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Pluripotent Stem Cell-Based Therapies in Combination with Substrate for the Treatment of Age-Related Macular Degeneration

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Abstract

Age-related macular degeneration (AMD) is the leading cause of blindness in the western world, which severely decreases the quality of life in the patients and places an economic burden on their families and society. The disease is caused by the dysfunction of a specialized cell layer in the back of the eye called the retinal pigmented epithelium (RPE). Pluripotent stem cells can provide an unlimited source of RPE, and laboratories around the world are investigating their potential as therapies for AMD. To ensure the precise delivery of functional RPE to the diseased site, some groups are developing a therapy composed of mature RPE monolayers on a supportive scaffold for transplantation as an alternative to injecting a single-cell suspension. This review summarizes methods of generating RPE from pluripotent stem cells, compares biodegradable and biostable materials as scaffolds, and describes the specific combination of human embryonic stem cell-derived RPE on Parylene-C membranes, which is scheduled to begin clinical trials in the United Sates in 2016. Stem cell-derived RPE monolayers on scaffolds hold great promise for the treatment of AMD and other retinal diseases.

Pathology of Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is the leading cause of blindness in people over 65 years of age and is predicted to cost the United States government at least $845 million annually as the population ages.[1,2] AMD manifests in 2 forms. Exudative or wet AMD is characterized by blood vessel invasion into the retina and accounts for about 10% of AMD cases.[3] Vision loss occurs due to the formation of fibrotic scars and an accumulation of fluid between the neural retina and its supportive retinal pigmented epithelium (RPE).[1] Currently, only palliative treatments are available for the wet form, which includes a regimen of intraocular injections of angiogenesis inhibitors, such as Lucentis®, Avastin®, or EYLEA®.

The other form of AMD, nonexudative or dry AMD, afflicts the majority of patients. In early stages of dry AMD, proteinaceous deposits called drusen accumulate in the subretinal space. Over time, this condition can advance to geographic atrophy, where RPE and photoreceptors degenerate and become dysfunctional within the macula. The macular area of the retina is responsible for high acuity, central vision, and although it only accounts for 4% of the retinal area, it perceives 10% of the visual field.[1] This is due to the fovea, a specialized region within the macula that contains the highest density of color-detecting cone photoreceptor cells in the retina. The fovea is just 2 mm in diameter, but its cones allow for 20/20 vision and the discernment of fine details.[1] Therefore, vision crucially relies on the photoreceptors in the macular region, and these cells are supported and maintained by the underlying RPE.

Located behind the retina, the RPE forms the blood–retina barrier.[4] The RPE is a polarized epithelial monolayer, meaning the 2 sides of the monolayer perform distinct specialized functions. The apical microvilli of the RPE interact with the photoreceptors, whereas its basal side attaches to the basal lamina in Bruch's membrane, which separates the RPE from the vascular choroid. Although it does not directly participate in the transduction of light sensation, which is the function of the neural retina, this epithelial layer performs a myriad of functions essential for vision. These include maintaining the health of the photoreceptors by transporting nutrients from the blood, removing old photoreceptor outer segments by phagocytosis, isomerizing all-trans retinol to 11-*cis*-retinal to perpetuate the visual cycle, limiting oxidation in the eye, and absorbing stray light.[1,4,5]

As the RPE age, accumulation of photo-oxidized chemicals and diminution of protective pigments are thought to

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contribute to the accretion of toxic concentrations of lipofuscin within the cells. This can precipitate an inflammatory feed-forward reaction, where stressed RPE shed debris as subcellular deposits, which in turn exacerbates the stressed state of the cells.[4] This cycle progresses until fatty, proteinaceous drusen deposits accumulate between Bruch's membrane and the RPE monolayer. These deposits can initiate immune reactions, disrupt the retinal architecture, and affect vision.

Drusen are associated with RPE dysfunction and degradation and are early signs of dry AMD.[6] Environmental factors such as smoking and genetic polymorphisms in complement factors H, B, and apolipoprotein E are associated with increased risk for drusen formation and development of AMD.[7–10] Although the molecular mechanisms that initiate AMD have not been fully elucidated, it is generally accepted that RPE dysfunction and death leads to deterioration of the photoreceptors, resulting in blindness.

Since the dry form of AMD accounts for 90% of cases and is due to RPE dysfunction, several efforts have been made to replace these damaged cells with viable RPE to rescue visual function.[3] Early efforts to restore healthy RPE to patients with a degenerating macular region involved detaching and rotating the retina, or transplanting an autologous, peripheral RPE–choroid graft to the affected macular area. Although some patients regained light sensitivity in the region of the graft, which demonstrates some modicum of proof of concept, the overall vision diminished and serious side effects such as retinal detachment were observed.[11]

Transferring RPE from one region of the eye to the diseased site also requires substantial surgical skills and is technically challenging. Furthermore, in these autologous graft cases, aged RPE comprise the therapeutic cells, and if genetic factors caused the AMD, then the graft will consist of similarly flawed cells.[12,13]

Sources of Stem Cell-Derived RPE

Geron Corporation's clinical trial for treating spinal cord injury in 2009 heralded the advent of human embryonic stem cell (hESC)-derived products as therapies for human maladies. In the infancy of this field, the eye is an excellent target organ for novel hESC therapies due to its potentially immune-privileged state, its excellent endpoint parameters with noninvasive imaging techniques, and sophisticated surgical protocols already in place.[14,15] Diseases affecting vision drastically reduce the patient's quality of life and present an economic burden to society. Therefore, developing effective stem cell-derived treatments for blindness has been an intense topic of research.

Current efforts to restore healthy RPE to patients use allogeneic RPE generated from hESCs and induced pluripotent stem cells (iPSCs), and both pluripotent sources can theoretically produce an unlimited supply of RPE for cellular therapies.[16,17] Clinical trials have already been initiated with RPE derived from both hESCs and iPSCs for the treatment of AMD[18–20] and are discussed in the delivery methods section of this review.

The hESCs are characterized as pluripotent cells, meaning they have the powerful ability to differentiate into any cell type in the body. During normal development, hESCs appear as the inner cell mass (ICM) region of the blastocyst, which is the embryonic structure 5 days postfertilization. This hollow cellular cluster contains about 100 cells, and these blastocysts are routinely generated outside of the body at *in vitro* fertilization (IVF) clinics.[21] Since only a subset of the created blastocysts at IVF clinics are selected for implantation, the remaining embryos can be cryopreserved for potential future implantations, donated to other couples, donated to research after informed consent, or permanently discarded.[22] If designated for research, the hESCs in the ICM of the donated blastocysts are collected, and due to their perpetual self-renewing capabilities, can be grown continuously in culture in the laboratory.[23]

Embryonic stem cells are not the only pluripotent source for generating RPE, for iPSCs are also capable of generating these specialized cells.[24] To accomplish induction of pluripotency in a differentiated somatic cell, a cocktail of exogenously applied stem cell transcription factors will commandeer the machinery maintaining the host cell's identity and forcibly reprogram the cell into an embryonic stem cell-like state. This technology was initially accomplished with murine cells in 2006[25] and recapitulated in human cells in 2007 by independent laboratories.[26,27] iPSCs rapidly revolutionized the approach to studying and treating diseases and earned their discoverer, Dr. Shinya Yamanaka, the Nobel Prize in 2012.

Early protocols for iPSC reprogramming involved virally inserting vectors in the host's genome, which could result in mutagenesis if the insertion disrupted the code for an essential gene. Newer methods use nonintegrating vectors, including episomal Epstein–Barr virus[28,29] or Sendai Virus.[30] Reprogramming has also been accomplished using mRNAs[31] and microRNAs.[32]

As the field progresses toward clinical applications, several attempts to replace viral integration with small molecules for induction have culminated in a successful virus-free reprogramming of murine somatic cells to pluripotency.[33] Exposing mouse embryonic, neonatal, and adult fibroblasts to a cocktail of 7 small molecules, consisting of a cAMP agonist, GSK, and TGF-β inhibitors and chromatin modifiers, was sufficient to reprogram the somatic cells into chemically iPSCs.[33] Reprogramming with small molecules and nonintegrating vectors avoids the risk of inserting exogenous genetic material into the host's genome, which could lead to adverse effects such as activation of oncogenes.

Since iPSCs originate from adult cells, fewer ethical and legal concerns hinder their progress toward the clinic. Additionally, iPSCs could be genetically matched to the donor cell type, thus providing the possibility of making patient-specific stem cells with theoretically reduced immunogenicity for cellular therapies.[34]

However, iPSCs are not identical to hESCs and may harbor genetic mutations and aging marks incurred by environmental insults on the original somatic cell.[35] Furthermore, the differentiation capability of iPSC lines may vary,[17,36] even when the lines originate from the same cell type. For example, Hu et al. generated 4 iPSC lines from RPE and found that only 2 of the lines preferentially differentiate back into RPE, while the other 2 iPSC lines produced less pigmented regions than the hESC controls.[37] Other reports also suggest a disparity in the function of iPSC versus hESC-derived RPE.[38] Although iPSCs should not be eliminated as a potential source of therapeutic cells, extra precaution and characterization should be performed on their derivatives before proceeding to the clinic.

Differentiation of Pluripotent Stem Cells into RPE

A plethora of protocols describe various ways of generating RPE from pluripotent stem cells. [18,24,36,39–48] It has been shown that stem cells grown as adherent monolayers or as 3D aggregates in suspension will spontaneously differentiate into a myriad of cell types, including RPE. Spontaneous differentiation is accomplished by removing molecules such as basic fibroblast growth factor from the stem cell culture conditions since these molecules are essential for maintaining hESC pluripotency *in vitro*. The differentiating adherent monolayer or free-floating, spherical embryoid bodies, will then require several weeks to months to make patches of pigmented RPE surrounded by other differentiating cell types.[41,42,49] These pigmented patches (Fig. 1A) may be manually isolated from contaminating cell types to obtain an enriched population of RPE (Fig. 1B).

Since spontaneous differentiation of stem cells into RPE requires an intense time investment, which would increase production costs of a cellular therapy, several laboratories have designed a variety of directed differentiation protocols to expedite this derivation. Furthermore, stimulating the directed differentiation of a particular cell type may discourage the growth of contaminating cells, thus increasing the final yield of the desired cell product. In these endeavors, natural signaling mechanisms that occur during *in vivo* development allow researchers to identify which molecular candidates should be exogenously applied to *in vitro* stem cells to accelerate their differentiation into RPE. The primary events of RPE development *in vivo* involve neural induction before gastrulation, specification of the eye field within the embryonic brain, and then maturation of specific ocular cells as the eye develops through the optic vesicle and optic cup stages.[5,50]

To initially guide stem cell differentiation toward a neural fate, as opposed to other fates from the mesoderm and endoderm germ lineages, directed differentiation protocols have applied neuralizing growth factors such as nicotinamide, Dkk-1, Lefty-A, and commercial supplements such as N2 or B27®.[36,45,46,48] Then, additional growth factors, such as Activin-A, retinoic acid, and TGFβ1, will predispose the neural cells to assume an RPE fate upon further differentiation.[46]

One report claimed to accomplish neuralization in 5 days, and upon addition of Activin-A achieved an RPE yield of 95% ± 1% by day 30.[51] To accomplish this efficient conversion of stem cells to RPE, cyst-like aggregates of undifferentiated hESC colonies were embedded in BD Matrigel™ hESC-Qualified Matrix, which is an extract rich in extracellular matrix proteins derived from the Engelbreth-Holm-Swarm murine sarcoma.[52] The embedded, 3-dimensional culture system mimicked the *in vivo* environment during neural tube formation by surrounding the entire cluster of cells with a matrix that supplied contacts for cellular adhesion. This approach facilitated the rapid attainment of a neural fate and varies from other

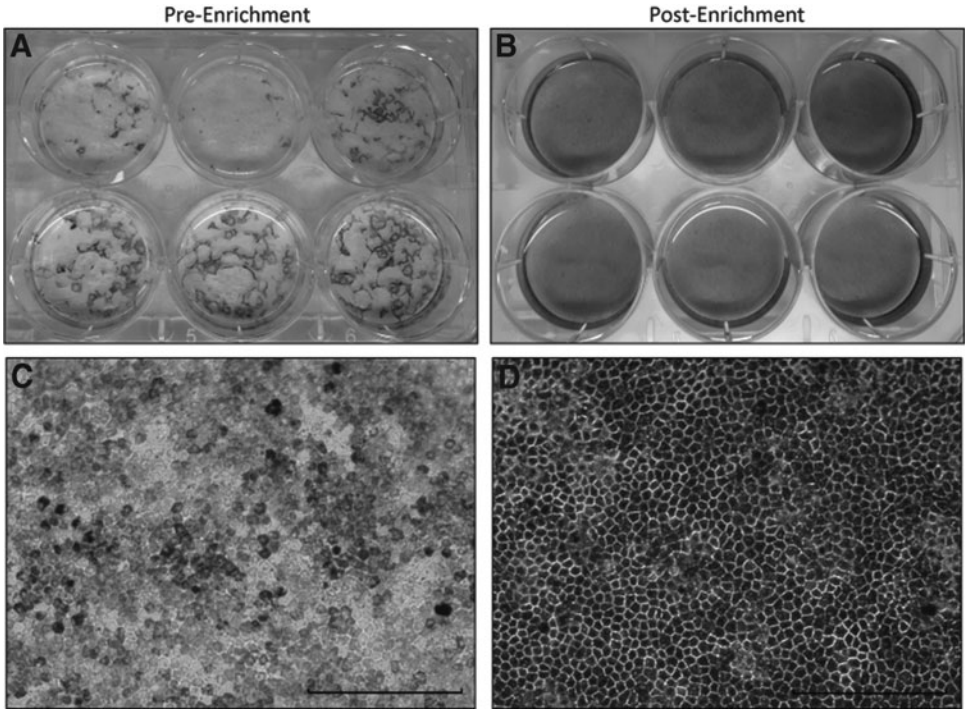

FIG. 1. Human embryonic stem cells (hESCs) differentiating into retinal pigmented epithelium (RPE) can be manually enriched to produce a more pure population of therapeutic cells. Upon removal of basic fibroblast growth factor, hESC cultures will spontaneously differentiate into a myriad of cell types, including RPE. After about 30 days in culture, pigmented foci will appear in the culture dishes. **(A)** After 100 days in culture, large pigmented regions can be observed within the well of the tissue culture vessel by the naked eye. These regions may be manually separated from nonpigmented cells in a process termed ''enrichment.'' **(B)** By 35 days postenrichment, a more pure population of pigmented hESC-derived RPE can be observed in the tissue culture vessel. **(C)** A brightfield and **(D)** phase contrast micrograph of an enriched hESC-derived RPE culture reveals the typical pigmented, cobblestone morphology. Scale bar = 200 μm.

culture methods that use free-floating spherical aggregates in solution or a 2D monolayer. The embedded neural progenitors, however, may produce both RPE and neural retinal derivatives. To promote an RPE identity, the 3D neural cysts were recovered from their Matrigel encasement and plated on a 2D culture system in the presence of Activin-A to foster the development of a polarized monolayer. By day 18, pigmented regions appeared followed by the acquisition of the typical cobblestone RPE morphology and expression of RPE-specific molecular markers such as Mitf, RPE65, and Bestrophin. These hESC-derived RPE effectively performed phagocytosis of photoreceptor outer segments when cocultured with murine retinal explants. This differentiation protocol demonstrates the value of combining exogenously applied growth factors, embedded 3D conditions, and 2D monolayers when directing the differentiation of hESCs to RPE.[51]

To date, the most rapid and efficient directed differentiation protocol generates progenitor RPE from pluripotent stem cells in 14 days. This approach first generates neural progenitor cells and then effectively promotes an RPE fate by 2 weeks, resulting in 97% of the stem cell derivatives being positive for PMEL17, a premelanosomal protein and an RPE marker.[39,40]

The first stage of this protocol establishes an eye field precursor population from pluripotent stem cells by adding nicotinamide and noggin to induce a neural fate,[46,53] the canonical Wnt inhibitor Dkk-1 to promote eye field development, and IGF-1 that stimulates forebrain and early eye field transcription factor expression.[5]

During the second stage of the protocol, addition of Activin-A and an FGF inhibitor SU5402 effectively promotes an RPE fate instead of neural retina. The final yield of PMEL+ cells can be increased from 80% to 97% by adding CHIR99021, a small molecule agonist of the canonical Wnt pathway, during the last 6 days of the protocol.[40] The progenitor RPE generated by the 14th day may be enriched and cultured as a monolayer for further maturation. After 30 days of maturation, these cells express RPE markers such as tyrosinase, PEDF, and CRALBP, and demonstrate functional phagocytosis of rod outer segments.[39,40]

In addition to rapidly generating RPE, this expedited protocol is useful for studying signaling mechanisms during RPE development. For the first time, activation of key components in the eye field transcription factor network and the timing of RPE gene and protein expression can be monitored over the course of 2 weeks in a human background. This protocol provides an opportunity to test the translational potential of eye development observations in model systems. Indeed, Leach et. al corroborated the importance of canonical/β-catenin Wnt signaling on human RPE development with the 14 day protocol, which previously had been observed during murine and chick ocular development.[40,54,55]

Directed differentiation of RPE from human pluripotent stem cells offers advantages such as increasing final RPE yield and reducing the time in culture, which lowers manufacturing costs. However, the majority of these protocols use Matrigel® in their procedures, which is a product derived from animals. To manufacture cells for clinical use, it is desirable for procedures to be performed under xeno-free conditions, which are defined culture conditions without animal products. Incorporating animal-derived components in RPE production methods introduces the risk of exposing therapeutic cells to nonhuman factors and viruses, which may cause an immune response posttransplantation.[56–58]

Therefore, efforts to optimize the clinical derivation of RPE from stem cells employ synthetic substrates such as vitronectin peptide Synthemax® plates as xeno-free alternatives to Matrigel and human feeder fibroblasts.[59–61] Also, synthetic small molecules could replace full-length recombinant proteins to avoid batch-to-batch variation in directed differentiation protocols. The small molecule chetomin, in combination with nicotinamide, yielded 67% of cells expressing a tyrosinase-GFP reporter after 35 days of iPSC differentiation.[59]

As the field of regenerative medicine progresses, so will the demand for stem cell-derived RPE production to be completed under defined conditions adhering to good manufacturing practices.

Engineering a Biocompatible Substrate for RPE Transplantation

A biomedical substrate or scaffold aims to provide a supportive, structural surface for cells to attach, proliferate, differentiate, and perform their normal functions after transplantation.[62] Results from preclinical animal studies indicate that monolayers of hESC-derived RPE on a substrate survive longer after transplantation than as single cells in a suspension.[63] The chemical composition and physical traits of the substrate may significantly influence how the transplanted cells operate in a diseased setting. Perhaps most importantly, the substrate must support the therapeutic cells' health and function.

To design an effective substrate for the transplantation of hESC-derived RPE, the qualities of the *in situ* RPE substrate should be considered. Naturally, the polarized RPE monolayer orients its apical side toward the photoreceptors of the neural retina, whereas the basolateral side sits upon the supportive, underlying Bruch's membrane. This pentalaminar membrane consists of 2 collagen strata that surround an elastin core layer that separates the basal membranes of the RPE from the underlying endothelial cells of the choriocapillaris blood vessels.[64]

When designing a substrate for hESC-derived RPE, the relative thickness of the transplant must also be considered. The Bruch's membrane is 1–4 μm thick, and substrates significantly exceeding this dimension could distort the contour of the overlying neural retina and result in a deformed visual perception.[65]

Purified proteins or modifications to constituents within the Bruch's membrane have been investigated as candidates for RPE scaffolds. Gelatin, which is made of single peptide chains from the denatured collagen triple helix,[66] has been investigated as a candidate substrate since it is a derivative of a protein that is naturally found in Bruch's membrane. However, when a gelatin substrate was used to transplant a porcine RPE sheet, an extra layer of RPE appeared after 28 days *in vivo*, possibly due to the sheet doubling back on itself.[67] Furthermore, employing natural products for substrate fabrication could lead to irreproducibility of results due to batch-to-batch variations in the acquisition of these proteins.

Synthetic polymers can be finely tuned to match the physical and chemical properties of the transplant's destination while also providing high reproducibility in large-scale manufacturing processes.[62] Both biodegradable and

biostable synthetic polymers have been investigated as substrates for RPE transplantation. The decomposition rate of biodegradable scaffolds can be controlled by varying the ratios of the constituents in the substrate and the types of bonds connecting them. Biodegradable scaffolds provide support to the therapeutic cells during and shortly after transplantation. These substrates do not leave a residual ectopic product in the eye since they are metabolized over time, which offers an advantage over biostable scaffolds. However, both the biodegradable polymer and its degradation products must not elicit an immune response.[62]

Several synthetic, biodegradable candidate materials have been investigated for supporting RPE health and function. Poly(D,L-lactic-co-glycolic acid) (PLGA) for example, is a food and drug administration-approved poly (α-hydroxy ester) whose biodegradable properties can be tuned by controlling the ratio of the lactide and glycolide constituents.[68,69] Certain ratios of high molecular weight PLGA can support the adhesion and proliferation of human fetal RPE *in vitro*.[68] Degradation of PLGA by hydrolysis of the ester bond between the 2 constituents results in molecules that are biocompatible and readily metabolized *in vivo*.[62]

However, to our knowledge, no biodegradable substrate has been used to transplant pluripotent stem cell-derived RPE into the subretinal space.[17] Conversely, biostable scaffolds would offer permanent support to the therapeutic cells, but must not interfere with the natural transport of nutrients.

Parylene-C is a member of the *para*-xylylene polymerization products, and this biostable polymer has been used in many biomedical applications, including transplanting hESC-derived RPE into the subretinal space.[70,71] This family of polymers consists of poly-*p*-xylylene, or Parylene-N, which is a linear chain of poly-*p*-xylylene whose aromatic rings are substituent free (Fig. 2).[70] Functionalizing the benzene ring with chlorine atoms produces polymer derivatives with varying mechanical, thermal, and electrical properties such as Parylene-C, which possesses 1 chlorine substituent per aromatic ring, and Parylene-D, which possesses 2 (Fig. 2).[70,72]

Initially, the electronics industry capitalized on Parylene-C's low water and gas permeability for producing protective coatings for circuit boards. Also, the chemical vapor deposition during the Parylene production process results in a conformal coat without pinholes.[70,73] Pinholes arise from bubbles that are trapped during the deposition process, which later burst to create defects in the film, thus rendering any underlying circuitry vulnerable.[74] This conformal coating property expanded Parylene's application to protecting delicate biomedical circuitry implanted into the relatively hostile environment of the body. Physicians have used Parylene-C to coat pacemakers, stents, and electrodes acting as neural prostheses.[73,75] Furthermore, Parylene-C is chemically inert and has a Young's Modulus measure of stiffness of 3.2 GPa, which confers recalcitrance to tearing.[72,73,76]

As a hydrophobic polymer, Parylene-C forms a protective barrier that isolates its cargo from the body and, therefore, must be modified to support adherent, therapeutic cells.[72,73,77] Like polystyrene, or tissue culture plastic, Parylene-C must undergo an etching treatment with oxygen plasma for 1–2 min to significantly increase its hydrophilicity to enable attachment of adherent cells.[72,78] Additionally, Parylene-C may be micropatterned into specific structures and stencils by photolithography, thus making it amenable to a range of cellular applications.[76,79,80] For example, layered Parylene-C stencils can support *in vitro* cocultures of stem cells with hepatocytes and fibroblasts. This allows for the regulated study of spatiotemporal effects of intercellular contacts between stem cells and their neighbors.[76]

Photolithography is essential to use Parylene-C as substrate for transplanting stem cell-derived RPE into the subretinal space. As mentioned above, Parylene-C is impermeable to water, gases, and small molecules, which would hinder the RPE function of transporting nutrients from the choriocapillaris below the Bruch's membrane to the photoreceptors in the neural retina. To overcome this obstacle, ultrathin permeable Parylene membranes (0.15–0.80 μm) have been designed and fabricated by the Tai group at the California Institute of Technology using a 2-step lithography approach.[80,81]

This lithography method results in a thick Parylene meshwork (6 μm) that supports the delicate, ultrathin regions. Synthesis of the transplantable scaffold starts with a thick coat of Parylene-C on a silicon foundation treated with hexamethyldisilazane (HMDS). HMDS affects Parylene's adhesion to other chemicals during the lithography process.[79,80] Then, an aluminum layer is deposited followed by a photoresist, which is a substance that confers protection to underlying layers until exposed to light. Lithography of the photoresist, wet aluminum etching, and reactive ion etching of the Parylene-C bores an array of 20 μm diameter cylinders through the underlying strata to the silicon base. This produces the 6-μm thick meshwork of the membrane. Finally, an application of an ultrathin Parylene-C layer over the pre-existing thick Parylene meshwork results in an array of ultrathin regions supported by the thicker polymer (Fig. 3A).[80,81] The finished substrate is a semipermeable, biostable support structure for the hESC-derived RPE (Fig. 3B).

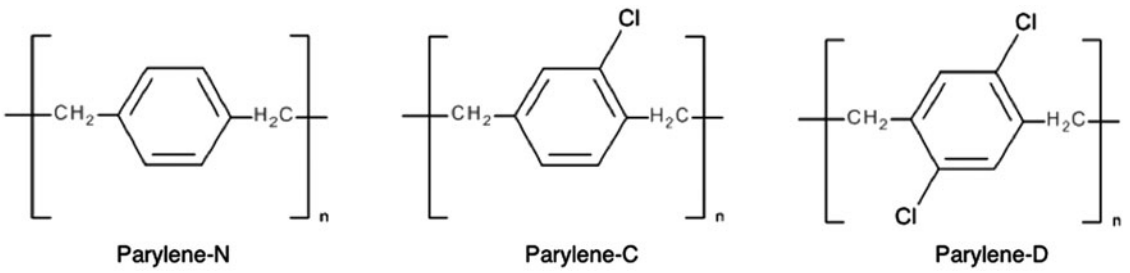

FIG. 2. The structures of members in the Parylene polymer family have different numbers and positions of chlorine substituents. This confers distinct physical properties, and each polymer can be used in various applications. Parylene-C has been implemented in coating electronics and biomedical devices and has been engineered into ultrathin, semipermeable membranes to serve as a substrate for hESC-derived RPE.

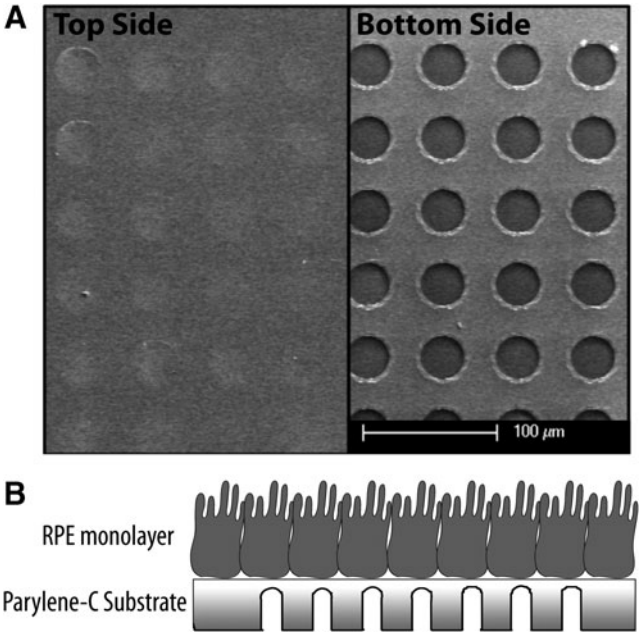

FIG. 3. Engineering Parylene-C for biocompatibility with hESC-derived RPE. **(A)** Transplantable scaffolds have been designed with a thick (6.0 μm) meshwork of Parylene-C that supports an array of ultrathin (0.3 μm) regions. The *top side* offers a continuous solid surface for adherent hESC-derived RPE, while an array of 20 μm pits make up the contour of the *bottom side*. The thicker regions confer mechanical support while the ultrathin regions permit diffusion of biomolecules. **(B)** A cartoon cross-section of the cellular therapy consisting of hESC-derived RPE cells on *top* of a Parylene-C substrate (illustration is not to scale).

Ultrathin regions of 0.15–0.50 μm thickness have been calculated to permit diffusion of chemicals up to MW ~1,302–291 KDa, respectively, which includes most of the serum proteins.[80] Vitamin A, a nutrient that must pass through the Bruch's membrane to perpetuate the visual cycle and its carrier have a MW ~75 KDa, which suggests that the ultrathin Parylene-C meshwork is a viable substrate for emulating the Bruch's membrane when transplanting stem cell-derived RPE.[80–83]

Having confirmed the ability of Parylene-C to permit nutrient transport, it is necessary to demonstrate its biocompatibility in the subretinal space. When compared to poly(imide), amorphous aluminum oxide-coated poly(imide), poly(vinylpyrrolidone) and poly (ethylene glycol) (PEG) after transplantation into the subretinal space of Yucatan pigs, Parylene-C and PEG did not significantly alter the retinal architecture nor did they instigate abnormal RPE behavior for 3 months.[84]

These studies demonstrate that Parylene-C can be fabricated with the proper dimensions and characteristics to support cell adhesion, provide appropriate diffusion properties, and to not distort the contour of the neural retina nor illicit an immune response.

Combination of Stem Cell-Derived RPE with Transplantable Parylene-C Substrate

Bringing a cellular therapy from the laboratory to the clinic requires astounding cooperation among diverse fields. Collaborations between cell biologists, material scientists, physicists, surgeons, and engineers reflect the truly interdisciplinary nature of regenerative medicine.[85] Designing a pluripotent stem cell-based product in combination with a synthetic substrate for subretinal transplantation to treat AMD exemplifies such synergy. This section discusses the combination of hESC-derived RPE with the synthetic Parylene-C substrate, surgical strategies to transplant this fabricated product, and the next progressive steps in the field.

Delivery methods for cell suspensions versus adherent cells on a substrate

An intense topic of interest in the field questions the efficacy of transplanting RPE as a single cell suspension or as a monolayer on a supportive substrate to treat AMD. Surgically delivering a cellular suspension inflicts less trauma than transplanting a scaffold, but RPE cells in suspension are distinctly disparate from mature monolayers. Both approaches have undergone preclinical animal studies and are being investigated in human clinical trials.[19,71,86,87]

Demonstrating efficacious delivery and functional recovery in an animal model are essential prerequisites before proceeding to clinical trials. Researchers have demonstrated the feasibility of transplanting hESC-derived RPE on Parylene-C into the subretinal space of the Royal College of Surgeons (RCS) rat, an important animal model of blindness.[71]

The RPE in the RCS rat cannot perform phagocytosis to remove the excess outer segments due to a mutation in the receptor tyrosine kinase gene *Mertk*.[88] Therefore, these animals start to become blind within 18 days after birth due to an accumulation of photoreceptor outer segments and experience total vision loss within 3 months.[88,89] Vision in RCS rats may be rescued by subretinal injections of fetal rat RPE,[90] adult human ARPE19, genetically modified h1RPE7,[91] hESC-RPE,[18] and iPSC-RPE.[92] However, the RCS rat does not emulate the disease phenotype of drusen deposits nor neovascularization as seen in AMD, but this strain does offer an animal model to test the viability, functionality, and potential immunogenicity of transplanted RPE cells on a substrate. The RCS rat model system has also been used to study retinitis pigmentosa, another degenerative retinal disease.

The first 2 human clinical studies using hESC-derived cells for the treatment of ocular ailments injected RPE suspensions into the subretinal space of patients with either dry-AMD or Stargardt's macular dystrophy. In both cases, many patients acquired slight visual improvement after 4 months.[19] Despite adverse effects from surgery complications and immunosuppression, safety issues arising directly from the injected cells have not been observed in the first 22 months.[93]

However, the degree of degeneration in diseased retinas will vary from patient to patient, therefore, the efficacy of injecting an RPE suspension as a viable therapy for the entire AMD population remains to be determined. Cells from a suspension could localize to any retinal area, and thus provide a randomized, patchy support for photoreceptors.[94] Furthermore, loose cells in a suspension may aggregate and preclude the formation of a polarized monolayer and possibly incite an immune reaction.

Clinical studies in Japan are investigating sheets of patient-specific iPSC-derived RPE monolayers as a treatment for exudative AMD.[95] The RPE sheets do not elicit

an immune response when transplanted into nonhuman primates.[96] The sheet's durability and orientation after surgery and the feasibility of producing iPSC lines for each patient is currently under investigation.

In contrast to single-cell injections and unsupported cellular sheets, solid transplantable substrates enable delivery of a mature monolayer to a specific destination while providing structure in a diseased environment. Parylene-C is a candidate substrate for transplanting therapeutic RPE, and hESC-derived RPE can adhere and grow into a confluent monolayer with characteristic RPE morphology and pigmentation on the ultrathin meshwork (Fig. 4).[80]

To compare the immunogenicity of hESC-derived RPE cell suspensions versus monolayers on Parylene-C, each condition was applied to the subretinal space of an athymic nude rat, an immunocompromised rat lacking T-cells.[63,97] Although neither resulted in tumor formation, the supportive Parylene substrate significantly improved the viability of the RPE 12 months postimplantation when compared to the injected suspension. Specifically, half of the 2,700 cells that were transplanted on the substrate were detected by human RPE markers 1 year postsurgery, while only 25% of the 100,000 suspension cells could be found in the eye.[63]

Additionally, the transplantable substrate significantly promoted the maintenance of a polarized monolayer while the suspension cells formed clumps in the subretinal space. Previous studies have demonstrated that once in suspension, the extent of RPE reattachment is directly proportional to cell survival, which is another advantage of employing a substrate.[98] Cells attached to a scaffold have an increased likelihood to survive, form a polarized monolayer, and to be precisely delivered to a specific destination.

Transplanting an intact hESC-derived RPE monolayer can be technically challenging, which has stimulated the optimization of surgical approaches to ensure reproducibility of the procedure with protection and proper orientation of the graft. To transplant hESC-derived RPE grown on an ultrathin Parylene-C membrane into the subretinal space of the RCS rat, the Hinton and Humayun groups at the University of Southern California have developed an implantation tool that supports and protects the delicate cargo and allows for specific orientation during delivery.

The implant consists of the cells and substrate, and it is secured on a thick, 10 mm parylene plate by 30 mm barriers. Together, this implantation device and its cargo remain sufficiently sturdy to endure the shear force of the surgery, but it is not too stiff to cause injury to the surrounding soft retinal tissue. The implantation plate and its therapeutic cargo are delivered into the subretinal space with forceps. Once in the desired location, forceps maintain the position of the implant while the tool is withdrawn.

In contrast to cumbersome transplantation devices that may interfere with the surgeon's view and result in accidental damage, this thin Parylene platform device is only slightly wider than the implant itself.[71] Proper placement of the transplant was confirmed by spectral-domain optical coherence tomography. Although the surgery caused a retinal detachment, which can be expected in this type of procedure, the tissue reattached within 1 week. Comparing the numbers of RPE attached to the substrate pre- and

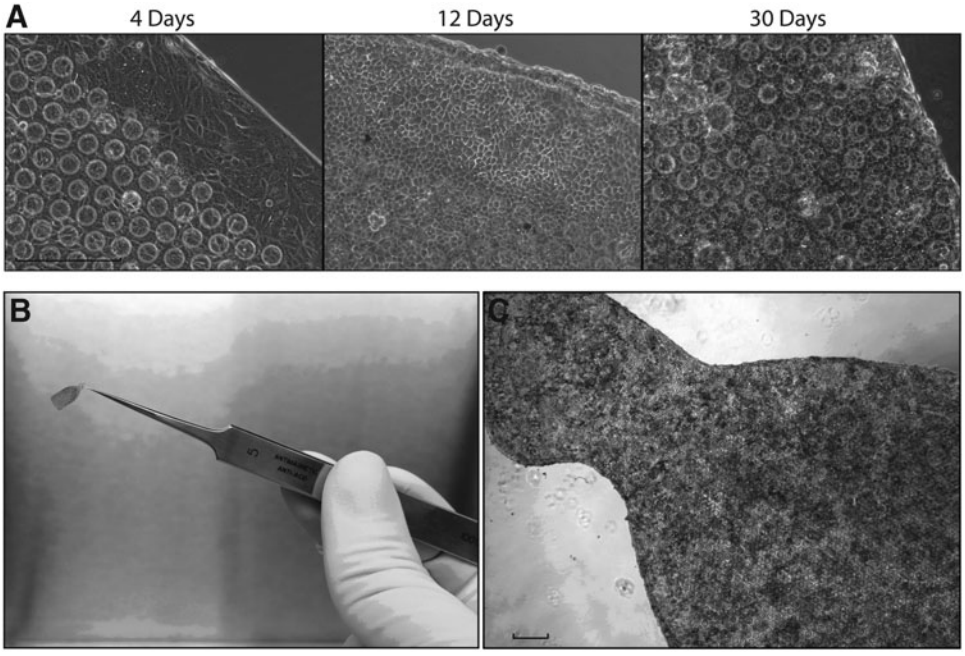

FIG. 4. Parylene-C scaffolds with ultrathin regions (0.3 μm) support hESC-derived RPE monolayers *in vitro*. **(A)** Four days after seeding hESC-derived RPE on Parylene, cells exhibit a fibroblastic morphology. Over the course of 1 month, the cells acquire the typical cobblestone morphology and form a pigmented monolayer, a hallmark of mature RPE. The ultrathin regions of the Parylene-C membrane appear as an array of *circles* in these phase-contrast micrographs. **(B)** The therapeutic product of hESC-derived RPE on a Parylene-C substrate allows for site-specific delivery of the mature monolayer. Adherent RPE therapies delivered directly to the relatively small region of the macula require fewer cells than an untargeted injection of cells in suspension. **(C)** Tabs on Parylene-C substrates (pointing *left*) enable surgeons to distinguish the *top* and *bottom side*s of the membrane, allowing the cell-side of the therapy to be transplanted with proper orientation. Scale bars = 200 μm.

posttransplantation revealed that <2% of the cells were dislodged during surgery. Specifically, the cell loss was observed primarily at the edges of the substrate, leaving the cells in the center of the patch seemingly undisturbed.[71] Optokinetic assays[92] that measure visual rescue by hESC-derived RPE on Parylene-C substrates *in vivo* are currently being investigated.

A fork-like implantation device has also been used to implant synthetic scaffolds of Parylene-C, silicon oxide, and iridium oxide into the subretinal space of RCS rats.[99] Before implantation, the scaffold slides between 2 prongs of the tool, which confers mechanical stability, and once in the subretinal space, the prongs slide away while a central bar keeps the implant in place. However, implants used with this device were thicker than 10 μm and had not been coated with cells. This approach may be more appropriate for transplanting thicker electrical retinal prosthesis rather than ultrathin membranes coated with therapeutic cells.[71,99] These bulky implantation tools may obstruct the surgeon's view, which could result in damage to the optic nerve and retina.

An alternate technique demonstrated protection of human fetal RPE grown on a rigid elastic polyester substrate made of polyethylene terephthalate by encapsulating with gelatin, which preserved the implant during an *in vitro* injection through a specially designed cannula.[87] However, to avoid batch-to-batch variation of naturally derived products such as gelatin, synthetic platform devices are preferred over organic substrates for clinical procedures. Additional tools must be designed when transplanting therapeutic cells on a substrate into larger mammals.

Future Directions

Monitoring stem cell-derived RPE posttransplantation is essential to fully assess their integration into host tissue. To this end, human specific markers, such as Tra-1-85 can be used to distinguish the hESC-derived cells from the host animal tissue in preclinical studies.[63] Furthermore, fluorescent reporters of genes of interest could be used in animal models. Novel methods for labeling the transplanted human cells are needed to allow researchers to evaluate proper monolayer orientation and integration as well as to detect any unwanted migration or dedifferentiation.

Transplantation of healthy hESC-derived RPE on Parylene-C may only treat early stages of AMD since degraded photoreceptors in late stages would be unresponsive to an RPE transplant. A plethora of protocols describe photoreceptor differentiation from hESCs, and hESC-derived retinal progenitors can integrate with host neural tissue and restore some visual response in blind mice.[36,45,100–102] However, photoreceptors cannot be restored in AMD patients unless the underlying RPE is also functional. Therefore, to rescue photoreceptors and RPE in late AMD patients, scaffolds supporting both cell types must be designed.

Pluripotent stem cell-based products are rising as a powerful tool in regenerative medicine. hESCs possess the ability to generate any cell type in the body, which offers an unlimited source of material for replacement therapeutics. Synthetic substrates may ensure the support and directed delivery of the hESC-derived cells in a myriad of diseases. If successful, pluripotent stem cell-derived products in combination with substrates may cure currently untreatable diseases, replace expensive palliative medications, and restore the quality of life to previously afflicted patients.

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Author Disclosure Statement

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