zweifel et al., 2010). One study investigated the effect of low-lipid and oxidized lipid media on primary human fetal RPE cells treated with cholesterol carriers including

high density lipoprotein (HDL) and apolipoprotein A-1 (APOA-1). They found that upon exposure of fetal RPE cells to oxidized LDL, apical cholesterol efflux increased significantly as compared to low-lipid media conditions whereas the basolateral cholesterol efflux was barely changed. These changes in vectorial lipid transport in stressed RPE cells suggests potential mechanisms underlying the formation of drusen versus sub-retinal drusenoid deposits (Lyssenko et al., 2018). Consistently Miller et al 2021 showed polarized APOE secretion in primary human RPE cells. Under oxidative stress conditions basolateral APOE secretion dropped favoring apically directed drusenoid formation (Miller et al., 2021). These culture conditions could potentially be used to evaluate the efficacy of drugs targeting apical versus basal RPE deposits.

Complement pathway-based assays

Genome-wide association studies have identified genetic variants in or near the *CFH*, *CFB*, *C3*, complement *C9*, *CFI* and vitronectin (*VTN*) genes, underscoring an important role for genetic variation in the complement system in the pathogenesis AMD (Fritsche et al., 2016; Jong et al., 2019). Various studies have evaluated the role of the complement system in RPE cells using various assays. Cultured RPE cells constitutively express various components of the complement system, including *C3*, *C5*, *CFB*, *CFH*, *CFI*, *CD46*, *CD59*, *CLU* and *VTN* (Anderson et al., 2010; Sugita et al., 2018). The effect of disease triggers or genotypes on the complement system in RPE cells can either be measured by quantifying the expression of complement gene expression or proteins, or by measuring deposition of complement activation fragments such as C3a, C3d, C5a or the membrane attack complex (MAC) using immunofluorescence (Jong et al., 2021).

The expression of complement factors is increased by exposure of RPE cells to inflammatory conditions such as interferon-γ treatment or in the presence of supernatant from T helper type 1 cells as well as by exposure to A2E and hypoxia (Fanelli et al., 2017; Parmer et al., 2018). Increased MAC deposition was observed in response to hypoxic conditions (Fanelli et al., 2017), exposure to serum (Galloway et al., 2017), and cells carrying the *CFH* Y402H genotype had significantly increased C3d deposition compared to *CFH* 402Y cells (Keir et al., 2017). RPE cells are, like host-cells in general, protected from MAC induced lysis via several mechanisms, and therefore MAC deposition on RPE cells is generally sub-lytic (Morgan 2016).

Knock down of *CFH* in hTERT RPE-1 cells results in increased *C3* expression and elevated C3 and/or C3b concentrations in the supernatant (Armento et al., 2020). In iPSC-RPE carrying high-risk genotypes at the *CFH* (Y402H) and *ARMS2/HTRA1* loci, expression of the *CFI*, *CFH* and *FHL-1* mRNA levels are significantly elevated while *C3* mRNA is reduced (Hallam et al., 2017). CFH and CFI secretion from iPSC-RPE were shown to be functional, as incubation with RPE supernatant results in degradation of purified C3b to iC3b. Deposit formation is observed in RPE cultures carrying high-risk genotypes, which was demonstrated by immunostaining for the terminal complement complex C5b-9 and for APOE (Sharma et al., 2021).

Several studies have examined the effect of exposing RPE cells to complement proteins. Treatment of primary RPE cells with the anaphylatoxin C3a increases VEGF expression

(Nozaki et al., 2006), and stimulation of fetal RPE with C3a leads to increased basal accumulation of collagens IV and VI, decreased proteasome activity and increased MMP-2 activity (Fernandez-Godino and Pierce, 2018). C3a and C5a treatment triggers an intracellular Ca2+ response (Busch et al., 2017). Exposure of fetal RPE cultures to human serum as a source of complement resulted in a selective, deposit-associated accumulation of additional known drusen components, including vitronectin, clusterin, and serum amyloid P (Johnson et al., 2011). Exposure of iPSc-derived RPE cells to complement competent human serum as a source of anaphylatoxins C3a and C5a leads to a dramatic increase in APOE-positive sub-RPE deposits, irrespective of *CFH* Y402 genotype (Sharma et al., 2021). APOE and lipid deposition are stimulated by C3a-C3aR1 and C5a-C5aR1 -induced NF-kB activation, autophagy downregulation, and dysregulated calcium homeostasis. The RPE can also generate biologically active C3a fragments and the consequent C3a-C3aR signaling leads to aberrant activation of mTOR, which inhibits autophagy and reprograms RPE metabolism (Toops et al. 2015; Kaur et al 2018). Sub-lytic MAC deposition on RPE cells can cause mitochondrial fragmentation (Tan et al., 2016) and increase secretion of IL-6, IL-8, and MCP-1 (Lueck et al., 2011), emphasizing a pro-inflammatory response of the RPE to complement stressors.

Cholesterol efflux assays

Given the notable accumulation of esterified cholesterol in Bruch's membrane with age and the abundance of lipids and cholesterol carriers in drusen and sub-RPE deposits observed in AMD donor tissue (Curcio, 2018), cholesterol assays are used in studying AMD pathobiology and assessing potential treatments. The most established assays involve measuring ^{3}H-cholesterol efflux via cholesterol acceptors including APOA1, APOE, and HDL (Biswas et al., 2017), fluorescent based assays of cholesterol efflux rate (Tsai et al., 2021), uptake of oxidized LDL by RPE cells (Gnanaguru et al., 2016), histochemical and immunohistochemical staining of RPE cultures evaluating intracellular and extracellular lipid distribution using filipin, Oil Red O, bodipy, and adipored (Choudhary et al., 2020; Pilgrim et al., 2017; Sharma et al., 2021; Toops et al. 2015; Tan et al., 2016), as well as protein and gene expression (Liu et al., 2014; PMID: 24393350).

AMD-associated genetic variants in lipid metabolism have been identified in or near the ATP-binding cassette transporter 1 (*ABCA1*), *APOE*, cholesteryl transfer protein (*CETP*) and hepatic lipase C (*LIPC*) genes (Fritsche et al., 2016; van Leeuwen et al., 2018). ABCA1 is strongly expressed in the RPE and is required for lipid metabolism and export (Storti et al., 2019). The expression of ABCA1 was found to be decreased in iPSC-RPE carrying the increased risk *ABCA1* genotype treated with a Liver X receptor (LXR) agonist, a regulator of cholesterol homeostasis, compared to iPSC-RPE cells carrying the reduced risk *ABCA1* genotype (Storti et al., 2019). A cholesterol efflux assay demonstrated that reduced ABCA1 expression hampered cholesterol efflux and led to lipid accumulation in iPSC-RPE cells (Peters et al., 2021). Increasing ABCA1 expression by a small molecule LXR agonist prevented cholesterol accumulation in primary porcine RPE cultures (Toops et al. 2015; Tan et al., 2016).

Redox status-based assays

Oxidative stress is common to all three non-genetic risk factors of AMD – aging, smoking, and high-fat diet (Datta et al., 2017). AMD disease processes are thought to be triggered when the redox status of RPE cells switches from an anti-oxidant stage to a pro-oxidant stage. Damaged mitochondria are a source of reactive oxygen species (ROS), if not removed by mitophagy and has been shown to be associated with AMD (Karunadharam et al., 2010). Mitophagy can be analyzed by measuring the expression of mitochondrial proteins, quantifying the levels of mitochondrial DNA, measuring the activity of enzyme Citrate Synthetase, and by electron microscopy (Williams et al., 2017). Downregulation of mitophagy and mitochondrial activity has been noted in AMD patient derived iPSC-RPE cells (Golestaneh et al., 2016). Another mechanism regulating cellular redox homeostasis is regulated by transcription factor NRF2 that is upstream of genes involved in managing cellular oxidative stress response (see Datta et al 2017 for more details). It directly regulates the expression of several key oxidative stress pathway genes, such as Catalase and SOD. In addition, NRF2 has been shown to regulate levels of cellular anti-oxidants glutathione and thioredoxin. NRF2 expression is shown to decrease with aging (Suzuki et al., 2008) and after acute oxidative stress in RPE cells (Sachdeva et al., 2014). Measurement of the expression of NRF2 target genes, and the levels of glutathione and thioredoxin provides a critical readout of cellular redox status and is directly associated with AMD pathogenesis.

CONCLUSIONS AND RECOMMENDATIONS

Due to space limitations, we were only able to report a fraction of the literature, featuring some of the known inducers of AMD in the context of developing *in vitro* models for AMD research. A very detailed review on the risk factors for progression and development of AMD was published recently (Heesterbeek et al. 2020) and can be used as a source for developing *in vitro* models using AMD inducers that have not been featured in this report.

The improvement in the morphology and function of non-human and human derived RPE cell lines have paved the way for their use in pre-clinical studies testing potential therapeutics and pharmaceuticals. These cell-based models have primarily included single cell platforms, but also more complex cocultures (RPE and choroidal ECs), as well as organoids. The advantages of culture systems are their potential reliability, consistency, and low cost compared to *in vivo* studies. Additionally, single cell model systems allow for testing the effect of drugs in a cell-focused manner (Ferrer et al., 2014; Sharma et al., 2021) whereas the more complex coculture and organoid systems are a step closer to the physiological microenvironment, in which there are heterotypic intercellular communications. The need for high throughput platforms to test potential therapies has driven the creation and characterization of *in vitro* model systems that recapitulate morphologic and functional abnormalities associated with human AMD.

Going forward, we provide recommendations for these *in vitro* models described here. The immortalized cell lines each were generated from a single individual, therefore they do not recapitulate natural variation that occurs in the general population. Moreover, their use in an undifferentiated state has frequently led to results that may not reflect RPE biology. However, knockouts or knock-ins of genes of interest can easily be generated in these cell

lines using genome editing/CRISPR technology. Furthermore, their ease of access makes them an attractive tool for several labs, and recent work focusing on metabolic conditions have now demonstrated simple methods for establishing better polarized ARPE-19 cell cultures. Primary RPE cells and iPSC-RPE cells have been validated in several labs for physiologically relevant RPE phenotype and can recapitulate natural variation of patients or genetics of mouse models. One major drawback of these models is that they require specialized training and handling to achieve consistent and high-quality cultures reducing their accessibility. Furthermore, not all AMD-associated genetic variants have been routinely genotyped (Jong et al., 2021). In order to compare results using different studies, and to interpret the effects of genetic variants in these cultures it might be ideal to genotype all possible AMD-associated genetic variants and rare variants (e.g. the *CFH* and *CFI* genes) (Fritsche et al., 2016). However, this may be time-consuming and expensive. Therefore, since the complement factor H – complement Factor H Related 5 (CFH-CFHR5) locus on chromosome 1q32 and the ARMS2)/HtrA serine peptidase 1(HTRA1) locus on 10q26 account for the majority of genetic susceptibility for AMD (Pappas et al., 2021), we recommend that at a minimum, these two loci be genotyped. To improve usability of the currently available in vitro models for different aspects of AMD research, it is critical to develop standardized protocols and quality control criteria such that results can be compared across labs.

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Highlights:

- *In vitro* models of the RPE provide a powerful tool to study RPE biology and AMD pathogenesis
- Key hallmarks of AMD pathophysiology have been replicated in several *in vitro* models
- Primary RPE and RPE cell lines are easily accessible and useful in biochemical and cell biology studies
- iPSC-derived RPE have been validated to present native-RPE like features and can provide patient-specific models
- Organ-on-chips and 3D organoids provide physiologically relevant models combining RPE with choriocapillaris and/or retinal photoreceptors

Table 1

Properties of RPE cell cultures from different source.

	ARPE19[a]	Mouse primary RPE[b]	Pig primary RPE[c]	Human primary RPE (fetal origin)[d]	Human primary RPE (adult origin)[e]	iPSC-RPE[f]
Accessibility	High	Moderate	Moderate	Low	Low	High
Culture methods	Easy	Intermediate	Intermediate	Intermediate	Difficult	Difficult, variable
Time to full differentiation	Short-Medium (2–4 weeks)	Short-Medium (<1–4 weeks)	Short (2 weeks)	Long (8 weeks)	Medium (4 weeks)	Long (10 weeks)
Cost	Low	Moderate	Low-Moderate	High	High	High
Expandability	High	Low	Low	Moderate	Low	Moderate (but can differentiate a new batch)
Cell phenotype						
- Hexagonality	Moderate	Moderate	High	High	Moderate	High
- Pigmentation	Low	Moderate	Moderate-High	High	Moderate	Moderate-High
- Junctional resistance	Low	Moderate	High	High	Low	High
- Polarity	Moderate	Moderate	High	High	Moderate	High
Developmental stage	N/A	High (fully differentiated)	High (fully differentiated)	Moderate	High	High (similar to adult RPE)
Connection to AMD genetics	Possible	Possible	Possible	Possible	Extensive	Extensive
Ability to model AMD pathology	Moderate	Moderate	Moderate	Moderate	High	High
Genetic manipulation	Possible	Possible	Possible	None-possible	None	Extensive
Usability						
- Biochemistry	High	High	High	Moderate	Low	High
- Cell biology	High	Moderate	High	Moderate	High	High
- High throughput screens	High	Moderate	Moderate	Low	Low	High

Scales used: (1) high, moderate, low; (2) easy, intermediate, difficult; (3) none, possible, extensive; (4) short, medium, long

[a] Davis et al., 1995; Dunn et al., 1996; Hazim et al., 2019

[b] Gibbs et al., 2003; Gibbs and Williams, 2003; Fernandez-Godino et al., 2016; Shang et al. 2021

[c] Toops et al., 2014; Georgiannakis et al., 2015; Klingeborn et al., 2017

[d] Hu and Bok 2001; Maminishkis et al., 2006; Sonoda et al., 2009

[e] Fernandes et al., 2018

[f] May-Simera et al., 2018; Sharma et al., 2019; Sharma et al., 2020