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Cellular and Biomaterial Approaches for Treating Age-Related Macular Degeneration

Ashrit Challa*, Srikar Sama, Selik Morrishetty
University of Pennsylvania, Philadelphia PA

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ABSTRACT: Tissue Engineering offers a novel, curative approach to treating Age-Related Macular degeneration (AMD), a disease characterized by excessive drusen deposition beneath the retinal surface and consequent vision loss. Preclinical studies in rats have shown that transplanted Retinal Pigment Epithelium (RPE) derived from human Embryonic Stem Cells (hESC) have not only slowed AMD but have also restored vision. There are two main methods of delivering RPE cells: direct injection and monolayer surgical insertion, the latter demonstrating long-term integration. Biocompatible scaffolds allow for better delivery of RPE cells, induced Pluripotent Stem Cells (iPSC), and Retinal Progenitor Cells (RPC). Unlike animal-derived extracellular matrix components, soft modulus biomaterials such as poly(lactic-co-glycolic acid) (PLGA) and poly(l-lactic acid) (PLLA) are ideal for AMD cell transplants because of fast degradation times, high cellular attachment proliferation, and strong adherence to Bruch's membrane. These biomaterials can also be created at a 10-100μm thickness so that vision is not distorted. Use of biomaterials could be improved by cross-linking them with anti-vascular endothelial growth factors (VEGFs) like Brolucizumab and retinal growth factors such as fibroblast growth factor (FGF). Similarly, hESC and iPSC cells can be genetically modified to secrete anti-VEGF factors.

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of irreparable blindness in the developed world, affecting approximately 170 million people worldwide. Over 11 million people in the United States suffer from AMD, with that number projected to reach over 22 million over the coming decades [1]. People affected by AMD experience a significant decrease in their quality of life due to impaired visual acuity at or near 20/200 (Figure 1) [2].

While methods such as complement therapy and neuroprotection are currently being researched, there are no efficacious treatments for dry AMD. AMD must progress into the later and more severe wet AMD stage for current treatments to be effective [3]. The prevailing therapies of photodynamic therapy and anti-vascular endothelial growth factor (VEGF) therapy are inadequate, as their focus is not on curing AMD and restoring sight, but on slowing down and preventing further vision loss [4].

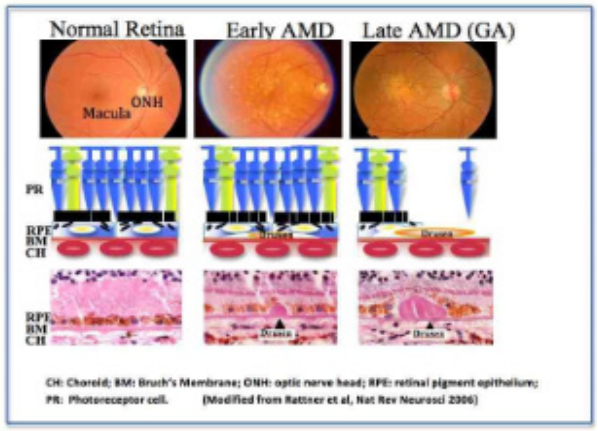

Figure 1. As AMD progresses, greater amounts of drusen disrupt the RPE-BM interface which leads to increased photoreceptor death.

As such, a tissue-engineered approach for treating AMD has impressive implications, potentially allowing for the reversal of a disease previously thought to be irreversible. This article reviews the clinical features of AMD, its current treatment options, stem cell and biomaterial tissue engineering therapeutic approaches, and pitfalls of and suggestions for such tissue-engineered approaches.

CLINICAL FEATURES OF AMD

The clinical trademark of AMD is the accumulation of fat and protein deposits, commonly known as drusen, in the macula, an area that is dense with photoreceptors responsible for high visual acuity [5]. Drusen accumulates underneath the photoreceptors beneath the retinal pigment epithelium (RPE), which functions as a source of nutrients and growth factors, as well as a photoreceptor phagocytosis mechanism. The RPE attaches to Bruch's Membrane (BM), which acts as a barrier between the retina and the choroid while regulating diffusion between the choroid and the RPE [6]. While the disease can be categorized into early, intermediate, and late stages based on the extent of drusen proliferation and vision loss, the most important distinction remains between dry and wet AMD. Dry, or non-exudative, AMD occurs when excess drusen is deposited between the RPE and BM. This causes gradual RPE and photoreceptor cell death, as well as central macular atrophy and blind spots. Wet, or neovascular/exudative, AMD usually follows dry AMD and is characterized by choroidal neovascularization (CNV), or the invasion of choroidal blood vessels into the RPE. Bleeding and leaking from these vessels result in RPE cell death and rapid progression of blurriness and loss of visual acuity [7].

CURRENT TREATMENTS AND MEDICATIONS FOR AMD

There are currently no effective curative treatments for AMD; however, existing therapies aim to manage the disease and stop its progression. Treatments for Wet AMD include laser photocoagulation and anti-VEGF therapy, such as Brolucizumab or Ranibizumab. Inhibiting vascularization in the eye prevents further progress of CNV, but it also contributes to chorioretinal atrophy due to less vascularization and potential narrowing of choroid capillaries. In fact, followups with anti-VEGF treated eyes indicate an extremely high level (98%) of macular atrophy, particularly in the fovea [8]. Photodynamic therapy (PDT) like Vertreporfin aims to simply stop the progression of destructive vascularization through laser-activated medication. However, there is no clinically significant improvement of visual acuity because of this therapeutic method [9]. As such, monthly anti-VEGF drug injections are currently prescribed and have had minimal to moderate success in restoring some vision to patients (about 30% of treated individuals), only maintaining the eye equivalent to its initial

state in which treatment first began. Even when PDT and anti-VEGF drugs are used in conjunction, a secondary approach when neither therapy is individually effective, there is a similar rate of visual acuity improvement (~11-13 letters) [10]. Recent advancements in pharmacology have led to the development of better-performing injections, such as Brolucizumab, which has a higher rate of visual acuity correction than Ranibizumab. Coupled with its small size, this compound allows for better vision improvement with fewer injections, but still holds the risk of injection-related complications and excessive drying [11]. Currently, there are no current treatments for dry AMD besides a surgical transplantation of a homologous donor retina. Transplantation has previously been shown to be ineffective due to a failure in synapse formation between fully differentiated tissue and the host. Surgical approaches of cleaning the debris near the retina and attempting to replace the degenerating retina with bolus injections have only offered temporary respite to the afflicted retina as it regresses back to its damaged form [12].

CELL THERAPY APPROACHES

Due to the difficulties of conventional therapies, recent years have witnessed much research and development in the use of stem cells and induced pluripotent cells as treatment or cures for AMD. In 1987, the first significant study on the use of transplanted RPE cells for treatment was conducted by Gouras et al, who placed transplanted rabbit RPE adjacent to the neural retina of a different rabbit [13]. Another landmark occurred ten years later, when Al-gvere et al. transplanted human fetal RPE patches into the subretinal space of human patients with wet or dry AMD. The results of this grafting indicated that RPE transplants would not be rejected and further degrade vision, even without immunosuppression, and that dry AMD had a lower graft rejection rate [14]. Since these landmark studies of RPE transplants, iris pigment epithelium (IPE) cells and human embryonic stem cells (hESC) have been transplanted in many animal models, showing vision improvement. Additionally, researchers have transplanted RPE cells, choroid-Bruch's-RPE explants, IPE cells and hESC in human patients with AMD, aiming to evaluate transplantation safety (Figure 2) [15].

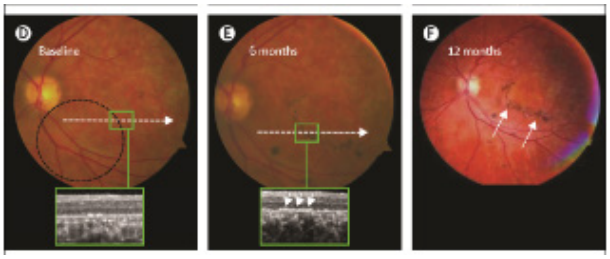

Figure 2: hESC-RPE cells injected into the subretinal space of human patients exhibit increased pigmentation and result in regeneration of the macula.

Preclinical studies conducted in rats have shown that using transplanted RPE cells derived from hESC have slowed retinal degeneration [16] and even improved visual acuity [17][18]. Additionally, hESC-RPE has formed a polarized epithelial layer in vitro, secreting growth factors such as pigment epithelium described factor and VEGF, all while expressing the barrier properties of normal adult human RPE cells [19]. Currently the two preferred methods for delivering hESC-RPE into the subretinal space are either injections of cells suspended in a fluid, which are inexpensive and simple but carry the risk of RPE cell dedifferentiation, or creating monolayers of hESC-RPE which can be surgically placed subretinally, necessitating a biologically compatible substrate [20]. Monolayers have shown higher rates of cell survival in comparison to injected

cell suspensions, as well as less clumping of cells [21]. Recent advancements with hESC-RPE therapy include a completed three-year and an ongoing clinical trial conducted in part by the London Project to Cure Blindness, with the former demonstrating the safety of long-term grafts while being the first to record longitudinal effects of hESC-RPE monolayer implantations. The completed study also showed lasting improvement in visual acuity of about 14-15 letters, as well as no adverse proliferative reactions such as teratoma formation, even after 37 months of observation. Similarly, the phase I results of the ongoing clinical trial, with interventions provided to two human patients, indicate hESC-RPE integration while presenting improvement of visual acuity and reading speeds [22][23]. While the former study was conducted using bolus cell injections of hESC-RPEs, the latter utilized a polyester sub-strate for monolayer insertion. Furthermore, there are also other clinical trials using cell sheets, such as a trial using induced pluripotent stem cell (iPSC)-RPE cell sheet transplantation in patients with dry AMD rather than wet AMD, although this trial has only very recently begun (NCT04339764). Though the retinal space in the eye is immunologically privileged, various studies have used immunosuppression (such as tacrolimus and mycophenolate mofetil) during their trials of implanted stem cells, while others have relied purely on the immunological status of the eye [24]. Both methods have had been successful in avoiding immunological rejection.

iPSCs have been studied for in vivo curative treatments and for the modeling of AMD and other eye-related diseases. This is because there is an information deficit in the exact mechanisms for the progression of AMD, specifically the non-exudative form. In response, the wide range of iPSC potential differentiation offers novel breakthrough methods in terms of replacement therapy and disease modeling [25]. To dedifferentiate human fibroblasts for use in iPSC-RPE procedures and other iPSC techniques, cells can be transfected with vectors such as the Venezuelan Equine Encephalitis RNA vector, inducing exogenous expression of pluripotency markers such as OCT4, SOX2, KLF4, and GLIS1. With a dedifferentiation rate of >95%, newly formed iPSC cells are then suspended to form embryoid bodies, which are then placed in RPE medium. This allows for iPSC-RPE cells from different lineages to express high levels of RPE genes and proteins, such as RPE65 and MERTK [26]. Already, iPSC-RPE cells have been used to better understand the molecular etiology of AMD, with one study identifying a single-nucleotide polymorphism near the VEGFA gene in AMD patients that decreases gene expression (Figure 3). In conjunction, another study has been able to identify genes that are differently regulated in AMD patients, alongside cell proliferation and localized immune response changes [27][28]. In this vein, iPSC-RPE research is exciting in its ability to better elucidate previously unknown mechanisms in AMD development.

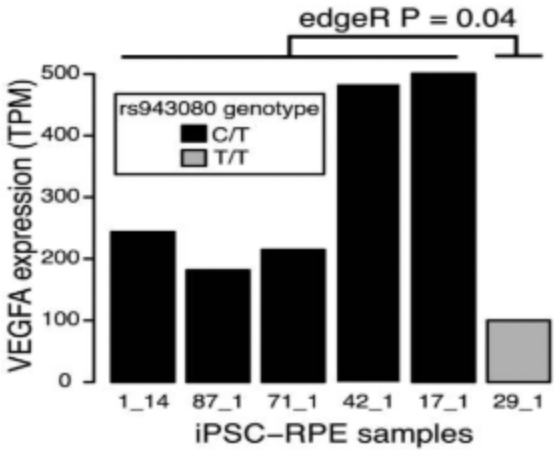

Figure 3. Expression levels of VEGFA for six iPSC-RPE samples, with the risk variant/ AMD SNP sample exhibiting significantly lower amounts of the gene

BIOMATERIALS APPROACHES

Surgeons currently do not have complete control over where the RPE or retinal progenitor cells (RPCs) are placed subretinally, with random clumps of cells not being conducive to regeneration. Additionally, without a way to ascertain the polarity and orientation of injected bolus or unoriented sheet cells, adherence to Bruch's membrane is drastically lowered. The use of biocompatible materials allows the parameters for delivery to be adjusted so that RPE cells, iPSCs, and other RPCs can be delivered subretinally to regenerate the damaged RPE cell layer, attaching to photoreceptors apically and BM basally. Thus, biomaterials must allow for cell attachment, proliferation, and correct orientation/polarization. To allow for RPE cell proliferation and BM attachment, the material must degrade by 2-3 weeks post-implant, and is ideally 10-100μm to allow for precise manipulation without retinal distortion and nutrient diffusion limitation [29].

Some biomaterials that have been explored as substrates for RPE monolayer insertion are collagen, Matrigel®, fibronectin, laminin, vitronectin, and oligopeptides. However, these organic, animal-derived extracellular matrix components were found to discourage cell proliferation and have variable degradation times based on individual enzymatic digestion rates [30]. Nanowires of poly(e-caprolactone) (PCL) may also be cast, but require precise construction in order to ensure porosity levels conducive to RPC polarization and attachment [31]. Poly(dl-lactic-co-glycolic acid) (PLGA), poly(glycolic acid) (PGA), poly(ethylene glycol) (PEG), and poly(dl-lactic acid) (PLA), are synthetic, thin, and degradable bio-materials. However, PLGA has an ideal degradation time of 2-3 weeks (whereas the others do not significantly degrade until about four weeks after initial cell seeding), can be 10-130μm thick, and has a feasible manufacturing process [32] [33]. Once solvated in chloroform or hexafluoroisopropanol (HFIP), these polymers are left to deposit on an even glass or Scaffdex surface for 8 hours while the solvent evaporates. The thin sheets are then left to dry, potentially stored in nitrogenous atmospheres over desiccators like calcium sulfate. They may then be crosslinked and sterilized using UV light [34][35]. The RPE, RPC, and iPSCs can thus be seeded. It has been found that there is 99-100% attachment of non-hESC human RPE cells to PLGA after 8 hours, and both PLGA and PGA sheets allow for RPE cell metabolism and protein expression. These polymers are therefore viable for cell attachment and proliferation, allowing for apical microvilli and basilar diffusion and molecule excretion [36][37]. However, these RPE cells do not attach significantly to PLA or PEG. After 3-7 days, cells become confluent, forming a multilayer or monolayer (depending on the cell type) of polarized RPE or RPCs and are ready for implantation.

PLGA sheets have been found to be the smoothest, thinnest, have the highest polarized RPE cell attachment, and most proliferation/material area repopulation. This is due to a 50:50 high molecular weight PGLA that contains an optimal ratio of lactic acid to glycolic acid. In comparison to previous substrates such as collagen, PLGA sheets are much smoother, thinner, and allow for the formation of an RPE monolayer with both correct orientation and polarity of cells. Additionally, multiple PLGA ratios of lactic acid to glycolic acid, such as high molecular weight (high MW) 50:50 and 75:25 PLGA, allowed for significant levels of cell proliferation. However, due to its faster degradation time, a high MW 50:50 PLGA blend is thought to be optimal [38]. The porosity of this blend allows correct adherence to BM so that as the biomaterial is degraded, the RPE cells attach

and are integrated into the eye. Studies also indicate that creating a polymer blend of PLGA and poly(l-lactic acid) (PLLA) may improve the porosity of the substrate, thus allowing for higher rates of cell proliferation. The modulus (hardness) of PLGA and poly(L-lactic acid) PLLA 50:50 is low enough to overcome the otherwise stiff composition of pure PLGA, with the flexibility provided by PLLA mitigating the risk of retinal damage. This 50:50 PLGA:PLLA was implanted in the rat model and the viability of the cells was monitored for 14 days [39]. Unlike previous attempts to directly inject RPC cells into the retina, the PLGA:PLLA bound RPC and RPE cells were still viable and expressed GFP, unlike transplanted cells, which only had a 10% survival rate 14 days after implantation (Figure 4). A more recent study shows that a 25:75 PLLA:PLGA blend might be more efficient, with a higher level of porosity and a lower elastic modulus (Figure 5) [40]. Soft modulus biomaterials of PLGA and PLLA are ideal for AMD cell delivery because they degrade fast, have high cell attachment, RPE and RPC cell polarization, cell proliferation, adherence to BM, and high viability after implantation.

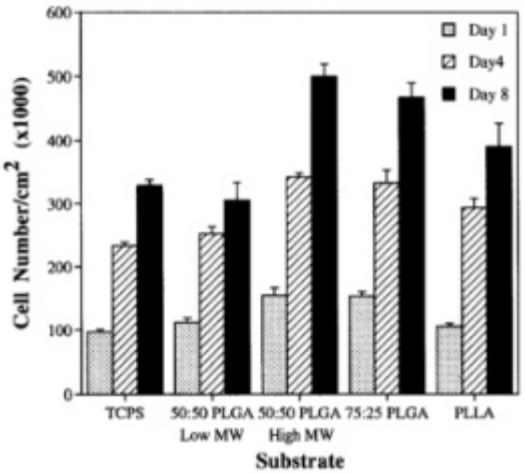

Figure 4. Attachment levels of RPE cells for different cell substrates, with three different compositions of PLGA, the most proliferative substrate

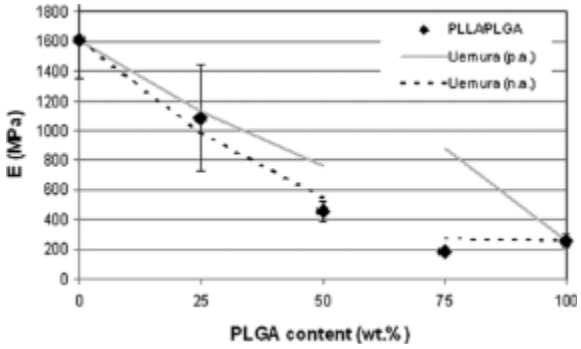

Figure 5: Predicted vs. Actual elastic moduli for various PLLA: PLGA ratios, with the 25:75 exhibiting the lowest modulus

SHORTCOMINGS

Although cell therapies have evolved greatly over the past few decades, regarding both transplantations and surgical monolayer integration, there are still many unanswered questions. Transplanted hESC-RPE cells demonstrated an increase in retinal pigmentation and visual acuity by about 14 letters in patients [41]. However, a 2008 study that surgically implanted fetal RPE into ten patients saw a four times improvement in visual acuity in one patient, from 20/800 to 20/200, which remained stable for five years [42]. This magnitude in visual improvement has not yet been seen from hESC or iPSC approaches, which are also marred by complications such as cataract formation and vitreous inflammation. And while iPSC-RPE clinical trial results are increasing in number, there is no published research on the longitudinal effects of iPSC-RPE cell transplantation in human patients with AMD. It is known that iPSC-RPE cells also have a faster rejection time than hESC-derived cells, triggering macrophage-mediated phagocytosis such that almost no iPSC cells remain after 13 weeks [43].

Given that donor retina scaffolds are difficult to procure, tissue engineering approaches provide alternative avenues. Regardless, the

use of cutting-edge stem cell engineering techniques and substrates like PLGA and PLLA still pose problems. Some of these problems include inflammation and injury due to the injection of cells, incomplete attachment of RPE cells to BM or a lack of subsequent proliferation, and the inability to replicate the true retinal environment. Cells may also dedifferentiate once attached to BM, which would only lead to the presence of more harmful debris in the retina and subretinal space. Both injected and surgically inserted cells have not yet shown complete restorations of vision and have only demonstrated peripheral and minor macular vision improvement. Finally, the toxicity of the material used as the cell substrate must also be considered, as degradation of these scaffolds will inevitably lead to the presence of small subunits in the macular region. With the use of PLGA, a potentially toxic and immunogenic substance in the eye, careful construction of scaffolding must be used in accordance with shape and size restraints. As more data comes out regarding biomaterial interactions with the actual human eye, rather than approximating animal models, it will be important to modify scaffold compositions and morphologies accordingly [44].

IMPROVEMENTS FOR CURRENT TECHNIQUES

Based on current research, there are many hypothesized methods of improving the treatment of AMD. Cell therapies utilizing stem cells can be further improved with genetically modified RPE cells, able to impede or revert the progression of AMD through genome and transcriptome modifications to counteract the changes of the disease. Neovascular AMD is due in part to an imbalance in growth factors such as VEGF. RPE cells naturally express many anti-VEGFs like pigment epithelium-derived factor (PEDF), but damage to these cells allows for excess vascularization. To further treatment, transplanted cells should be genetically modified to upregulate the production of anti-VEGFS, such as endostatin, PEDF, basic fibroblast growth factor (bFGF), brain-derived neurotrophic factor (BDNF), or ciliary neurotrophic factor (CNTF). To perform the necessary genetic modifications for the aforementioned suggestions, possible transfection methods include the Sleeping Beauty (SB100X)^ transposon system or the CRISPR/Cas9 system. During the later stages of AMD when damage has occurred to both photoreceptors and RPE cells, another improvement would be to transplant autologous ESC or iPSC which differentiate under a wider range of factors. This would allow for the reconstruction of the entire BM-RPE-photoreceptor complex, potentially leading to greater improvements in vision. In addition to therapeutic uses, it would also be helpful to use iPSC-RPE cells to model dry AMD alongside wet AMD in humans, as these cells could uncover more about dry AMD while finding use as high throughput drug screens. Gene therapy efficacies could also be tested in this way. This knowledge could lead to not just curative methods, but perhaps preventative interventions as well.

In using biomaterials as a substrate for RPE cells, endogenous factors can perhaps be crosslinked to PLGA or PLLA. As protases break the crosslinks in PLGA scaffolds, anti-VEGFs such as Ranibizumab and retinal growth factors like FGF could be freed, allowing for retinal regeneration [45]. Delivery of Ranibizumab via nanoparticles has already been demonstrated, so it is now a matter of delivering these types of medications on PLGA scaffolds for AMD patients (Figure 6) [46].

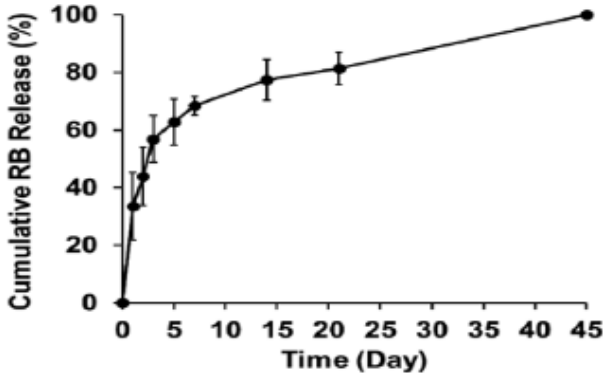

Figure 6: Cumulative Release percentage of Ranibizumab by PLGA nanoparticle system; if modified into PLGA scaffolding, could be used to deliver anti-VEGFs to integrating RPE cells

Because RPE cells must be polarized, incorporating PLGA with this cell type alongside RPCs and iPSCs may allow for differentiation into RPE and other retinal cells. In this manner, the eye can heal any damage done by the degradation or delivery of the substrate biomaterial and cells in the first place. Similarly, the use of drugs to modulate calcium systems has shown down-regulating effects on proliferation, which could be used in the case of teratomacreating iPSC-RPE cells. If cross-linked in PLGA scaffolds, these inhibitors could be used as a form of cell control [47]. In regard to surgical placement, current methods require the rolling up of PLGA/PLLA scaffolds. These rolled-up scaffolds are then delivered to the subretinal space. An improvement would be to develop a method by which a higher modulus material can be delivered to the eye directly in between the BM and the photoreceptors, perhaps via a small incision. This higher modulus material could then be removed to leave softer biomaterials, which are less harmful to retinal health and vision. Finally, cleaning up dedifferentiated cell and degraded scaffolding debris with lasers may also allow for better vision and healing.

CONCLUSION

Age-related macular degeneration is one of the leading causes of blindness in aging adults and is prevalent in today's population. Tissue engineering solutions for AMD have improved upon conventional treatments, thanks to their ability to not just stop the progression of AMD, but also result in visual improvement. The use of hESC-RPE and iPSC-RPE, coupled with a thin PLGA/PLLA scaffold, may allow for effective and safe integration of RPE cells in the macula. With more research on polymer toxicity, molecular crosslinking, and methods of efficient insertion, these cellular engineering techniques may become the best way to reverse AMD damage and fully regenerate the patient's eye.

AUTHOR INFORMATION

Corresponding Author

*Ashrit Challa, University of Pennsylvania, Department of Biology; achalla@sas.upenn.edu, 937-750-1530

Author Contributions

Ashrit Challa – Text
Srikar Sama – Figures and Proofreading
Selik Morishetty – Figures and Proofreading

ABBREVIATIONS

AMD – Age-Related Macular Degeneration
VEGF – Vascular Endothelial Growth Factor
RPE – Retinal Pigment Epithelium
BM – Bruch's Membrane
CNV – Choroidal Neovascularization
PDT – Photodynamic Therapy
IPE – Iris Pigment Epithelium
hESC – Human Embryonic Stem Cells
iPSC – Induced Pluripotent Stem Cells
RPC – Retinal Progenitor Cell
PLGA – Poly(dl-lactic-co-glycolic acid)
PGA – Poly(glycolic acid)

PEG – Poly(ethylene glycol)
PLA – Poly(dl-lactic acid)
HFIP - Hexafluoroisopropanol
PLLA – Poly(l-lactic acid)
PEDF – Pigment epithelium-derived factor
bFGF – Basic fibroblast growth factor
BDNF – Brain-derived neurotrophic factor
CNTF – Ciliary neurotrophic factor

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