Abstract

Currently, there is no cure for blindness. Pharmacology can only slow down the progression of a blinding disease, aiming at minimizing the symptoms. Stem cell regenerative therapy is a novel and very promising approach to overcome blindness. It has shown some potential in the eye but there remain major obstacles to be overcome before it can turn into a clinical reality, namely, the integration and survival of transplanted cells. In this work, we follow a two-pronged approach to address these challenges.

Studies of photoreceptor development and degeneration could previously only be performed in vivo, due to the extremely poor survival of isolated photoreceptors in culture. Here, we describe a three-dimensional culture platform that enables isolated mouse postnatal day 11 rod photoreceptor survival and maturation in vitro and investigate its mechanism of action. We show that material stiffness does not affect photoreceptor survival and instead identify hyaluronic acid (HA) as its bioactive component. We investigate the molecular pathways activated by HA on photoreceptors and find that the Wnt, RhoA and mTOR pathways are its downstream effectors.

The photoreceptors and the retinal pigmented epithelium (RPE) of the retina have a well-established mutual relationship in development, homeostasis and degeneration. In the second part of this work, we assess whether co-transplanting human embryonic stem cell-derived RPE
and mouse postnatal day 11 rod photoreceptors in an HA hydrogel can improve behavioral recovery and cell survival compared to transplanting each cell type alone. We first validate the NaIO$_3$ mouse model, which recapitulates advanced retinal degeneration. We next demonstrate that visual recovery in this model can be achieved only when RPE and photoreceptors are co-transplanted, as evidenced by three different outcome measures. This is accompanied by superior cell survival for both cell types in the co-transplant group. Our findings will guide future research and translation for retinal degenerative diseases.
Acknowledgments

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I dedicate this thesis to Beatrice Ballarin, who recently became my fiancée. I couldn’t have made it without your patience and understanding.
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1 Introduction

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1.1 Rationale

Degenerative diseases of the retina affect millions worldwide and are currently incurable. In the majority of such diseases, the RPE and photoreceptors are the first cells to die, while the rest of the retinal circuitry and architecture remains relatively intact. This has given rise to the idea that replacement of the deceased cells with functional ones might achieve lasting improvements in vision. Approaches to perform cell replacement have shown some efficacy [1-3], yet are hindered by the marginal survival and integration of transplanted cells.

Studying photoreceptor development, degeneration and their interactions with other cell types can currently only be performed in vivo, due to the poor survival and maturation of photoreceptors in vitro [4,5]. A culture system that enables isolated photoreceptor survival in
vitro would provide a valuable tool to address this problem. Designing such a system can be performed by drawing on knowledge from the role of the extracellular matrix (ECM) and tissue mechanics in retinal biology. Improving photoreceptor survival in vitro would provide hints of possible ways to improve their survival in vivo.

Despite extensive knowledge on the mutual relationship between RPE and photoreceptors and their coordinated development and degeneration in disease, cell transplantation approaches have so far focused on replacing either the RPE or photoreceptors separately [2,3]. Even though this has demonstrated some efficacy in genetic animal models of disease, cell survival is extremely limited [6-8]. Biomaterials have been employed to improve transplanted cell survival, but the majority are either non-injectable, increasing the potential of retinal damage during transplantation, or non-biodegradable, which raises concerns about long-term immune responses [9]. The derivation of RPE from human pluripotent stem cells [10,11] led to the commencement of a number of clinical trials employing RPE suspension transplants or RPE sheets for age-related macular degeneration or Stargardt's disease [12-14]. Preliminary data show some promise, but it is still too early to conclude whether these transplants lead to a significant vision improvement in patients. In addition, advanced stages of retinal degeneration in patients would require replacement of both RPE and photoreceptors. Providing photoreceptor and RPE delivery in an injectable biomaterial would combine the minimal invasiveness of dissociated cell transplants with improved cell survival, while replacing both the cell types that degenerate in disease.

1.1.1 Hypothesis and Objectives

The overarching hypothesis of this work is:

*Co-transplantation of human embryonic stem cell-derived RPE and mouse postnatal day 6 rod photoreceptors in the subretinal space of a rodent model of retinal degeneration will result in better survival of the cells and behavioral recovery of the animals than with either of those cell types alone.*

To test this hypothesis, this work was divided into two primary objectives:
1. To identify a culture platform that enables isolated postnatal day 11 mouse rod photoreceptor survival and maturation in vitro.

In chapter 2 of this work I demonstrated that culturing FACS-sorted Nrl-GFP+ rod photoreceptors in HA-based hydrogels dramatically improved their survival in vitro. I then investigated the mechanism of action mediating this pro-survival effect and demonstrated that material stiffness does not play a role. Instead, HA was necessary to improve photoreceptor survival. I then assessed the downstream signaling targets of HA in rods and identified mTOR as its main molecular mediator. Based on inhibitor experiments, I found that the canonical Wnt and the RhoA pathways are the upstream signals connecting HA to mTOR. Lastly, I demonstrated the Nrl-GFP+ rods undergo a partial maturation when cultured in the HA-based hydrogels, evidenced by marker upregulation at the RNA and protein levels.

2. To evaluate the effect of co-transplantation of human embryonic stem cell-derived RPE and postnatal day 6 mouse rod photoreceptors in terms of survival and restoration of visual function.

In chapter 3 of this work I differentiated human embryonic stem (hES) cells into RPE and co-transplanted them with Nrl-GFP+ rod photoreceptors into the NaIO3-injured mouse model of retinal degeneration. I first validated the NaIO3-injured mouse model by characterizing the temporal onset of blindness by behavioral assays and verifying retinal degeneration at the tissue level. I next performed a transplantation experiment at a late stage of the degeneration where I compared transplantation of both cell types together to each cell type alone, vehicle controls and uninjected animals. The vehicle used was an injectable HA-based hydrogel comprised of HA and methylcellulose (MC). I found that visual recovery by optokinetic head tracking and light avoidance assays were only achieved when RPE and photoreceptors were transplanted together. In addition, electrophysiological recordings showed the presence of detectable scotopic b-waves only in animals that had been transplanted with RPE and photoreceptors together. I assessed donor cell survival 2 months after transplantation and found a 2-fold increase in both RPE and photoreceptor survival when transplanted together compared to the respective single cell type controls.
1.2 The Retina

The retina is the light-sensitive tissue of the eye. It consists of 7 different cell types: rod and cone photoreceptors, horizontal cells, bipolar cells, amacrine cells, Muller glia and retinal ganglion cells organized in a laminated structure (Fig. 1.1). Due to the developmental sequence in which retinal cells are generated, the retina is arranged in an inside-out fashion, whereby light traverses the retinal ganglion cells and inner cell layers before it reaches the photoreceptors, which are located towards the outside of the retina. The photoreceptors detect light and convert it into an electrical and eventually chemical signal that gets propagated onto the bipolar cells, the amacrine cells and eventually the retinal ganglion cells which transport the information to the brain. The horizontal and amacrine cells are largely responsible for center-surround inhibition, which enhances contrast and sharpens visual perception. Horizontal cells receive input from photoreceptors and depending on their excitation status they deliver feedback to both photoreceptors and bipolar cells. Through synaptic modulation, they enhance neuronal activity on photoreceptor-bipolar columns that are directly targeted by light (“center”) and inhibit activity in neighboring columns (“surround”) [15]. Similarly, amacrine cells participate in synaptic modulation of both bipolar and ganglion cells, contributing to center-surround inhibition in the inner retina [16].
1.2.1 The Photoreceptors

The photoreceptors are the light-sensitive neurons of the retina. Rods, constituting approximately 97% of photoreceptors in the mouse and 95% in humans, are responsible for low-light (scotopic) vision, while cones are responsible for high acuity photopic vision.

Photoreceptors are compartmentalized in 3 compartments: the cell body, the inner segment and the outer segment. The cell body contains the nucleus, while the inner segment contains the organelles necessary for performing functions such as energy production and protein synthesis. The outer segment is a specialized structure optimized for light reception. It consists of membranous stacks that maximize the light-receiving area and contains all the enzymes participating in the visual cycle. Rod and cone outer segments differ in their structure and the
visual cycle enzymes employed; it is believed that the latter is responsible for the differential sensitivity of rods and cones to light [17]. Outer segment development is an indication of photoreceptor maturation and begins after the photoreceptors become specified, at approximately postnatal day (P)10-11 in the mouse [18]. This coincides with eye opening and the initiation of light detection.

Electrical signal transduction in photoreceptors is atypical with respect to other neurons [19]. The photoreceptor resting state is characterized by depolarization due to cation influx (mainly Na\(^+\)), which leads to continuous glutamate secretion. This gives rise to the so-called “dark currents”. Light absorption leads to activation of the G-protein coupled receptor opsin (rhodopsin in rods, cone opsin in cones) and conversion of a molecule of 11-cis retinal to all-trans retinal. The activated opsin activates transducin, which then activates phosphodiesterase, which in turn hydrolyzes cyclic guanosine monophosphate (cGMP). The decrease in cGMP concentration leads to closure of the cGMP-gated cation channels on the plasma membrane and hyperpolarization of the photoreceptor. Hyperpolarization interrupts the dark current of glutamate release, and this activates the downstream bipolar neuron. Hence, unlike most neurons, photoreceptors respond to a stimulus by hyperpolarization as opposed to depolarization.

1.2.2 The Retinal Pigmented Epithelium (RPE)

Retinal photoreceptors are juxtaposed by the RPE, a pigmented epithelial monolayer performing a plethora of functions that are essential for vision [20]. It was originally believed that the RPE’s only function was, by virtue of their pigmentation, to absorb stray light from reflecting back onto the photoreceptors, hence augmenting visual acuity. Interestingly, the association of pigment and vision is ubiquitously observed in biology: even the simplest light sensitive organisms combine pigment and photoreception within the same cell [21]. Classic developmental studies [22,23] established that the RPE hold an indispensable role in photoreceptor development, maturation and function (Fig. 1.2).

The RPE are located between the choroid, which constitutes the blood supply for the outer retina, and the photoreceptors. Choroidal vessels are fenestrated; tight junctions between RPE cells establish a diffusion barrier between the retina and the blood supply known as the outer blood-
retinal barrier. This ensures protection of the sensitive retinal tissue from pathogens and potential toxins that are circulating in the body, while at the same time bestowing the RPE with the role of gatekeeper. Nutrients entering the retina and metabolic byproducts being excreted to the circulation are actively transported through the RPE. In addition, the RPE secrete an array of pro-survival factors in a polarized manner [24], the most well-studied being pigment-epithelium derived factor (PEDF) and vascular endothelial growth factor (VEGF), which are pivotal to the health and homeostasis of both photoreceptors and choroid.

Perhaps one of the most intriguing functions of the RPE is its active participation in the visual cycle of the photoreceptors. 11-cis-retinal, the form of vitamin A required by the photoreceptors to initiate the visual cycle, is converted into all-trans-retinal upon photon absorption. The RPE uptake the all-trans-retinal and through an enzymatic complex isomerize it back into 11-cis-retinal and provide it to the photoreceptors. This ensures the responsiveness of photoreceptors to novel visual stimuli.

The photoreceptor outer segments are exposed to continuous illumination during the day which, in combination with their high metabolic activity [25], generates oxidative damage. To preserve their integrity, photoreceptors shed approximately 10% of the length of their outer segments daily [18]. The RPE function as phagocytes, uptaking the shed outer segments and digesting them [26]. This function is perturbed in the royal college of surgeons (RCS) rat, a well-established model of retinal degeneration, leading to photoreceptor death and blindness by postnatal day 30 [27,28]. The mutation responsible for this phenotype was mapped in the tyrosine-protein kinase mer precursor (MERTK) gene, and genetic correction of the mutation rescued the degeneration [29,30].

In vitro experiments of retinal explants demonstrated that the photoreceptors would survive and mature only when the RPE were present [31]. Experiments using modern genetic tools also showed that targeting mutations specifically to the RPE would lead to photoreceptor degeneration [32,33], while mutating the photoreceptors would result in RPE impairments [34]. Collectively, these findings established that the RPE and photoreceptors constitute one functional unit that is necessary for vision; alterations in one of the two partners of the unit results in degeneration of both and loss of vision.
1.2.3 RPE and Photoreceptors in Disease

Notwithstanding the invaluable contributions of basic research studies to our understanding of the mutual relationship between RPE and photoreceptors, similar observations can be made in human disease. Age-related macular degeneration (AMD) and retinitis pigmentosa are examples of such diseases.

1.2.3.1 Age-Related Macular Degeneration

AMD is the leading cause of blindness in the developed world, currently affecting approximately 170 million people globally [36]. Even though the exact etiology of the disease is still debated, AMD is believed to originate in dysfunctions of the RPE that culminate in degeneration of photoreceptors and blindness.

The first observable clinical signs of AMD are accumulation of extracellular deposits, called drusen, below the RPE and intracellular RPE deposits, termed lipofuscin. Drusen comprise aggregates of proteins, lipids and immune factors while lipofuscin mainly consists of undigested photoreceptor outer segment material [37]. These deposits are believed to indicate metabolic dysfunction and senescence of the RPE; they become more abundant with age and RPE
degeneration ensues. The RPE dysfunction and degeneration induces photoreceptor degeneration and blindness, leading to the phase of AMD known as dry AMD, the most advanced stage of which is termed geographic atrophy. AMD occurs in the macula of the retina, which is the cone-dominated area responsible for high acuity central vision only found in primates. The RPE and photoreceptor degeneration occurring in the macula leads to loss of central vision, while peripheral vision is initially maintained. In a minority of cases (approximately 10%), dry AMD is succeeded by blood vessel sprouting from the choroid into the subretinal space. These blood vessels are unstable and leaky, leading to blood accumulation in the subretinal space—a condition termed wet AMD. Even though recent pharmaceutical developments have achieved significant improvements in the management of wet AMD by inhibiting VEGF [38], no treatments exist for dry AMD, which constitutes 90% of cases.

It is worth noting that this sequence of events is debated by some experts in the field. Observations of rod degeneration in the periphery of the macula before the onset of RPE degeneration [39] have suggested that the initial deficit leading to AMD might lie in the photoreceptors. In congruence with this hypothesis, macular translocation studies demonstrated that when the retina is detached from the degenerating RPE in AMD patients and rotated to be positioned on top of healthy RPE, degeneration of the latter RPE ensues [40].

An additional hypothesis for the pathological origins of AMD, initially formulated by Friedman [41], classifies AMD as a vascular disease and states that degeneration of choroidal blood vessels precedes RPE dysfunction. The ensuing starvation of RPE leads to its degeneration. This hypothesis has been supported by histological analyses of patients’ retinas, where areas with degenerated choroid but intact RPE could be identified [42,43]. However, multiple groups have found the opposite phenotype both in experimental models and humans: regions with degenerated RPE but seemingly intact choroidal vessels [44-47]. Gerald Lutty and colleagues attempted to solve this conundrum by grouping wet and dry AMD patients separately and analyzing RPE and choroidal vessel integrity in cadaver eyes [48]. Interestingly, their results suggested that the RPE die first in dry AMD while the choroid dies first in wet AMD. They proposed that choroidal vessel demise in wet AMD induces hypoxia on the RPE, which leads to VEGF upregulation and neovascularization [49]. These data raised the possibility that dry and wet AMD may in fact initiate by different mechanisms, which leads to their disparate manifestation in patients.
1.2.3.2 Retinitis Pigmentosa

Retinitis pigmentosa (RP) is a rare genetic disorder, affecting approximately 1 in 4000 people, that commences with mutations in rod photoreceptors [50]. The disease is remarkably diverse; mutations in more than 50 different genes have been identified as the underlying cause.

RP manifests in degeneration of rod photoreceptors, which are mainly found in the periphery of the retina. This leads to night blindness and tunnel vision. Eventually, the cone photoreceptors at the center of the retina degenerate as well and the patients lose all vision. Interestingly, the demise of photoreceptors in RP is followed by alterations in the RPE, which de-differentiate and migrate along the blood vessels of the inner retina [51].

1.2.4 HA in the Subretinal Space

The subretinal space, which is the space between the RPE and photoreceptors, contains a plethora of proteins, growth factors and extracellular matrix (ECM) molecules [52]. HA constitutes a major component of that ECM, and is posited to function as a scaffold for the tethering of other ECM molecules such as chondroitin sulphate proteoglycans and growth factors such as PEDF [53]. Other components of the ECM include heparan sulphate proteoglycans, vitronectin, and small amounts of fibronectin and laminin.

HA is a glycosaminoglycan molecule that is abundant in the central nervous system as well as peripheral tissues. HA consists of repeating units of the dimer N-acetyl-glucosamine and D-glucoronic acid HA, and the chain lengths of the polymer can vary widely [54]. It is synthesized by HA synthases, enzymes which are anchored to the plasma membrane, and catalyze the addition of sugar units to an extending polymer chain, which is then secreted into the extracellular space [54]. In the retina, HA expression is high during development and remains present in adulthood [55]. Even though the cell-specific expression pattern of HA synthases in the retina has not been extensively studied, the RPE produce HA and secrete it in a polarized manner towards the photoreceptors [56].
Rather than being a mere space filler of the extracellular space, HA performs significant biological functions, that are mediated by signaling through HA receptors. The main HA receptors are CD44 and RHAMM (receptor for hyaluronan-mediated mobility), while other proteins have also been found to bind HA [54]. CD44 signals through the HER2 tyrosine kinase and Src kinases to induce cell survival, proliferation or motility [57]. The RHAMM downstream pathway operates through MAP kinases [57]. In the retina, the RPE express RHAMM while the Muller glia express CD44 [58,59]. Interestingly, neither of these proteins is expressed on photoreceptors; Hollyfield and colleagues identified two HA-binding glycoproteins, namely, SPACR and SPACRCAN, which are expressed on photoreceptors [60-62]. Interestingly, both SPACR and SPACRCAN mutations have a causative role in retinitis pigmentosa patients (Christian Hamel, personal communication and [63]). However, the downstream signaling pathways activated by these proteins upon HA binding remain unexplored.

1.3 Cell Transplantation Approaches to Reverse Blindness

Despite the presence of some remodeling [64], the loss of RPE and photoreceptors in retinal degenerative diseases leaves the rest of the retinal circuitry largely intact [65]. This finding has generated impetus for cell transplantation approaches aiming to replace the lost cells with the eventual goal of re-establishing vision.

Efforts to transplant cells in the posterior retina began in the 1980s with the innovative work of Peter Gouras [66,67] who transplanted human RPE in the monkey retina. This work was followed by the demonstration that RPE transplants could rescue the photoreceptors of the RCS rat [68]. The advent of stem cells ushered in an era of hope for regenerative medicine, due to the seemingly inexhaustible cell supply that they can provide. RPE were differentiated from hES cells in 2004 [10], and have been extensively demonstrated to be genetically and phenotypically similar to human fetal RPE [10,69]. Various labs have demonstrated visual rescue in models of RPE dysfunction by transplanting pluripotent stem cell-derived RPE [3,70], and clinical trials are currently ongoing across the world with both hES and induced pluripotent stem cell (iPS)-derived RPE in AMD patients [12].
These trials have either focused on performing RPE transplantation in solution [71], or more recently on sheets [14,72]. Both approaches are associated with advantages and limitations. Transplants of RPE grafts as a sheet lead to better RPE survival [73] and afford more control over the localization of the transplant, since no significant outgrowth of the transplanted RPE is usually observed [13]. In addition, RPE transplanted as a monolayer can be delivered in their polarized, mature state, while dissociated transplants will have to undergo maturation in vivo. In theory, this entails risks related to uncontrolled RPE proliferation, even though this has not been observed in animal models or humans after dissociated RPE transplants. Last but not least, dissociated RPE must adhere to an aged and potentially compromised Bruch’s membrane mature and function. Even though studies have shown that hES-RPE adhere to aged Bruch’s membrane more efficiently than aged RPE [74,75], RPE grafts provide a Bruch’s membrane substitute with the cells hence obviating the attachment issue. However, a significant drawback of RPE sheet transplants is that due to their size they cannot be injected through a fine needle, and instead require invasive surgery and custom-made delivery tools [76,77]. This increases the risk of damaging the already compromised retinal tissue. Increased immune responses have been observed at early time points after implantation of RPE grafts, compared to RPE suspensions [78]. Furthermore, clinical complications potentially associated with the graft transplantation surgery have been observed in patients [72,79]. Therefore, both suspension and graft transplants of RPE are accompanied by advantages and drawbacks. A strategy that combines the improved survival afforded by graft transplantation with minimal invasiveness would address these challenges.

Similar approaches have been undertaken in photoreceptor replacement. Since a demonstration in 2006 that early postmitotic rod photoreceptors could survive and integrate in host retinas contributing to visual recovery [80], various labs have demonstrated similar results with both primary and stem cell-derived rods [6,81-83]. Importantly, rod photoreceptors have also been derived from human pluripotent stem cells [1,84] and shown to improve visual function in animal models of photoreceptor degeneration. However, more research is needed to identify a developmental stage in those human stem cell-derived photoreceptors that exhibits a high integration potential, similar to the early postnatal mouse photoreceptors. Marius Ader, Jane Sowden and colleagues are spearheading investigations into identifying markers for such photoreceptors [85-87]. Notwithstanding the significant advancements made in the photoreceptor
transplantation field in the last ten years, the majority of such research has depended on GFP expression to track transplanted cells and determine survival and integration. Recent reports [88-90] by multiple laboratories have demonstrated that transplanted cells can transfer material to host photoreceptors, leading to GFP fluorescence in the host. The transfer appears to be predominantly occurring between donor and host photoreceptor cells, suggesting that advanced degeneration models which lack most of their photoreceptors at the time of transplant would be less vulnerable to misinterpretation of the results.

At the same time, a different school of thought, partly led by Turner, Aramant and Seiler [91-93] pursued whole neural retinal sheet transplantation (i.e. containing all 7 cell types of the retina) with the absence or presence of the RPE [94-96]. Cells that are transplanted as a sheet tend to survive better, as the continuous adherence to their matrix protects them from anchorage-dependent cell death, known as anoikis [97]. Additionally, the polarity of the cells is maintained, and it has been shown that sheet transplants exhibit greater immune tolerance compared to cell suspensions [98,99]. However, retinal sheet transplantation is plagued by issues relating to embryonic tissue sourcing, graft integration and complexity of the surgery. After transplantation, the recipient retinas contain two sets of inner nuclear layers and ganglion cell layers which is non-physiological.

Clinical trials focusing on cell transplantation for retinal degeneration will be discussed below, in section 1.3.2.

1.3.1 Biomaterials for Retinal Transplantation

The obvious advantages of transplanting cells as a graft as opposed to a cell suspension gave birth to the idea that the use of biomaterials could ameliorate the outcome of cell transplantation. Materials can provide an adhesion surface for the cells, and afford a more even distribution, decreasing cell aggregation [100]. They can also be used as vehicles for the incorporation of additional factors, with the purpose of enhancing cell survival, differentiation, or integration [101].

Biomaterials used for cell transplantation can be broadly classified into biodegradable and non-biodegradable materials. Ideally, a material will support early survival of the transplanted cells but eventually degrade and be replaced by native ECM. In the long term, non-biodegradable
materials can present a physical barrier to the integration of transplanted cells with the host circuitry [102], as well as induce fibrotic immune reactions [103]. Furthermore, subretinal implantation of a non-degradable material would cause persistent retinal detachment, which could lead to complications such as photoreceptor degeneration, gliosis, and proliferative vitreoretinopathy [104-106].

Early studies of cell transplantation into the retina focused on biomaterial biocompatibility. Bhatt et al [107] reported no gross evidence of rejection using sheets of collagen I to transplant human RPE into the rabbit subretinal space. Moreover, transplantation of biodegradable gelatin membranes containing neural retina sheets into the eyes of rabbits did not elicit an inflammatory response [108] and material degradation was investigated. These early reports demonstrated the feasibility of biomaterials for cell transplantation into the retina.

The mechanical properties of biomaterials used for cell transplantation are key to ensure minimal retinal tissue damage after transplantation. For example, Lavik et al [109] demonstrated that scaffolds composed of a blend of poly(L-glycolic acid) PLGA and poly(lactic acid) (PLA), prepared via solid–liquid phase separation, resulted in reduced elastic modulus and increased maximum strain at failure compared with PLA alone. The reduced elasticity of the scaffolds means that they are more compliant to the sensitive retinal tissue (i.e., the scaffolds can more readily alter their conformation to match the structure of the retina), and thus reduces the physical damage to the retina upon transplantation. In addition, the increased maximum strain at failure means that the scaffolds are less likely to break during the transplantation procedure.

Mouse retinal progenitor cells (RPCs) were efficiently and uniformly dispersed along the surface of PLGA/PLA scaffolds. RPCs adhered to the scaffolds, differentiated into Müller glia, and were implanted into the rat subretinal space. However, no evidence of photoreceptor or other neuronal differentiation is shown, which would have been preferable for transplantation into animal models of retinal degeneration. Müller glia transplantation has not been shown to have a regenerative effect in the retina, and usually impedes transplanted cell integration in the retina [110]. Thus, the authors suggest that future studies should also utilize chemical cues delivered in these PLGA/PLA scaffolds to promote differentiation into functional photoreceptors. Using the PLGA/PLA scaffolds, ~50% of transplanted cells were estimated to be alive after 14 days [109], which is significantly greater than that achieved by bolus injections, where typically ~1–2% of cells survive [80]. Importantly, a subsequent study demonstrated that biomaterials increase cell
survival after retinal transplantation compared with bolus injections [111]. Transplantation of RPCs into the subretinal space with laminin-coated PLGA-PLA scaffolds promoted a 10-fold increase in cell survival over bolus injection. At 2 and 4 weeks after transplantation, RPC differentiation was observed, as evidenced by the expression of neurofilament 200 (NF-200, a neuronal marker), PKC-α (a bipolar cell marker), recoverin and rhodopsin (photoreceptor markers). Some cell migration into the retinal layers was also observed. Probing the mechanism of increased survival conferred by the biomaterial, the authors showed that EGF, which is used in the culture medium of the RPCs, had adsorbed to the biomaterial and may have contributed to the increased survival observed in the biomaterial group. An advantage of this particular approach is that the biomaterial allows for more precise control over the placement of the transplanted cells in the host tissue such that it can be positioned at the site of the degenerated area (for example, in the macula for AMD patients). A potential caveat is that although PLGA-PLA scaffolds promote cell survival and differentiation upon transplantation, the implantation surgery is highly invasive and the biomaterial, although degradable, is still present after 4 weeks, and thus may lead to retinal detachment. Even though the molar mass and shape of the biomaterial and implantation site influence its degradation rate, PLGA is known to be a slowly-degrading material within the eye. For example, PLGA microspheres have been detected in the eye 6 months after transplantation [112]. Additionally, the number of cells that are shown to leave the graft and migrate into the retina is low, and one could argue that using a thick, solid biomaterial, while increasing the percentage of surviving cells, decreases their integration with the host tissue. This hypothesis is validated by results published by the same group, in pig transplantation experiments [102]. Retinal progenitor cells were transplanted either in single cell suspension, as spheres, or in the PLGA-PLLA material. Integration into the host retina was only observed in the cell suspension group. Collectively, these studies demonstrated that cells transplanted with biomaterials showed increased cell survival; however, there were still shortcomings that needed to be overcome.

Subsequent research focused on improving the migration of cells out of the biomaterial and integration into the host tissue. Tucker et al [113] transplanted RPCs using a scaffold consisting of PLGA encapsulated with matrix metalloproteinase-2 (MMP2). MMP2 is known to degrade neurocan, a CSPG, and CD44, a hyaluronan-binding protein [114]. Neurocan and CD44 can form fibrous networks in the degenerating retina (and in other CNS regions), and have been shown to impede neurite outgrowth, ultimately causing cell death [115-118]. RPCs that were
delivered from PLGA-MMP2 scaffolds exhibited greater migration into the host outer nuclear layer of the retina compared to PLGA-bovine serum albumin (BSA) scaffolds. Furthermore, the migrating RPCs differentiated into photoreceptors as determined by the expression of the photoreceptor markers recoverin or rhodopsin. Importantly, the presence of MMPs did not negatively affect retinal morphology. This study shows that functionalization of biomaterials with factors that neutralize inhibitory clues of the host microenvironment can improve the integration of RPCs into the retina.

Another technique to promote the survival, migration, and integration of transplanted RPCs into the retina was developed by Tao et al [119], who used microelectromechanical system technology to create ultrathin (6 μm) poly(methyl methacrylate) (PMMA) sheets with or without pores of defined sizes. The survival and proliferation of murine RPCs cultured on porous and non-porous PMMA sheets in vitro were comparable to cells cultured on tissue culture polystyrene. Interestingly, RPC transplantation with porous PMMA sheets into the mouse subretinal space demonstrated significantly more surviving cells after 4 weeks compared with non-porous PMMA sheets. The difference was hypothesized to be due to an inferior retention of the RPCs in the non-porous PMMA during the transplantation process, leading to increased cell reflux. Substantive cell migration into the host retina was also observed with the porous scaffolds; migrated donor cells expressed glial fibrillary acidic protein (GFAP), NF-200, or recoverin as evidence of differentiation towards Müller glia, neurons, and photoreceptors, respectively. The retinal detachment created during transplantation appeared to resolve itself within 4 days, possibly due to the thin, porous material used; however, although PMMA is biocompatible and has been used as a lens replacement for many years [120], it is a stiff, non-degradable material [121]. Therefore, it would either ultimately require a second surgery for its removal or, if left within the eye indefinitely, would likely result in a long-term immune response or other complications associated with the intraocular use of PMMA [103,122,123].

Non-biodegradable materials are currently being investigated in the clinic for RPE transplantation in AMD. RPE normally adheres to the Bruch’s membrane, a pentalaminar semipermeable structure located between the RPE and choroid [124]. Bruch’s membrane alterations and deposit accumulation are observed in AMD, which impairs RPE adhesion [124]. This finding led to investigations into generating synthetic non-biodegradable Bruch’s membrane substitutes to combine with RPE transplants. Parylene-C, a clinically used polymer which is
biocompatible and chemically inert, was found to exhibit similar permeability to healthy human Bruch’s membrane when its thickness is reduced to 0.3 um [125]. These thin parylene-C membranes were combined with a 6 um-think mesh to generate a scaffold with improved mechanical strength which was shown to support hES-RPE survival and polarization in vitro. Subsequent in vivo studies demonstrated that the parylene-C scaffold led to significantly improved survival of hES-RPE after transplantation in nude rats, as compared to hES-RPE injections in suspension, but was also accompanied by a stronger fibrotic response in early time points [78]. The hES-RPE loaded parylene-C scaffold improved visual acuity in the RCS rat [8], and after a preclinical safety study in minipigs with no significant adverse events reported [126] was used in a phase I clinical trial for dry AMD. No safety issues were identified in the 4 patients treated, while some indications of visual improvement were noted, even though the sample size was too small to draw conclusions on efficacy [14]. A fifth patient was initially included in the trial but could not undergo implantation of the scaffold-RPE construct due to subretinal scarring, the relation of which to the transplantation surgery is unclear. A separate group used polyester membranes to transplant hES-RPE in pigs before proceeding to a phase I clinical trial on 2 patients for wet AMD [72]. 1 of the 2 patients experienced proliferative vitreoretinopathy which may have been caused by the transplanted RPE or the transplantation procedure and needed surgical correction. Preliminary data on visual fixation indicated a potential beneficial effect of RPE-scaffold construct which would need to be validated on more patients.

The use of materials that cause the least amount of damage to the eye during the transplantation procedure is a significant consideration, and has led to the development of injectable biomaterials. All of the aforementioned materials were transplanted in the subretinal space through a scleral incision, using custom-made surgical tools, which entails significant trauma to the eye. In an effort to resolve this issue, Redenti et al [127] used microfabricated poly(glycerol sebacate) (PGS) scaffolds that were 45 μm thick with 50 μm diameter pores, and coated them with laminin. Mouse RPCs cultured on PGS showed increased adhesion and proliferation compared with those cultured on glass. This highly elastic material was injected through a 25-gauge needle, requiring only a 1–2 mm scleral incision. PGS is biocompatible and degrades within ~1 month after subretinal transplantation. Subretinal transplantation of mouse RPCs in PGS showed that mouse RPCs migrated into the inner and outer nuclear layers of the host retina, with few differentiating into photoreceptor and bipolar cells; however, the efficacy of this matrix for cell delivery in vivo is unclear, as no control group consisting of cells in the absence of PGS
was included. Interestingly, Ballios et al studied HAMC, an injectable hydrogel consisting of HA and methylcellulose (MC) [100], for retinal stem cell (RSC) transplantation, first showing its benefit in vitro and then in vivo. Importantly, the HAMC hydrogel was injected through an even finer (than the PGS scaffold), a 34 gauge needle with fast gelation after injection. An in vivo degradation assay in the subretinal space demonstrated resorption of the gel after 7 days, thereby minimizing risks of adverse retinal detachment while maximizing cell migration and host-tissue integration. After subretinal injection in mice, RPCs delivered in HAMC were evenly distributed along the Bruch's membrane whereas RPCs delivered in conventional saline controls were aggregated. Importantly, the retinal architecture was not affected by the hydrogel injection. A follow-up study using retinal stem cell-derived rod photoreceptors demonstrated superior integration of these cells into the photoreceptor layer of genetically blind mice [6], when injected in HAMC vs saline. The cell transplants in HAMC partially restored the pupillary reflex to light in those blind mice. Together these data demonstrate that HAMC is a compelling biomaterial for cell delivery to the retina while obviating the complicated surgery associated with transplanting biomaterial sheets.

1.3.2 Clinical Trials for Retinal Degeneration

Recently, a major breakthrough was achieved in retinal disease treatment using gene therapy. A clinical trial in Leber congenital amaurosis (LCA) patients demonstrated significant improvements in vision after delivery of the wild-type RPE65 allele using an adeno-associated virus (AAV), and the product was approved by the FDA [128,129]. The vision improvements were maintained for at least 3 years [130]; however, it was later discovered that, despite the initial improvements in vision after RPE65 gene replacement, retinal degeneration can continue [131]. The (albeit partial) success in this particular trial created hope that more monogenic retinal diseases, such as RP, might be treatable using gene therapy [132]. However, there are more than 50 candidate genes [133] whose mutation is known to cause the disease. A new clinical trial would be needed for each specific gene, which in conjunction with the relative rareness of the disease, renders this gene therapy strategy unworkable.
Neuroprotective strategies have also been pursued to promote repair in retinal disease patients. For example, ciliary neurotrophic factor (CNTF) expressing cells, encapsulated to avoid immune rejection, were implanted in the vitreous of RP patients [134]. The capsules were biostable, composed of polyether sulfone, polycaprolactam, and polyvinyl pyrrolidone whereas their lumen contained polyethylene terephthalate, which was found to support cell survival. Six months after implantation, no adverse effects or complications were observed. In a second trial with more participants, the effects of the CNTF-expressing implant were assessed for visual acuity [135,136]. Although no significant benefit was observed in vision tests, the implants led to an increase in macular volume and cone density. The same encapsulated cell implant was used in a clinical trial in dry AMD patients, with similar results: no significant improvements in vision were found, but macular thickness increased in the treated eyes compared to controls [137].

These challenges underline the need for cell transplantation strategies, focusing on replacing the RPE and/or photoreceptors that are lost because of retinal degeneration. Most of the clinical trials performed to date focus on replacement of the RPE, because of the relative ease in isolation and culture of viable RPE from the human eye, as well as its location at the outside of the retina. These trials are primarily focused on AMD, in which RPE degeneration is believed to be pivotal to disease progression [138]. Early trials attempted to rotate the retina to bring the macula over a patch of healthy RPE [139-141] and some improvements in vision could be observed [142,143]. However, the risk for complications was relatively high [144] and later studies found that RPE degeneration is recurrent in the novel patch of RPE over which the macula is placed [40]. These issues obviated the widespread use of macular translocation in the clinic [145].

A more straightforward approach to cell replacement has been RPE transplantation. After showing success in animal experiments [146,147], RPE patch transplantation of autologous cells harvested from a healthy area of the affected eye or homologous cells from an adult enucleated eye was used in the clinic [148]. The autologous transplantation gave positive results, whereas the homologous one showed no improvements. When human fetal RPE were transplanted as patches in AMD patients, no visual improvements were reported and macular edema occurred, probably because of an immunological reaction to the cells [149]. Notwithstanding that some consider the retina to be an immunoprivileged site, these studies underline that immune rejection of allografts is an important consideration in transplantation approaches to the eye. To overcome this issue, Binder et al. transplanted autologous RPE in solution, harvested from the same eye, in wet AMD
patients [150] in combination with submacular surgery for removal of the neovascu- lature. Vision improvements were observed in 57% of patients, with no recurrence of the neovascularization at least 1 year. These positive results led to a clinical trial were submacular surgery alone was compared with submacular surgery combined with autologous RPE transplantation [151], which concluded that RPE transplantation led to improvements in visual acuity and retinal thickness; however, vision was still impaired and patients could not read [152]. In contrast, a different study that also performed autologous RPE transplantation combined with submacular surgery detected no improvements in vision and concluded that the particular approach is not beneficial [153]. Even though there were differences between the two protocols, such as number of cells transplanted, that may account for the discrepancy observed, this variability underlines the delicate surgical procedures needed in submacular transplants which limits reproducibility. In a different trial, van Meurs et al. transplanted autologous patches of RPE containing Bruch membrane and choroid in wet AMD patients [154]. Short- and long-term follow up examinations, of up to 7 years after surgery [79], showed that the transplants were able to preserve macular function in most patients, and even increase visual acuity in a small subset. Falkner-Radler et al compared the two different approaches for RPE transplantation (i.e., cell suspension or RPE-choroid graft transplantation) and found them to be similar in terms of visual outcomes and tissue correlates [155].

Overall, the RPE transplantation studies performed to date have shown that vision acuity improvements can be achieved, and the complications observed are not as frequent as in the macular translocation experiments. The extent of vision improvement needs to be increased for these approaches to become clinically relevant and to justify the complex surgical procedures required. One potential reason for the limited amount of vision improvement is that the autologous RPE used are already aged, and therefore exposed to significant oxidative damage and lipofuscin deposits. hES-derived RPE offer the potential to overcome this issue, because they are similar to fetal RPE in terms of gene expression [10], yet lack the limitations of older cells. In addition, RPE attachment to the aged Bruch membrane is known to be problematic [156,157], and constitutes a potential reason for the limited success of transplantation approaches. Interestingly, fetal and hES-derived RPE attached better than adult donor RPE to old human Bruch membranes in vitro [74,75]. The superior quality of RPE attachment (and survival) when derived from ES cells than adult autologous RPE, coupled with the fact that hES-derived cells are more readily available than fetal RPE, led to the commencement of a clinical trial with hES-derived RPE in dry AMD patients [71]. With the use of immunosuppression, the rejection problems that were observed in the past with
homologous RPE appear to have been resolved. This is the second clinical trial using hES cell–derived progeny in the United States after the Geron trial that used ES-derived oligodendrocyte precursor cells for spinal cord injury repair, which was interrupted and eventually taken over by Asterias [158]. More trials based on pluripotent stem cell–derived RPE transplantation have started in the United States, Europe and Japan [12]. 3 of these trials, namely, the London project to cure blindness [72], the California project to cure blindness [14], and the trial led by Masayo Takahashi in Japan [159] are performing RPE transplantation in sheets. The latter is the first clinical trial using induced pluripotent stem cell–derived progeny.

The main issue that RPE transplantation faces is that if photoreceptor degeneration has occurred, just replacing the RPE will not re-establish vision as functional photoreceptors are also needed [160]. Therefore, RPE transplantations must be performed early in disease progression, preferably at the dry AMD stage, before photoreceptor degeneration begins. At later stages, both cells should be replaced. An interesting approach in this direction was followed by Radtke et al. [161] who transplanted fetal retinal sheets containing the entire neural retina with its RPE in RP patients. The sheets survived up to at least 6 months, which was the latest time point examined, and there was no evidence of rejection or edema. In a second study, fetal retinal sheets were transplanted in RP and dry AMD patients, and seven of ten patients had improved vision [162]. Significantly, the improved vision was maintained for between 1 and 6 years postsurgery—that is, until the last time point surveyed per patient. Although this approach is limited by the supply of fetal tissue and ethical considerations, it demonstrates promise and lays the foundation for future studies.
2  Hyaluronic acid hydrogels enable rod photoreceptor survival and maturation in vitro through activation of the mTOR pathway

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2.1  Abstract

The culture of isolated photoreceptors in vitro has remained elusive in neuroscience. By using defined hyaluronic acid (HA) hydrogels, we dramatically increase photoreceptor survival and maturation in vitro, as evidenced by upregulation of outer segment markers at the RNA and protein levels. While substrate stiffness is known to be a key factor influencing cell survival in vitro, we show that isolated photoreceptors do not respond to modifications in hydrogel stiffness modifications but depend, instead, on HA for survival. While the molecular pathways that are induced by HA on photoreceptors are unknown, we identify mTOR activation as the molecular mechanism underlying the pro-survival effect, and demonstrate that the canonical Wnt and RhoA pathways are intermediaries. This work establishes a valuable method for isolated photoreceptor culture in vitro, which will be useful in translational and basic retinal research. The pathways identified herein may be useful targets in retinal degeneration.

2.2  Introduction

The retina is the light sensitive tissue of the eye that is responsible for vision. Photoreceptors are the light-detectors of the retina and their degeneration has led to the onset of blindness in numerous diseases, including retinitis pigmentosa and age-related macular degeneration [163,164].
Replacing the lost photoreceptors in the eye with transplanted cells holds great promise; however, the culture of isolated photoreceptor cells in vitro continues to elude neuroscientists [165-167]. In addition to transplantation, culturing photoreceptors would provide an invaluable tool for in vitro studies, such as drug screening, cell interaction studies and disease modeling. Although whole retinas can be maintained in culture for several days, [168] when photoreceptors are isolated and cultured in vitro they undergo morphological changes and profound apoptosis [165,166]. The few surviving cells do not resemble photoreceptors as they lose their outer segments and downregulate the expression of visual cycle proteins.

Three-dimensional (3D) constructs have enabled the culture of cells not normally possible [169] and were instrumental to the derivation of retinal tissue from mouse and human embryonic stem cells [170,171]. We wondered if the beneficial effects of 3D culture could be extended to primary photoreceptors too. Unlike conventional 2D culture conditions, 3D hydrogels can be designed with mechanical and topographical cues that are normally present within the cell niche [172]. In designing a biomimetic hydrogel for photoreceptors, we chose to base it on hyaluronic acid (HA) as it is present in the subretinal space and serves as a scaffold for the sequestration and presentation of biomolecules to photoreceptors [53]. While HA itself does not form a gel, HA-furan can be chemically crosslinked with poly(ethylene glycol)-bismaleimide by a Diels-Alder click reaction, resulting in a crosslinked HAPEG [173] (Figure 2.1).

The chemical and mechanical properties of HAPEG can be tuned to create a permissive environment for cell culture [173]. HAPEG gel stiffness can be tuned by either percent solids (i.e. HA concentration) or percent furan substitution on HA. On the one hand, changing the HA concentration enables greater changes in stiffness than percent furan substitution, but the ligand concentration is varied simultaneously. On the other hand, changing the furan substitution on the HA polymeric backbone allows gel stiffness to be varied without changing ligand density, thereby decoupling the effects of ligand density from those of mechanical properties.

HA has been demonstrated to activate an array of signaling pathways [174] such as RhoA, PI3 kinase and canonical Wnt in various tissue types. While little is known about HA signaling in mammalian photoreceptors, mTOR signaling has been shown to promote cone photoreceptor survival in mouse models of retinal degeneration [175]. To gain greater insight into the signaling pathway mediated by HA, mTOR was investigated.
We demonstrate, for the first time, that FACS-purified rod photoreceptors from postnatal Nrl-GFP mice can be maintained in culture for at least two weeks. We identify hyaluronic acid as the bioactive component of our HAPEG hydrogels, decipher the molecular pathways induced by HA in the rod photoreceptors and identify mTOR as a key mediator of this pro-survival effect. Interestingly, partial maturation of rod photoreceptors was observed when cultured on hyaluronic acid based hydrogels, as evidenced by the upregulation of outer segment markers at the RNA and protein levels. Together, these data show that HA-based hydrogels enable the successful culture of rod photoreceptors in vitro and suggest its benefit in vivo.
Figure 2.1. Schematic overview of the experiments performed in this work. Photoreceptors were isolated by FACS from the eyes of Nrl-GFP mice expressing GFP only in rod photoreceptors. The sorted rods were cultured in HAPEG gels and analyzed for their survival and maturation. The HAPEG gels were formed by crosslinking HA-furan chains with PEG-maleimide₂ by Diels-Alder chemistry. To investigate the mechanism of action of the HAPEG gels, the effects of stiffness and HA presence were independently assessed. The
gel stiffness was modified by either varying the furan substitution of the HA or altering the HA concentration. The effect of HA presence was probed by using MCPEG gels, which are similar to HAPEG but lack HA, and by adding soluble HA in the medium of photoreceptors cultured in standard 2D conditions.

2.3 Materials and Methods

2.3.1 Mouse strain

The Nrl-GFP [176] transgenic mice were generously donated by Dr. Swaroop. Experimental procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals and approved by the Animal Care Committee at the University of Toronto.

2.3.2 Media optimization, cell isolation and sorting

The eyes of P11 Nrl-GFP mice were enucleated and the surrounding muscle tissue removed under a microdissection microscope. The eyes were then dissected in half through the optic nerve and the neural retinas were isolated taking care not to include any ciliary epithelium tissue in the preparation. The neural retinas were treated with a papain-DNAse I solution (Worthington Biochemical) for 15 min, and subsequently rinsed with ovomucoid trypsin inhibitor (Worthington Biochemical). The cells were resuspended in DMEM/F-12 containing the B-27 (2%), N-2 (1%) supplements and 2% penicillin/streptomycin (all from Life Technologies). 7-AAD (Life Technologies) was added to the cell solution before cell sorting to exclude dead cells. The cells were fluorescently sorted in a BD FACS Aria sorter. Sorting was done for GFP+ / 7-AAD- cells. The collection tubes contained DMEM/F-12 supplemented with B-27, N-2, 2% penicillin/streptomycin and 20% Hyclone fetal bovine serum (Thermo Scientific). The sorted photoreceptors were then spun down and resuspended in culture medium. To achieve an n=4 for each experiment, eyes from 4 littermates were dissected, sorted and cultured separately.

The sorted Nrl-GFP+ photoreceptors were cultured in DMEM/F-12 containing the B-27, N-2 supplements, 1% non-essential amino acids, 1% Glutamax, 1% sodium pyruvate, 1% penicillin-streptomycin and 0.1% β-mercaptoethanol (all from Life Technologies), 20 ng/ml bFGF (R&D
Systems), 5 ug/ml heparin (R&D Systems) and 5% Hyclone FBS (Thermo Scientific). Medium was replenished every other day. In cultures on gels, the cells were plated directly on top of gels that had been prepared and washed in advance. The standard culture was done on Geltrex™ (Life Technologies)-coated wells, which were coated for 1 h at 37° C with 1% Geltrex™ in DMEM/F-12. In the case of the Geltrex™ gel cultures, undiluted Geltrex™ was incubated in the wells for 1 h at 37° C to gel before plating the cells. Poly-D-lysine (Sigma Aldrich) was coated at 50 ug/ml for 3 h at 37° C. Inhibitors: Rapamycin (Sigma Aldrich) was used at 100 nM, Y27632 (Sigma Aldrich) at 20 uM and IWR1e (Millipore) at 10 uM. All the inhibitors were resuspended in DMSO (Sigma Aldrich), according to the manufacturers’ instructions. In the inhibitor experiments, DMSO of equal volume was used in the control cultures.

We initially wanted to optimize the culture medium for our FACS-sorted Nrl-GFP rods. Based on previous literature on photoreceptor culture in vitro [165-167], we supplemented our cultures with bFGF and heparin, as well as the neural cell supplements B27 and N2. We found small increases in cell survival upon bFGF addition, while the addition of serum led to a significant increase in cell survival by day 7 (Figure 2.2). In either case, 2D culture of photoreceptors entailed pronounced cell death, in agreement with the literature, and few cells were present by day 14 (Figure 2.2, 2.4).
Figure 2.2. Effect of medium composition on photoreceptor survival in standard, 2D culture. A 2-way ANOVA identified treatment and time as significant main effects, \( p<0.001 \) and a significant time \( \times \) treatment interaction, \( p<0.001 \). Data with different letters are statistically different: a vs b \( p<0.05 \); a vs c \( p<0.001 \); b vs c \( p<0.05 \); d vs e \( p<0.001 \); d vs f \( p<0.001 \). (\( N=4 \) mice per group, mean ± standard deviation; Bonferroni post hoc analysis)

### 2.3.3 HAPEG gels

HAPEG hydrogels were prepared as previously described [173]. Briefly, hyaluronic acid (289 kDa, StemCore) was first chemically modified to incorporate a furan functional groups: HA was dissolved in 0.1 M MES buffer (pH 5.5) and DMT-MM was added for 30 min. Furfurylamine (Sigma) was then added, and the reaction was stirred for 24 h at room temperature. The reaction mixture was then dialyzed extensively against 0.1M NaCl and then distilled water to remove unreacted reagents and byproducts. HA-furan was then sterile filtered through a 0.22 μm membrane, and lyophilized to afford an amorphous white powder. Furan substitution was quantified by \(^1\)H-NMR as previously described and shown below.
Figure 2.3. $^1$H NMR of 50% furan-substituted hyaluronan (289 kDa) in deuterium oxide. Substitution of hyaluronan is calculated from the ratio of furan integrals at 6.46 and 7.51 ppm to the N-acetyl signal at 2.03 ppm of native hyaluronan. In this instance, \( \frac{(0.17+0.33)}{1.00} \times 100\% = 50\% \) substitution. Spectra obtained using a 500 MHz $^1$H NMR spectrometer, 256 scans.

To prepare the gels, HA-furan (50% furan degree substitution, 40 mg/mL unless otherwise stated) and bis-maleimide PEG (Rapp Polymere, 3 kDa, 81.9 mg/mL) were dissolved separately in sterile 0.1 M MES buffer (pH 5.5). To prepare 100 µL of 1% HA gel with PEG-bismaleimide crosslinker (79.8 mol% relative to total number of furans in the gel mixture) used for the cell culture experiments, 25 µL of the HA solution, 17 µL of the PEG solution, and 58 µL of 0.1 M MES buffer were mixed together, and then vortexed to ensure adequate mixing. 30 µL of the gel mixture was then transferred into each well of a 96 well plate and incubated at 37 °C overnight. The gels were then washed with sterile 0.1 M MES buffer (PH 5.5) 3 times, PBS 3 times,
followed by DMEM/F-12 media 3 times. Unless otherwise indicated, all HAPEG gels used here had 1% HA-furan/PEG-bismaleimide (PEG-MI₂), with a 50% furan substitution of the HA.

2.3.4 Immunostaining and Live/Dead staining

The primary antibodies used in this study are: anti-ABCA4 (Abcam ab77285, used at 1:200), anti-Peripherin2 (Proteintech 18109-1-AP, used at 1:500) and anti-pmTOR (Abcam ab109268, used at 1:100). The specificity of the antibodies was confirmed in tissue sections before they were used for experiments.

Cell cultures were fixed for 30 min with ice cold fresh 4% PFA (Sigma Aldrich) to ensure diffusion in the gels. After washing 3 times in PBS for 10 min each, cells were permeabilized with 0.3% Triton-X for 5 min (ABCA4, Peripherin staining) or 10 min (pmTOR staining). Blocking was performed with 5% normal goat serum (Jackson Immunoresearch Laboratories) for 2 h at 4 °C. Primary antibodies were diluted in 1% normal goat serum and 0.05% Triton-X in PBS and incubated overnight at 4 °C. After three 10-min washes with PBS containing 0.05% Triton-X, secondary antibodies (1:400, highly cross-absorbed, Life Technologies), diluted in 1% normal goat serum and 0.05% in PBS, were incubated for 3 h at 4 °C. Three washes with 0.05% Triton-X in PBS (10 min each) followed, in the second of which Hoechst 33342 (Life Technologies) was added (2 ug/ml) for nuclear staining.

Live/Dead staining was done in live cultures, using 4 uM ethidium homodimer-1 (Life Technologies) as a dead stain and 2 ug/ml Hoechst 33342 as a nuclear stain. Live photoreceptors constitutively express GFP because of the Nrl-GFP transgene, therefore no live cell dye was used. The dyes were added directly to the cell culture medium and imaging started after a 40 min incubation at RT. Imaging was completed within 3 h after the addition of the dyes to each batch of cells to ensure that there is no uptake of ethidium homodimer-1 by live cells.

Imaging was done with an Olympus FV1000 confocal microscope. For each experiment, negative control samples stained with only the secondary antibodies were used to adjust the exposure power and to quantify accurately. Quantification was done by manual counting of cells using the ImageJ software. Three fields of at least 100 cells each were counted in each well (typically, an imaging field would contain approximately 300 cells for the HAPEG gel fields),
and wells for each mouse were run in duplicate. In the 3D cultures the selection of fields was random, while in standard cultures the fields had to be manually picked due to the scarcity of cells (in the standard cultures, 90-100% of the cells within a well were imaged). The pictures shown for the 3D cultures are Z-projections.

2.3.5 Quantitative PCR

RNA was isolated using Trizol (Life Technologies), following the manufacturer’s instructions. For extracting RNA from the cells cultured in gels, the gels were resuspended in trizol and homogenized in a bead homogenizer using zirconium beads. The samples were treated in 6 cycles of 30 s homogenizations followed by 1 min incubations on ice to prevent heating. The bead homogenization was also performed in the cells cultured on Geltrex™ coating, to ensure consistency. After RNA extraction, DNase treatment was done with TurboDNAse (Ambion). Only RNA that gave a 260/280 ratio >1.8 was used. RNA was reverse transcribed using the superscript VILO cDNA synthesis kit (Life Technologies). qPCR amplification was done in an Applied Biosystems 7900HT instrument using sybr green (Roche). Melting curves were performed for each experiment and negative controls (minus RT controls and no template controls) were always included to ensure the accuracy of the results. In certain experiments, the amplified products were also analyzed by agarose gel electrophoresis to ascertain the size of the amplicons. The primer pairs used were as follows: abca4 FP ACGGCTGTCTAGGAGTGTGG, abca4 RP GGGCACCCTACAGTGGATAG, prph2 FP CATCCACGTTGCTCTTGATT, prph2 RP TCGGGACTGGTTTGAGATTC, gapdh FP AGGTCGGTGTGAACGGATTTG, gapdh RP TGTAGACCATGTAGTTGAGTCA. Quantification was done using the ΔΔCt method [177] and GAPDH was used as a housekeeping gene. The relative expression shown is 2^{ΔΔCt}.

2.3.6 Mechanical compression testing

The Young’s moduli were determined for all HAPEG hydrogels as described previously [173]. Briefly, the hydrogels were pre-swollen in PBS for a day and formed into cylindrical samples with a diameter of 5 mm. 100 ul of hydrogel were used for each test, which, depending on the swelling of the hydrogels, ranged from 2200 to 2800 um in height. Samples were placed
between two impermeable flat platens connected to a DAQ-Nano17 force transducer (ATI Industrial Automation) on a Mach-1 micromechanical system (Biomomentum). Samples were subjected to an initial tare force of 0.01 N to even out surface defects, and the platento-platen separation was taken as the initial sample height. Uniaxial, unconfined compression was performed at 37 °C at a deformation rate of 10 μm/s until an applied strain of 20% was reached. The Young’s modulus was taken as the slope of the resultant stress versus strain chart for each sample.

2.3.7 Statistical analysis
All data are presented as the mean of an n≥4 eye dissections and the error bars represent standard deviation. Statistical analysis was done in Graphpad Prism version 5. Unpaired student’s t-test was used to compare 2 groups, and one-way ANOVA with Tukey’s posthoc to compare multiple groups. Two-way ANOVA was used to investigate the effects of time, treatment and their interactions in time course experiments. Graphs are annotated with p values represented as *p≤0.05, **p≤0.01 and ***p≤0.001.

2.4 Results

2.4.1 Hydrogel culture drastically improves photoreceptor survival
The culture of sorted Nrl-GFP rod photoreceptors in HAPEG gels resulted in a profound increase in their survival over a period of at least 14 days (Figure 2.4a, b). Interestingly, the absolute number of photoreceptors per well drastically decreased in the standard 2D culture from day 3 onwards, with only a few clusters of cells remaining (a schematic of each well is depicted in Figure 2.4c and quantification is shown in Figure 2.4d). Unlike 2D cultures, photoreceptors cultured in hydrogels remained distributed throughout the wells. In order to compare our HAPEG gels with a commonly used, albeit an undefined 3D matrix, we also cultured the photoreceptors in Geltrex™ gels. We found similar survival in the two different gels, even
though Geltrex™ is derived from a mouse sarcoma and is comprised of numerous extracellular matrix proteins and growth factors [178] whereas HAPEG is well-defined and comprised only of HA and PEG. These data indicate that 3D culture may be sufficient to promote photoreceptor survival and prompted us to investigate the mechanism by which 3D culture promoted rod survival. Since the Geltrex™ gels are ill-defined, we focused our investigation on the defined HAPEG hydrogels.

![Figure 2.4. Culture of Nrl-GFP+ photoreceptors in HAPEG or Geltrex™ hydrogels drastically improves their survival. a. Representative imaging fields over 14 d of culture in standard conditions, Geltrex™ or HAPEG gels. Green shows the Nrl-GFP fluorescence and red shows ethidium homodimer 1, which is taken up by dead cells. Scale bar is 50 um. b. Quantification of cell survival as a percentage of live cells over total cells per well. A 2-way ANOVA identified treatment and time as significant main effects, p<0.001, and a significant time x group interaction, p<0.01. (n=4 mice per group, mean ± standard deviation; *p<0.05, ***p<0.001 between cultures in standard conditions vs HAPEG, Bonferroni post hoc analysis) c. Schematic demonstrates the vast loss of photoreceptors only in the standard culture condition vs. their relatively homogeneous distribution](image-url)
throughout the HAPEG-containing wells. d. Quantification of the absolute number of photoreceptors per well in the three different culture conditions. A 2-way ANOVA identified treatment and time as significant main effects, p<0.001 and a significant interaction of p<0.05. (n=4 mice per group, mean ± standard deviation; *p<0.05; ***p<0.001 between cultures in standard conditions vs HAPEG, Bonferroni post hoc analysis.)

2.4.2 Gel stiffness does not mediate the pro-survival effect

Extracellular matrix stiffness is a well-established parameter influencing cell survival and fate [179]. The stiffness of native retinal tissue has been measured between 1 and 15 kPa [180], which is similar to the 5.95 kPa stiffness of the 1% HAPEG gels that we used (Figure 2.5a), as measured by mechanical testing. We hypothesized that if hydrogel stiffness was the underlying mechanism for the pro-survival effect, we should observe a difference when photoreceptors are cultured in stiffer versus weaker hydrogels.

By controlling both the percent concentration of HA and the percent furan substitution, we were able to tune the HAPEG gel stiffness over a 50-fold range, from 1.79 to 87.79 kPa (Figure 2.5a). In order to achieve the maximum stiffness of 87.79 kPa, we had to simultaneously increase the HA concentration and the furan substitution. Interestingly, stiffness had no effect on photoreceptor cell survival; cell survival was largely consistent in all hydrogels at approximately 80 to 85% on day 5, irrespective of modulus (Figure 2.5b, c). These data led us to conclude that stiffness does not mediate the pro-survival effect of the HAPEG hydrogels. We acknowledge that altering stiffness beyond the range examined here may have an effect on photoreceptor survival.
Figure 2.5. Gel stiffness does not mediate the pro-survival effect of the HAPEG gels. a. Young’s moduli of the various HAPEG gels. Gels are labeled as the percentage of HA in HAPEG with percent furan substitution on the HA in parentheses. (n=4 gels per group, mean ± standard deviation; *p<0.05; ***p<0.001, one way ANOVA, Newman-Keuls post hoc analysis). b. Quantification of cell survival of Nrl-GFP+ photoreceptors after 5 days of culture in the various gels. (n=4 mice per group, mean ± standard deviation) c. Representative images of photoreceptors grown on the various hydrogels on day 5, scale bar is 50 um.

2.4.3 HA is necessary for the pro-survival effect

Since HA is naturally present in the subretinal space, we asked whether the pro-survival effect of these HAPEG hydrogels was mediated by HA. To answer this question, we pursued two studies: (1) we added 1% of soluble HA to the medium of rod photoreceptors cultured in 2D polystyrene – the standard culture condition in which we had observed poor survival; and (2) we cultured the
photoreceptor rods in a 3D, chemically defined hydrogel that lacks HA. A 3D hydrogel of similar stiffness to the 1% HAPEG gels was synthesized by crosslinking the same PEG-bismaleimide with methylcellulose-thiol, resulting in MCPEG crosslinked gels [181].

In the absence of HA, in the MCPEG hydrogels, the rod photoreceptors did not survive as well as they had in HAPEG gels, indicating that the 3D geometry alone is insufficient for cell survival. Interestingly, adding 1% HA to the MCPEG gels reversed that effect (Figure 2.6a), suggesting that HA is key to cell survival. The importance of HA to photoreceptor rod survival became even clearer when the medium in which photoreceptors were cultured in 2D was supplemented with 1% soluble HA. Here we observed a remarkable result of significantly greater cell survival after 5 days in culture in the presence vs. absence of HA in standard 2D culture conditions, and similar survival to that observed on HAPEG gels (Figure 2.6b, c). These results suggest that HA is necessary and sufficient to induce the observed pro-survival effect on rod photoreceptors. Of note, the photoreceptor survival in the MCPEG gels averaged 68.9%, which is higher than the standard 2D culture value of 51.6% (Figure 2.6a, b). This suggests that 3D culture itself may have a prosurvival effect on photoreceptors. While the benefit of 3D culture was not observed in HA, the pro-survival effect of HA on photoreceptors may have masked the benefit of 3D observed with MCPEG gels.
Figure 2.6. HA mediates the pro-survival effect of the HAPEG gels. a. Quantification of photoreceptor survival after 5 days of culture in HAPEG gels, standard culture conditions or standard culture with 1% HA in the medium shows that the addition of HA has a profound effect on survival. b. Quantification of photoreceptor survival after 5 days of culture in HAPEG gels, MCPEG gels, or MCPEG gels with 1% HA added to the gel mixture shows that 3D hydrogel is insufficient for cell survival in the absence of HA. c. Representative images. Scale bar=100 um. (n=4 mice per group, mean ± standard deviation, ***p<0.001, one way ANOVA, Tukey’s post hoc analysis.)

2.4.4 HA acts through the mTOR pathway in rod photoreceptors

In order to decipher the molecular mechanisms that HA initiates in rod photoreceptors, we explored the mTOR pathway. We found that culturing rod photoreceptors in the HAPEG gels induces mTOR activation, as evidenced by immunostaining for the active phosphorylated form
of mTOR (Figure 2.7a). On day 1, immunostaining for phospho-mTOR is evident for 27.2% of rod photoreceptors cultured in HAPEG yet only 1.53% of the rods cultured in the standard conditions (Figure 2.7a, b). Surprisingly, the increased activation of mTOR persisted to day 5, with 11.6% of the rods staining positive for phospho-mTOR in HAPEG vs 0.62% in the control culture (Figure 2.7a, c). To further test whether HA was acting through the mTOR pathway, rapamycin, a well-established inhibitor of mTOR signaling[182] was added to HAPEG cultures and abolished the pro-survival effect of HAPEG (Figure 2.7d). Importantly, no further decrease in cell survival relative to standard culture conditions was observed, indicating that rapamycin was not generally toxic to cells, but rather blocking the mTOR pathway through which HA was likely acting. Similarly, when rapamycin was added to the 2D culture of rod photoreceptors, no further cell death was observed, confirming that rapamycin did not have a significant toxic effect (Figure 2.8).

2.4.5 HA induces mTOR through combined Wnt and RhoA activation

Given that the mTOR pathway is a downstream target of many signaling cascades[183], we wanted to delineate which pathways are induced by HA and lead to mTOR activation. The upstream signaling pathways for mTOR activation include both the canonical Wnt pathway and the RhoA pathway. We found that inhibiting either the canonical Wnt signaling by IWR1e or the ROCK kinase signaling (a major effector of RhoA) by Y27632, diminished the pro-survival effect of the HAPEG gels on rod photoreceptors (Figure 2.7e, f). Importantly, there was no generic toxicity associated with these inhibitors when added to the standard 2D culture of rod photoreceptors, indicating that their effect was pathway dependent (Figure 2.8). Neither Y27632 nor IWR1e are as effective as rapamycin in decreasing the pro-survival effect of HA in HAPEG (Figure 2.7e, f). We hypothesized that this may be due to the fact that both Wnt and RhoA pathways act by activating mTOR, and therefore individually blocking either of them only provides a partial inhibition of the mTOR pathway. Indeed, combining each of either Y27632 or IWR1e with rapamycin did not produce any additional decrease in cell survival on the HAPEG gels, indicating that ROCK and canonical Wnt mainly activate mTOR in this context (Figure 2.7e, f). Furthermore, combining Y27632 and IWR1e abolished the pro-survival effect of the
HAPEG gels to the same extent as rapamycin (Figure 2.7e, f), suggesting that canonical Wnt and RhoA activation are the upstream mediators of mTOR activation in the rod photoreceptors.
Figure 2.7. HA acts by inducing the mTOR pathway through the canonical Wnt and RhoA pathways. a. Activation of the mTOR pathway as evidenced by phospho-mTOR immunostaining in rod photoreceptors cultured in HAPEG for 1 and 5 days. Scale bar=50 um. b-c. The phospho-mTOR is significantly greater when rod photoreceptors are cultured on HAPEG vs. standard 2D conditions for (b) 1 and (c) 5 d (n= 4 mice, mean ± standard deviation, ***p<0.001; *p<0.05, Student’s t-test, unpaired, 2-tailed.) d. Quantification of rod photoreceptor survival after 5 d of culture in HAPEG gels with and without rapamycin vs. standard culture conditions. There are significantly more cells surviving on HAPEG gels than either on HAPEG+rapamycin or standard culture conditions (n= 4 mice, mean ± standard deviation, ***p<0.001, one-way ANOVA, Tukey’s post hoc.) e. Quantification of photoreceptor survival after 5 d of culture in HAPEG gels in the presence of the indicated inhibitors. Data with different letters are significantly different: a vs b, p<0.01; a vs c, p<0.001; b vs c, p<0.05 (n= 4 mice, mean ± standard deviation, one-way ANOVA, Tukey’s post hoc.). f. Representative images of photoreceptors in HAPEG gels show NrlGFP+ live (green) cells, ethidium homodimer dead (red) cells and the overlay with Hoechst (nuclei). Scale bar is 50 um.
Figure 2.8. Normalized survival of photoreceptors cultured for 5 days in standard conditions in the presence or absence of the indicated inhibitors reveals no generic toxicity of the inhibitors. N=4 mice per group, mean±standard deviation, one way ANOVA, Tukey’s post hoc.

2.4.6 Culture in HAPEG induces partial maturation of rod photoreceptors

In order to assess in vitro photoreceptor maturation in a quantifiable manner, we performed immunostaining for the outer segment markers ABCA4 and peripherin (PRPH2), which are only expressed in mature photoreceptors. In the standard 2D culture condition, approximately 10% of cells expressed ABCA4 and 15% expressed peripherin over the course of 14 days (Figure 2.9a, b). In contrast, rods cultured in the HAPEG hydrogels exhibited a sharp increase (of 75%) in protein staining for ABCA4 starting from 1 day after plating (first time point examined) up to 7 days of culture (40% expression), which was significantly higher than the control cultures (Figure 2.9a). Surprisingly, increased expression of either outer segment marker was not observed in Geltrex™ gels for either ABCA4 or peripherin and, in fact, there was significant variability in the data. Peripherin was upregulated by 35% on day 7 in HAPEG cultures (Figure 2.9b); however, by day 14, both ABCA4 and peripherin had dropped to baseline levels (of approximately 20%). Notwithstanding the loss of markers at day 14 in the HAPEG gels, most of
the positive cells detected were double positive for peripherin and ABCA4, which was not observed in the standard 2D culture.

Quantitative PCR for *abca4* and *prph2* (Figure 2.9c-f), which encodes peripherin, corroborated the immunostaining data. At day 5, we observed a significant increase in *abca4* expression (Figure 2.9c) over controls in the HAPEG gels (8.75 fold), while *prph2* showed no differences in expression (Figure 2.9d). Interestingly, the Geltrex™ cultures showed a great degree of variability, which may result from its ill-defined composition and batch-to-batch variability. At day 7, we found significant increases in the expression of both *abca4* (4.5 fold) and *prph2* (5.6 fold) over controls in the HAPEG cultures (Figure 2.9e-f). We therefore conclude that rod photoreceptors undergo a partial maturation upon culture in the HAPEG gels. Consistent with these data, we often observed rod photoreceptors with small processes that stained positive for peripherin and/or ABCA4 when cultured in HAPEG (Figure 2.9g). Such processes were very rarely observed in the Geltrex™ cultures (an example is shown on day 3 for Geltrex™ in Figure 2.9g) and were never found in the standard culture.
Figure 2.9. Nrl-GFP+ photoreceptors undergo partial maturation when cultured in HAPEG gels. a-b. Quantification of immunostaining for ABCA4 and peripherin by manual counting of immunostained positive cells over 14 days of culture in HAPEG, GeltrexTM gels or standard culture. A 2-way ANOVA identified treatment and time as significant main effects, p<0.001 and a significant time x treatment interaction, p<0.001. (n=4 mice per group, mean ± standard deviation; ***p<0.001 between cultures in standard conditions vs HAPEG, Bonferroni post hoc analysis.) c-d. qPCR for the expression of abca4 and prph2 transcripts after 5 days of culture in the indicated conditions. “After sort” stands for d0, immediately after cell sorting. (n=4 mice per group, mean ± standard deviation; *p<0.05 between cultures in standard conditions vs HAPEG and between “after sort” vs HAPEG. Student’s t-test, unpaired, 2-tailed.) e-f. qPCR for the expression of abca4 and prph2 transcripts after 7 days of culture in the indicated conditions. There is a significant increase in abca4 expression in HAPEG gels vs standard culture and between “after sort” vs HAPEG (n=4 mice per group, mean ± standard deviation; **p<0.01 based on one way ANOVA, Tukey’s post hoc). For prph2 expression, data with different letters are significantly different: a vs c, p<0.001; b vs c, p<0.05; a vs b, p>0.05, not significant. (n=4 mice per group, mean ± standard deviation, one-way ANOVA, Tukey’s post-hoc analysis) g. Representative images for the immunostaining; Hoechst (blue); ABCA4 (red); peripherin (green). Scale bar is 50 um for the images and 5 um for the higher magnification images.

2.5 Discussion

We demonstrate a novel and efficient way to maintain isolated mouse rod photoreceptors in vitro. There is robust survival for 7 days, and while there is only 20% survival after 14 days in HAPEG gels, this is significantly greater than what is observed in standard 2D culture strategies. After an initial decrease in survival in the first 3 days, which we attribute to cellular stress due to the retinal dissociation and cell sorting, the photoreceptors maintain steady levels of survival until day 7, which we attribute to hyaluronan. The HAPEG hydrogel is well-defined and comprised of only HA and PEG, making its pro-survival effect remarkable. There are no cell-adhesive peptides or proteins, no additional cell types and no added growth factors, except for
what is normally used in the cell culture medium. This provides an alternative to Geltrex™, which, due to its derivation from a mouse sarcoma, suffers from inconsistent results. The pro-survival effect attributed to HA and confirmed by its addition to standard 2D culture, enables photoreceptor biology to be studied in vitro.

Surprisingly, stiffness of the HAPEG gels did not affect rod photoreceptor survival. While this is in contrast to the effects that others have observed of stiffness on different cell types, such as neurons [184], neural stem cells [185] or mesenchymal stromal cells [186], it demonstrates that either the ECM modulus is not a universal signaling cue or that our hydrogels were not in a range where this difference would be observed. The latter is unlikely given the 2 orders of magnitude range that we investigated, but possible. Other cell types in the retina, such as the Müller glia, may be responsible for mechanosensation and these may, in turn, affect other cell types. For example, Davis et. al. [187] found that an immortalized Müller glia cell line modified its cell morphology and gene expression in response to changes in stiffness.

Interestingly, this pro-survival effect of HA is reflected in its use in both ophthalmic surgery [188], and photoreceptor transplantation research [189,190]. While mouse retinal stem cell-derived rod photoreceptors were shown to interact with HA via CD44 [190], the receptor for HA on native rod photoreceptors remains largely elusive. The main HA receptors, CD44 and RHAMM, have not been detected in photoreceptors [58,191,192], and were also undetectable in our P11 rods (data not shown). There is evidence for the existence of two glycoproteins, SPACR and SPACRCAN [60-62], on rod photoreceptors that can bind HA; however, the intracellular pathways induced by SPACR or SPACRCAN are unknown.

We demonstrate, for the first time, that mTOR mediates the pro-survival effect of HA on the rod photoreceptors and that this occurs in a cell autonomous manner. In contrast, Punzo et. al. [175] reported that insulin activates mTOR in a non-cell autonomous manner, thereby rescuing cone photoreceptors in a mouse model of retinitis pigmentosa; yet, the exact cell types that activated mTOR and promoted cone survival remain unidentified. It is known that rod photoreceptors secrete survival factors [193] that are important for the maintenance of cones. In retinitis pigmentosa, rod cell death results in the demise of cone photoreceptors [194], which corroborates the existence of these survival factors. Thus, our finding that mTOR can mediate the survival of rod photoreceptors in a cell autonomous manner may explain (and be consistent with) Punzo et
al’s observations on cones. Recently, the same group demonstrated that genetically activating mTOR specifically in cone photoreceptors can also improve cone survival and function in mouse models of retinitis pigmentosa [195]. As further evidence for the importance of the mTOR pathway on survival, Tsang et al. [196] reported that silencing tuberin, an endogenous inhibitor of mTOR, enhanced photoreceptor survival in a mouse model of retinitis pigmentosa, although the exact cell types in which mTOR was activated were again unclear. A recent phase I/II clinical trial using subconjunctival sirolimus, an mTOR inhibitor, for geographic atrophy reported deterioration of visual acuity in treated eyes [197]. Whether sirolimus had an effect on rod photoreceptor viability remains to be elucidated.

Consistent with the literature on the molecular cascades induced by HA [174], we too found that HA induced multiple pathways in rod photoreceptors. The canonical Wnt pathway has been shown by others to have a pivotal role in the generation of new photoreceptors by Müller glia in fish [198] and has been implicated in mammalian photoreceptor degeneration [199,200]. We also found that inhibiting the canonical Wnt pathway partially abolishes the pro-survival effect of HA on photoreceptors. Surprisingly, others have reported RhoA/ROCK to be detrimental to cell survival in many cases [201]. For example, ROCK inhibition protected retinal ganglion cells from cell death in an optic nerve crush model [202,203] and a bovine retinal explant model of hypoxia [203]. In contrast, we found that the RhoA/ROCK pathway promotes rod photoreceptor survival. It is possible that the effects of ROCK activation and its downstream targets are context-dependent, as has been seen in other signaling cascades [204]. These pathways may constitute compelling targets for future research in treating retinal degeneration.

Since we were able to culture the rod photoreceptors in vitro for longer than previously possible, we were able to probe them for expression of outer segment markers. The rods used in this study were sorted at P11 and since outer segments are typically generated at P10-P12 through to P21 [205], these rods would have produced outer segments had they remained in vivo. In fact, both peripherin and ABCA4 are detectable at P11 in vivo (Figure 2.10). As ABCA4 is a membrane protein [206], which may be damaged during retinal dissociation and cell sorting, the significantly greater expression on day 1 in HAPEG gels vs. both Geltrex™ and standard 2D, suggests maintenance of pre-existing ABCA4 proteins. Interestingly, we observed an increase in protein expression of peripherin after 7 days in culture, indicating that the photoreceptors
continued along their natural developmental program, despite being isolated and grown in an in vitro environment. This further underscores the importance of the 3D HAPEG hydrogel as a biomimetic environment for effective cell culture. The qPCR data confirm this result with increased expression of both \textit{abca4} and \textit{prph2} transcripts during in vitro culture in HAPEG vs. the expression immediately after sorting. While ABCA4 and peripherin protein and gene expression are clear in these rod photoreceptors cultured in HAPEG hydrogels, and small processes stained positive for peripherin and/or ABCA4 expression (Figure 6g), we recognize that we cannot conclusively argue for outer segment formation in the absence of electron microscopy images, which is technically challenging in these highly (98+%) hydrated systems.

We designed a tool for photoreceptor culture in vitro, discovered HA as a pro-survival factor, and identified mTOR as playing a key role in rod photoreceptor viability. We can now study photoreceptor biology in vitro and use this strategy for more effective transplantation strategies of photoreceptors. Moreover, we can now imagine how to further enhance photoreceptor culture in vitro. We ascribe the eventual decrease in survival to the lack of additional niche cues. With the inclusion of additional factors and cells, the cell niche may be better-emulated and likely result in even greater survival or maturation. The use of our platform will enable such experiments to be conducted in the future.

![Immunohistochemistry of P11 Nrl-GFP retinas for Peripherin and ABCA4 demonstrate that both proteins are expressed in budding outer segments (arrows) at this age. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar=25 um.](image)

**Figure 2.10.** Immunohistochemistry of P11 Nrl-GFP retinas for Peripherin and ABCA4 demonstrate that both proteins are expressed in budding outer segments (arrows) at this age. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar=25 um.
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3 Co-transplantation of RPE and photoreceptors restores vision in an animal model of advanced retinal degeneration

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NM, DvdK and MSS designed research; NM and SH performed research; NM and YS analyzed data; NM, DvdK, AN and MSS interpreted data; NM and MSS wrote the manuscript.

3.1 Abstract

Degenerative diseases of the retina typically commence with degeneration of either the photoreceptors or the retinal pigmented epithelium (RPE), but, regardless, both cell types are eventually affected. Approaches to replace the deceased RPE or photoreceptors separately have shown some promise; however, restoring vision in an advanced stage of retinal degeneration requires both RPE and photoreceptors to be replaced. Here, we used sodium iodate (NaIO$_3$) injections in mice to degenerate both RPE and photoreceptors, thereby recapitulating advanced retinal degeneration, which resulted in complete blindness, similar to what is observed in humans. We then co-transplanted, for the first time, RPE and photoreceptors using a hyaluronic acid-based hydrogel as the delivery vehicle. After transplanting RPE, derived from human embryonic stem (hES) cells, and rod photoreceptors, derived from the Nrl-GFP mouse retina, we evaluated vision restoration by three different metrics and show that vision was partially restored only when RPE and photoreceptors were co-transplanted. To gain a better understanding of why, we examined the retinal tissue and found that both RPE and photoreceptors survived significantly better when co-transplanted than in their respective single cell type controls. We demonstrate a novel approach to reverse advanced stages of blindness.
3.2 Introduction

The RPE and the photoreceptors of the retina have a well-established symbiotic relationship [207]. Classic studies [22,23] and observations of disease pathology demonstrate that their development and function are coordinated, and that deficits in either cell type lead to degeneration of both and the onset of blindness. Age-related macular degeneration (AMD) is believed to originate in dysfunction of the RPE [37], which eventually compromises the photoreceptors and leads to blindness. Conversely, retinitis pigmentosa is caused by mutations in rod photoreceptors, causing cell death and leading to alterations in the RPE [51]. Advanced stages of blindness are characterized by impairment of both RPE and photoreceptors.

The retina comprises a well-organized laminar structure of seven distinct cell types, which largely remain intact [65], even after RPE and photoreceptor degeneration in diseased retinas. This has generated the impetus for cell transplantation to replace the exact cells lost at the correct retinal layer with the goal of re-establishing vision [80,208]. To date, the focus has been on transplanting either cell type in isolation, employing animal models that exhibit dysfunctions only in the respective cell type [82] or performing transplants at early time points along the course of degeneration, before the onset of blindness [3]. These models do not recapitulate end-stage human retinal degeneration and have suffered from material transfer, which has confounded the interpretation of previous results [88-90].

Here, we use the sodium iodate (NaIO₃) mouse model, which exhibits advanced retinal degeneration compromising both RPE and photoreceptors and resulting in complete blindness [209,210]. We first validate the model by characterizing the temporal onset of blindness, and then use it to test the hypothesis that co-transplanting RPE and photoreceptors is superior to transplanting either cell type alone. We use primary rod photoreceptors derived from the post-natal day 6 (P6) Nrl-GFP mouse retina, which exhibit a high potential for survival and integration after transplantation [2,81]. We use RPE derived from hES cells, which rescue vision in genetic (but not degenerative) models of RPE dysfunction [3] and show limited benefit in clinical trials [71]. We employ a hydrogel cell delivery vehicle consisting of a blend of hyaluronic acid and methylcellulose (HAMC), which enhances photoreceptor survival in vitro and in vivo and results in a better distribution of cells that have been injected directly into the sub-retinal space [6,211].
3.3 Materials and Methods

3.3.1 Animals

Experimental procedures were performed in accordance with the guide to the care and use of experimental animals and approved by the animal care committee at the University of Toronto.

Animals were randomized before the initiation of the studies to minimize within-cage effects and all experiments were performed in a blinded fashion.

The Nrl-GFP [212] transgenic mice were generously donated by Dr. Swaroop. NaIO₃ (Sigma) was administered intravenously via the tail vein in 8-10 week old female C57Bl/6J mice (Charles River) as a 1% sterile-filtered (0.22 µm syringe filter, Millipore) solution in saline, at 70 mg/kg. Control mice were injected with saline. Even though mice tolerate NaIO₃ at this dose relatively well, some animal death (approximately 10% of the animals) is consistently observed soon after injection. Using multiple (2 or 3) 70 mg/kg doses delivered on alternate days did not cause more animal death (i.e. the animals that survived the first injection also survived the second or third). When a higher, 100 mg/kg dose of NaIO₃ was delivered, 3 out of 5 animals died.

To prevent weakness and dehydration of NaIO₃-treated animals treated with CsA, mice were kept on a 9% fat diet (Teklad 2019, Envigo) with daily mash (made with hydrated powder of Teklad 2019) provided for 2 weeks after NaIO₃ administration and during immune-suppression. Animal weight was monitored frequently and animals that were below 20 g were not used for the cell transplantation studies. CsA was administered by osmotic minipumps (as opposed to bolus injections), since we found that bolus CsA injections in NaIO₃-treated animals led to high morbidity and death rates. Osmotic minipumps (Alzet 2006 or 1004) were loaded with CsA (LC laboratories) in a solution of 65% ethanol, 35% Cremophor-EL (Sigma) and implanted in mice subcutaneously, 4 days before cell transplantation. Both the NaIO₃-treated and wild type animals were implanted with CsA pumps. The concentration of CsA loaded in the pumps was calculated using the Alzet Drug concentration calculator to deliver 10 mg/kg/day. The pumps were replaced according to the indicated release duration provided by Alzet (6 weeks for Alzet 2006 and 4 weeks for Alzet 1004). For pump implantation, the animals were brought to a surgical plane of anaesthesia with isoflurane. The pumps were implanted subcutaneously in the lower back area of the animals through a small mediolateral incision. Ketoprofen (5 mg/kg) was administered once
daily for 2 days after the surgery. After pump implantation, and until the end of the study, animals were monitored daily for signs of distress or weakness. Animals that appeared dehydrated were provided with subcutaneous fluids (lactated Ringer’s solution) 3 times daily (this was required for 3 animals).

3.3.2 Rod Photoreceptor Sorting

Rod photoreceptors were isolated from P6 Nrl-GFP mice as described previously [211]. Briefly, eyes were dissected and the retinas were dissociated into a single-cell suspension by using a papain kit (Worthington). Fluorescent sorting was then performed for GFP+/7-AAD− cells on a BD FACS Aria sorter.

3.3.3 RPE Differentiation

RPE were differentiated from H9 hES cells as described previously [213]. Briefly, the hES cells were grown in feeder-free conditions on Geltrex™ (ThermoFisher Scientific)-coated plates to confluence for 10-12 days in mTESR medium (Stem Cell Technologies), at which point the culture medium was switched to a differentiation medium containing 13% knock-out serum replacement (KSR), 1% Glutamax, 1% non-essential amino acids and 0.1% β-mercaptoethanol in Knock-out DMEM (all from ThermoFisher Scientific) in the absence of bFGF. The cell medium was replaced on alternate days. After approximately 1 month of culture, pigmented clusters started to appear in the wells. After an additional month of culture, these clusters became enlarged and more numerous. The pigmented clusters were manually isolated with a scalpel under a dissecting microscope, and plated in new Geltrex™-coated wells in medium containing 5% Hyclone FBS (ThermoFisher Scientific), 7% KSR, 1% Glutamax, 1% non-essential amino acids, 0.1% β-mercaptoethanol and 10 ng/ml bFGF (R&D Systems). For additional purity, these pigmented clusters were allowed to grow in culture for 1 additional month and the centers of the clusters, which exhibited the strongest RPE-like cobblestone morphology, were manually isolated for a second time. From this point onwards, the cells were considered to be RPE. For transplantation studies, only cells that had been passaged twice (P4) were used. The cells used for transplantation were kept at confluence for 1 month after their last passage in order for them to reacquire a cobblestone morphology.
3.3.4 HAMC Preparation

A physical blend of hyaluronan (HA, 1200-1900 kDa; Novamatrix) and methylcellulose (MC, 300kDa, Shin-Etsu) was used to prepare HAMC, as previously described [6]. Briefly, 24 h before use, sterile filtered HA and MC were dissolved into Hank’s Balanced Salt Solution (HBSS) without calcium chloride, magnesium chloride and magnesium sulfate (ThermoFisher Scientific) at a concentration of 1% w/v, mixed in a SpeedMixer (DAC 150 FVZ; Siemens) for 30 s and left at 4°C overnight. HAMC was mixed with cell solutions (in HBSS) to bring the final concentration to 0.5% w/v for both HA and MC. Prior to use, the HAMC-cell mixture was kept on ice for all experiments.

3.3.5 Cell Transplantation

Subretinal transplantation was performed by a trans-vitreal approach. The cells were washed with HBSS and resuspended in HAMC at a final concentration of 12,500 cells/µl for RPE and 25,000 cells/µl for Nrl-GFP photoreceptors. Injections were performed into NaIO₃-treated animals that had been fitted with CsA minipumps. Animals were brought to a surgical plane of anesthesia with isoflurane. Using a 34-gauge beveled needle attached to the Nanofil submicrolitre injection system (World Precision Instruments), 2 µL of cell suspension was injected into the sub-retinal space of the animals using a Möller Hi-R 900C surgical microscope (Innova Medical Ophthalmics). The injection was performed at a rate of 0.03 µl/sec and the needle was kept in place for an additional minute after injection completion to limit backflow. Our subretinal transplantation success rate was 80%. Subretinal transplantations were considered unsuccessful when cell backflow into the vitreous or excessive bleeding was observed. Animals with unsuccessful transplantations were not included in the analysis. After transplantation, the animals received Ketoprofen (5 mg/kg) once daily for 2 days.

3.3.6 Optokinetic Head Tracking

Visual thresholds were measured by using the OptoMotry system (Cerebral Mechanics) [214]. Vertical sine gratings are projected on monitors as a virtual cylinder which surrounds an unrestrained mouse, placed on a platform. The rotation of the cylinder (12°/sec) elicited tracking behavior that was scored by a blinded observer watching through live video. For visual acuity measurements, the grating contrast was kept at 100%. Spatial frequency was measured for each eye separately using a staircase paradigm provided by the OptoMotry software, which varied the
spatial frequency from 0.003 c/d to 0.642 c/d. The left eye tracks rotation in the clockwise direction while the right eye tracks rotation in the counter-clockwise direction [215]. The experimenter was blinded to the direction of rotation and the spatial frequency of sine gratings being projected on the screens. The luminance level inside the OptoMotry apparatus was measured at ~200 lux, which falls well into the photopic range [216]. Unless otherwise stated, OKT was performed in photopic conditions. Scotopic illuminance levels can vary depending on the behavioral assay used. For scotopic conditions testing, the animals were dark adapted for 2-10 hours and the experiments were performed in a completely dark room (no red lights). Neutral density filters (Lee filters) were used to decrease the luminance level inside the OptoMotry apparatus to <1.5 lux, which falls in the scotopic range for this assay [216]. To assess the scotopic tracking behavior, an infrared-sensitive camera was used (Sony Handycam DCR-HC28; Sony).

Each animal was tested until a reliable visual threshold could be established. In the complete absence of tracking, animals were tested for at least 7 minutes or 4 times in each direction of rotation, whichever was longest. Most sessions lasted between 10 and 20 minutes per animal. Each testing session consisted of multiple trials, during which the animal was presented with rotating sine gratings for up to 20 seconds or until tracking was observed. After each trial the sine gratings were turned off and the screens projected 50% gray for 4 s before the next trial began. For each weekly time point, each animal was tested at least 2 times on different days for each lighting condition. The OptoMotry chamber was thoroughly cleaned with Preempt cleaning solution (Virox Technologies) before and after each test.

3.3.7 Light Avoidance Assay

The light avoidance assay was performed on a custom-modified place conditioning apparatus (SOF-700RA-25 Two Chamber Place Preference Apparatus; Med Associates). One environment was black and the other was white, both with a metal rod floor. A removable partition containing a small aperture was positioned between the 2 environments such that the animals could move freely. The mice were placed in the middle compartment facing towards the wall of the chamber so as not to influence their preference. Mice were dark-adapted for 2-10 hours and received a drop of 1% tropicamide (Mydriacyl, Alcon) on each eye 5-10 minutes before being placed in the chambers. The testing was performed in a completely dark room (no red lights). The animals
were initially familiarized with the chambers by being placed in them for 10 minutes without any recording. On a different day, the animals were placed in the chambers for 10 minutes and their background preference for white or black compartment was recorded, in the absence of any stimulus. 4 weeks after cell transplantation, a single 10-min preference test session was performed and the time spent in each compartment, the average activity in each compartment, the number of transitions and the fecal boli produced by the mice were recorded. The white compartment was illuminated from above with a white LED lamp (Sylvania) that was covered with neutral density filters (Lee Filters) resulting in an illumination level of ~10 lux at the chamber floor. This illumination level has previously been used for scotopic testing in light avoidance assays [84]. No illumination could be detected in the black compartment. Both compartments were covered with custom-made light-impermeable lids, and the lid of the white (lit) compartment contained a transparent, circular area of 6 cm in diameter through which the light seeped into the chamber. For the background testing, the transparent circular area was covered so that the lids of both compartments were completely light-impermeable. No temperature differences were detected in the lit vs dark compartments after 8 h of testing. The chambers were thoroughly cleaned with 70% ethanol before and after each test.

The light avoidance index presented in the results was calculated as:

\[- \{(% \text{ of time spent in lit white chamber}) - (% \text{ of time spent in unlit white chamber by background preference})\}\]

### 3.3.8 Electroretinography

The animals were dark-adapted overnight (12-15 h), and prepared for bilateral ERG recordings under dim red light. Anaesthesia was induced with a mixture of ketamine (150 mg/kg) and xylazine (10 mg/kg), injected intraperitoneally. The head was secured with a stereotaxic holder and the body temperature was maintained at 38°C, using a homeothermic blanket. Pupils were dilated using 1% Tropicamide (Mydriacyl, Alcon). A drop of 0.9% saline was applied on each cornea to prevent dehydration and to allow electrical contact with the recording electrode (gold wire loop). A 25-gauge platinum needle inserted subdermally behind the eyes served as reference electrode. Simultaneous bilateral recording was achieved with active gold loop electrodes placed on each cornea. Amplification (at 1–1000 Hz bandpass, without notch filtering), stimulus presentation, and data acquisition were performed by the Espion E² system (Diagnosys). Stimuli
consisted of single white (6500 K) flashes (10 µs duration), repeated 3–5 times to verify the responsiveness reliability. For scotopic responses, stimuli were presented at 19 increasing intensities varying from −5.2 to 2.9 log cd/m² in luminance. To allow for maximal rod recovery between consecutive flashes, inter-stimuli-intervals were increased (as the stimulus intensities were progressively increased) from 10 s at lowest stimulus intensity up to 2 min at highest stimulus intensity. After the scotopic recordings were completed, a 30 cd/m² background stimulus was applied and photopic responses were studied. Single flashes with intensities ranging from −1.6 to 2.9 log cd/m² along 11 steps of incremental intensities were presented.

3.3.9 Immunohistochemistry and Quantification

Animals were sacrificed by an overdose of sodium pentobarbital, followed by transcardial perfusion with ice cold PBS and 4% paraformaldehyde (PFA), sequentially. The eyes were enucleated and kept in 4% PFA overnight at 4 °C. The following day, the eyes were thoroughly washed with PBS and placed in a 30% sucrose solution overnight at 4 °C. After being embedded in Tissue-Tek OCT compound (Sakura), the eyes were flash-frozen in dry ice-cooled 2-methylbutane and serially sectioned to 14 µm using a Leica CM3050S cryostat. Every fifth section from each eye was used for staining and quantification. On average, 45 sections from each eye were analyzed. The following antibodies were used: STEM121 (1:2000, Takara Bio Y40410), anti-GFP (1:500, Rockland 600-101-215), anti-PRPH2 (1:750, Proteintech 18109-1-AP), anti-RPE65 (1:250, Millipore MAB5428). For in vitro immunostaining of the RPE cells, the following antibodies were used: anti-RPE65 (1:200, Novus Biologicals NB100-355), anti-Bestrophin (1:200, Novus Biologicals NB300-164) and anti-ZO-1 (1:100, Life Technologies 40-2200).

Images for quantification were acquired using a Zeiss AxioScan.Z1 slide scanner. Quantification was conducted by using Fiji [217]. For each Regions of interest (ROIs) were drawn to encompass either the outermost layer of the retina (inner nuclear layer and occasional patches of remaining outer nuclear layer) for transplanted photoreceptor quantification or the subretinal space (between the outermost layer of the retina and the choroid) for RPE quantification. Transplanted cells found in other areas such as the vitreous or the choroid, were rare and not included in the analysis. The injection site was also excluded from the analysis. The number of cells per eye was determined by counting the number of STEM121 or GFP-positive pixels in each eye and dividing this value by the average number of pixels in one STEM121 or GFP-positive cell, respectively. Pixel quantification was performed using Fiji algorithms (“moments”
for STEM121 quantification and “Huang” for GFP quantification) that were chosen based on their effectiveness at discriminating between positive signal and background staining in control slides. By following this approach, we could maximize the tissue area analyzed since we were using the entirety of the retinal area of ~45 sections for each calculation, as opposed to imaging randomly selected fields of each section, which may introduce bias. Images for demonstration were acquired on an Olympus Fluoview FV1000 confocal microscope.

ONL thickness was quantified within a ~1000 um region from the optic nerve head. In each section the thickness was measured in 3 random areas within the 1000 um region. At least 8 sections were used for each eye.

### 3.3.10 Quantitative Real-Time PCR

RNA was isolated using Trizol (Life Technologies), following the manufacturer’s instructions. After RNA extraction, DNAse treatment was done with TurboDNAse (Ambion). The quality of the purified RNA was assessed by measuring the 260/280 ratio on a spectrophotometer (ND-1000, Nanodrop). Only RNA with a 260/280 ratio >1.8 was used. RNA was reverse transcribed using the superscript VILO cDNA synthesis kit (Life Technologies). qPCR amplification was done in an Applied Biosystems 7900HT instrument using SYBR Green Master Mix (Roche). Melting curves were performed for each experiment and negative controls (no RT enzyme controls and no template controls) were always included to ensure the accuracy of the results.

The primer pairs used were as follows:

- **MITF FP** TTGTCCATCTGCCTCTGAGTAG, **MITF RP** CCTATGTATGACCAGGTTGCTTG, **OTX2 FP** ACCTTGAACCTCCACCTCTGC, **OTX2 RP** GCTTCTCTTCTCTGACTCTCTTTTG, **RPE65 FP** TACAGAAAGCACTGAGTTGAGC, **RPE65 RP** CCATTAGTAAGTCACATCCATTCTTGAGC, **CRALBP FP** AGATCTCAGAGATGATGGGAC, **CRALBP RP** GAAGTGGATGGCTTTGAACC, **PEDF FP** TATACCTAAAAACGGCTTCTGGAG, **PEDF RP** GGTCGAGATCTTGCCATTAG, **PMEL17 FP** GTGTGATGGCCCTGTGCTTTCATG, **PMEL17 RP** CAGTGACTGCTATGGTGGG, **TYR FP** GTGTAGGCTTCTTCTTCCAACCTTAG, **TYR RP** GGTCCATTACCACAATAGCATCC, **NANOG FP** CCCTCCTCCATCCCTCTATG, **NANOG RP** TCGGTGGTATTAGGCTTCAACC, **OCT4 FP** CTGTCTCTCCACCTCAGTGTGTGGTCTCTCTCCTTAG, **GAPDH FP** AGCAAGAGCACAAGGAGGAAGAG, **GAPDH RP** GAGCAGAGGTACTTTATGGATGG, **HMBS FP** TGCTATCATTGGAGTGATT, **HMBS RP**
Quantification was performed using the ΔΔCt method; GAPDH and HMBS were used as housekeeping genes. The relative expression shown is the average of the $2^{-\Delta\Delta C_t}$ values calculated for each housekeeping gene.

### 3.3.11 Statistical Analysis

All data are reported as mean ± standard error of the mean unless otherwise indicated. Statistical analysis was performed using GraphPad Prism software. N represents number of animals, n represents number of eyes, where appropriate. For comparisons between multiple groups, an analysis of variance (ANOVA) followed by Tukey’s (for 1-way ANOVA) or Bonferroni (for 2-way ANOVA) post-hoc test was used. For comparisons between two groups, a Student’s t-test was used. For comparison between groups with unequal variances, a non-parametric Mann Whitney test was used to compare two groups. Three statistically significant outliers by Grubb’s test (1 eye in HAMC and 1 eye in the uninjected group for the OKT data, 1 eye in the RPE+PR group for the RPE quantification data) were excluded from the analysis. A p-value of < 0.05 was regarded as statistically significant (*p<0.05, **p<0.01, ***p<0.001).

### 3.4 Results

#### 3.4.1 Sodium Iodate Induces Retinal Degeneration

We injected NaIO$_3$ intravenously and assessed mouse visual function over time by optokinetic head tracking (OKT) and light avoidance assays (Figure 3.1A). OKT is based on an innate reflex, whereby mice track the movement of rotating stripes by moving their head in the direction of the stripes [214]. OKT revealed a biphasic loss of visual acuity after NaIO$_3$ administration, with complete blindness evident by day 35 post-injection (Figure 3.1B). The light avoidance assay is based on the natural aversion that mice exhibit towards lit spaces [218]. Mice are placed in a 2-compartment chamber with a connecting aperture where one compartment is lit and the other is kept dark. The decrease in time spent in the lit compartment upon provision of the light stimulus is a measure of light sensation, and was plotted as “light avoidance index” (see Materials and Methods). This assay, performed on day 36 after NaIO$_3$ administration, corroborated the OKT results: NaIO$_3$-treated animals exhibited significantly (4.9-fold, p<0.001) decreased aversion to light, demonstrating diminished visual function (Figure 3.1C).
We next analyzed the tissue histology of the NaIO$_3$-treated animals, 64 days after treatment. We found a complete absence of RPE cells (Figure 3.1D) and a significant thinning of the outer nuclear layer (ONL) in treated animals (Figure 3.1E): from 57 ± 1 µm in saline-treated controls to 16 ± 1 µm in NaIO$_3$-treated animals (p<0.001, Figure 3.1F); and from 11.0 ± 0.1 cell layers in saline-treated controls to 3.5 ± 0.1 cell layers in NaIO$_3$-treated animals (p<0.001, Figure 3.1G), indicating widespread photoreceptor death. The rest of the retinal structure exhibited no apparent morphological differences from the controls (Figure 3.1E). These data demonstrate that the NaIO$_3$ mouse model exhibits a phenotype that resembles advanced retinal degeneration in the human at both behavioral and tissue levels.
Figure 3.1. NaIO₃ induces retinal degeneration at the behavioral and tissue levels. (A) Experimental timeline. OKT, optokinetic head tracking assay; Light Avoid, light avoidance assay. (B) Visual acuity over time after NaIO₃ or saline injection, measured by OKT (mean ± SEM, n=16 for NaIO₃, n=6 for saline). Statistical significance was evaluated by 2-way ANOVA, with a Bonferroni post-hoc. Treatment p<0.001, time p<0.001, treatment x time interaction p<0.001. ***p<0.001. (C) Light avoidance assay, demonstrating significantly decreased aversion to light after NaIO₃ administration (mean ± SEM, N=9 for NaIO₃, N=3 for saline). Statistical significance was evaluated by a 2-tailed, Mann Whitney test. **p<0.01. The light avoidance index shown is the decrease in time spent in the lit compartment after provision of the light stimulus (see Materials and Methods). (D) Representative immunohistochemistry images of the subretinal space, RPE and choroid 64 days after NaIO₃ or saline injection, stained for the RPE marker RPE65 and the outer segment marker PRPH2. ONL, outer nuclear layer; OS, outer segments; Cho, choroid. Scale bar is 15 µm. (E) Representative immunohistochemistry images of the retinal layers 64 days after NaIO₃ or saline injection. The arrows demonstrate the boundaries of the ONL that were used for quantification. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar is 50 µm. (F) ONL thickness in µm or (G) in cell layers, 64 days after NaIO₃ or saline injection (mean ± SEM, n=26 for NaIO₃, n=6 for saline). Statistical significance was evaluated by a 2-tailed, unpaired t-test. ***p<0.001.

3.4.2 RPE Differentiation From hES Cells

We chose hES cells as an RPE source because they provide an unlimited and clinically relevant cell source. We followed the overgrowth protocol [10] with small modifications (Figure 3.2A). The differentiated RPE (hES-RPE) established cobblestone monolayers that are pigmented, express the tight junction marker ZO-1, and the RPE-specific markers RPE65 and Bestrophin-1 (Figure 3.2B).

Real-time quantitative PCR revealed a progressive upregulation of the RPE markers MITF, OTX2 and RPE65 at the RNA level over the course of the differentiation (Figure 3.2C). A comparison to the ARPE19 cell line, which is often used as a human RPE substitute [219], demonstrated that the hES-RPE expressed 25-fold more MITF (p<0.01, Figure 3.2C), 10-fold
more OTX2 (p<0.001, Figure 3.2C) and 270-fold more RPE65 (p<0.001, Figure 3.2C). To investigate these differences further, we assessed the expression of the RPE markers CRALBP and PEDF, the RPE-melanocyte markers PMEL17 and TYR, and the pluripotency markers NANOG and OCT4 in hES, hES-RPE and ARPE19 cells (Figure 3.2D). All four RPE markers were significantly upregulated in the hES-RPE compared to both hES cells (50 to 660-fold, p<0.01) and ARPE19 cells (190 to 1620-fold, p<0.01) (Figure 3.2D). On the contrary, the pluripotency markers were significantly downregulated (310 to 580-fold, p<0.001) in hES-RPE compared to the originating hES cells, to levels similar to those found in ARPE19 cells (relative expression of NANOG: 1 ± 0.1 for hES-RPE vs 0.7 ± 0.1 for ARPE19; relative expression of OCT4: 1.2 ± 0.2 for hES-RPE vs 1.5 ± 0.6 for ARPE19) (Figure 3.2D). Together, these data show that RPE can be readily derived from hES cells and that they express higher levels of RPE markers than the ARPE19 cell line.

### 3.4.3 Photoreceptor Isolation from Nrl-GFP mice

We used P6 Nrl-GFP rods as our source of photoreceptors because these cells have been extensively studied in the past [2,80] and possess high integration capacity into recipient retinas. Nrl is the fate-determining transcription factor of rod photoreceptors [212], hence GFP is specifically targeted to newborn rods in these mice (Figure 3.2E). A flow cytometry purity check after fluorescence-activated cell sorting (FACS) for GFP revealed that our photoreceptor population was 97.2% GFP+ (Figure 3.2F).
Figure 3.2. RPE derivation from hES cells and photoreceptor isolation from Nrl-GFP mice. (A) Timeline of RPE differentiation from hES cells. KSR, knock-out serum replacement; bFGF, basic fibroblast growth factor. (B) Representative immunostaining pictures of hES-RPE, stained for the RPE markers Bestrophin-1, RPE65 and the tight junction marker ZO-1. Scale bar is 20 µm. (C) qPCR for the expression of the RPE markers MITF, OTX2 and RPE65 over the course of the differentiation protocol and in the ARPE19 cell line (mean ± SEM, n=3-6). Statistical significance was evaluated by 1-way ANOVA with a Tukey’s post-hoc. **p<0.01; ***p<0.001. (D) qPCR for the expression of the RPE markers CRALBP, PEDF, the melanocyte markers PMEL17, TYR and the pluripotency markers NANOG, OCT4 in hES cells, hES-RPE and ARPE19 cells (mean ± SEM, n=3-6). Statistical significance was evaluated by 1-way ANOVA with a Tukey’s post-hoc. **p<0.01; ***p<0.001. (E) Representative immunohistochemistry image of the retinal layers in an Nrl-GFP mouse, demonstrating GFP expression only in the ONL. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar is 40 µm. (F) FACS plots demonstrating expression of GFP and the dead cell stain 7-AAD in P6 Nrl-GFP retinal preparations before and after FACS. MFI, mean fluorescence intensity.

3.4.4 RPE and Photoreceptor Co-transplantation Induces Visual Recovery at the Behavioral Level

We performed transplantation experiments at a late time point (8 weeks) after NaIO3 treatment, in mice that were completely blind (Figure 3.3A). We transplanted 50,000 photoreceptors and/or 25,000 RPE per eye in a total volume of 2 µl in HAMC into the subretinal space. These numbers were chosen based on: i) previous experiments with single cell type transplants [3,6,80], ii) technical cell density limitations (>50,000 cells/µl in HAMC caused needle clogging and cell death), and iii) pilot studies where we determined that a photoreceptor to RPE ratio of 2:1 is superior to higher ratios in terms of visual recovery (data not shown). A HAMC-injected group and a non-transplanted group (“Uninjected”) served as vehicle and negative controls, respectively.

We assessed behavioral recovery after transplantation by OKT and light avoidance assays. Rod photoreceptors mediate scotopic visual responses while cones are responsible for photopic
vision. To distinguish between the two, we performed OKT at both scotopic (<1.5 lux) and photopic (~200 lux) conditions. Interestingly, only the co-transplant group exhibited significant visual recovery 4 weeks after transplantation in scotopic conditions (p<0.001, co-transplant vs uninjected, co-transplant vs HAMC, co-transplant vs photoreceptors alone) (Figure 3.3B). No significant differences (p>0.05) were identified between the groups at any time point after transplantation in photopic conditions (Figure 3.3C).

To corroborate our OKT data, we performed light avoidance testing in scotopic conditions (~10 lux) 4 weeks after transplantation. We analyzed the average mouse activity in the lit vs dark chambers. Mice are a nocturnal species and are normally more active in the dark [220]. The only groups that were significantly more active in the dark chamber than the lit chamber were the co-transplant group and the wild type animals (p<0.05, Figure 3.3D). Like the wildtype animals, the co-transplant group avoided the lit compartment significantly more than the photoreceptor alone group (Figure 3.3E). Importantly, no differences were observed between the groups in the number of entrances into the lit chamber (p>0.05, Figure 3.3F) or the number of fecal boli produced (p>0.05, Figure 3.3G). These data indicate that the differences observed in mouse activity are not mediated by anxiety [221,222], but rather demonstrate increased visual perception in the co-transplanted animals, which is similar to that of wildtype. Together, these data demonstrate that RPE and photoreceptor co-transplantation restores some functional vision in previously blind mice at an advanced stage of degeneration.
Figure 3.3. RPE and photoreceptor co-transplantation leads to visual recovery. (A) Experimental timeline. CsA, cyclosporin A; OKT, optokinetic head tracking assay; Light Avoid., light avoidance assay; ERG, electroretinography. (B) Visual acuity in scotopic conditions over time after transplantation, assessed by OKT (mean ± SEM, n=9-12). Statistical significance was evaluated by 2-way ANOVA with a Bonferroni post-hoc.
***p<0.001 for Photo+RPE vs Uninjected, Photo+RPE vs HAMC, and Photo+RPE vs Photo. (C) Visual acuity in photopic conditions over time after transplantation, assessed by OKT (mean ± SEM, n=9-12). As expected, a 2-way ANOVA with a Bonferroni post-hoc identified no significant effects. (D) Average mouse activity in the dark and lit chambers during the light avoidance assay, one month after transplantation (mean ± SEM, N=5-7). Statistical comparisons between dark and lit chamber activity within each group were performed by 2-tailed, paired t-tests. *p<0.05. (E) Light avoidance index, one month after transplantation (mean ± SEM, N=5-7). Statistical significance was evaluated by 1-way ANOVA with a Tukey’s post-hoc. *p<0.05 for Wild Type vs Photo, Photo+RPE vs Photo. (F) Average number of entrances into the lit chamber during the light avoidance assay, one month after transplantation (mean ± SEM, N=5-7). A 1-way ANOVA with a Tukey’s post-hoc identified no significant effects. (G) Average number of fecal boli produced during the light avoidance assay, one month after transplantation (mean ± SEM, N=5-7). A 1-way ANOVA with a Tukey’s post-hoc identified no significant effects.

3.4.5 RPE and Photoreceptor Co-transplantation Leads to Detectable ERG B-waves

To assess whether cell transplantation afforded any changes in the electrical responses of the retina to light, we performed electroretinography (ERG). It has been previously demonstrated that photoreceptor transplants can lead to the light-driven trans-synaptic activation of depolarizing (ON) bipolar cells as reflected by recordable b-waves [1]. In wild type animals, the amplitude of b-waves grows proportionally to flash stimulus strength, saturating at 1.89 log cd/m² (Figure 3.4A). Detection of ERG signals has been limited in cell transplantation experiments essentially due to insufficient critical mass potential [2]. The co-transplant group showed an initial increase in b-wave amplitudes starting at -2.82 log cd/m², followed by saturation at 0.37 log cd/m² and gradual extinction at higher light intensities (Figure 3.4B). This pattern is consistent with the occurrence of functional, albeit weak, phototransduction (and ON bipolar cell trans-synaptic activation) that gets saturated at higher flash strengths in the co-transplanted animals. One possible explanation could be related to ineffective retinoid cycling by the co-transplanted RPE. B-waves did not exceed the 40 µV criterion amplitude in any other groups (Figure 3.4C-F); notably, no detectable b-waves were observed in the uninjected, NaIO₃-
treated animals (Figure 3.4F). As expected, and consistent with the lack of behavioral recovery in photopic conditions by OKT, no differences were observed among the groups for photopic ERG recordings (Figure 3.5).

![Graphs showing scotopic b-waves](image)

**Figure 3.4.** RPE and photoreceptor co-transplantation leads to detectable scotopic ERG b-waves. B-wave amplitudes intensity response in: (A) wild type animals (mean ± SEM, n=10), (B) NaIO$_3$-treated animals transplanted with RPE and photoreceptors, (C) NaIO$_3$-treated animals transplanted with RPE alone, (D) NaIO$_3$-treated animals transplanted with photoreceptors alone, (E) NaIO$_3$-treated animals transplanted with HAMC vehicle or (F) NaIO$_3$-treated uninjected controls. Recordings were done 7 weeks after transplantation.
(mean ± SEM, n=9-12). The dashed line demarcates the 40 µV criterion amplitude, above which a signal reflects a true ERG b-wave and below which a signal reflects background.

Figure 3.5. RPE and photoreceptor co-transplantation do not lead to changes in photopic ERG b-waves. (A) Photopic b-wave amplitudes of wild type animals over a range of light intensities (mean ± SEM, n=10). (B) Photopic b-wave amplitudes of NaIO₃-treated animals transplanted with RPE and photoreceptors, (C) RPE alone, (D) photoreceptors alone, (E)
HAMC vehicle or (F) uninjected controls, over a range of light intensities, 7 weeks after transplantation (mean ± SEM, n=9-12).

3.4.6 Co-transplantation Leads to Superior Survival for Both RPE and Photoreceptors

To probe the mechanism underlying the observed behavioral effects, we assessed transplanted cell survival 2 months after transplantation by immunostaining for human-specific STEM121 to detect the hES-RPE and GFP to detect the donor photoreceptors. At this time point, the majority of the ONL was absent in most animals, with the exception of occasional patches of host photoreceptors.

Both donor RPE and photoreceptors were more abundant when transplanted together than each alone; however, the overall percent survival was still low for both. Donor RPE formed monolayer-like structures that juxtapose the outermost layer of the host retina (Figure 3.6A). Interestingly, co-transplantation led to a 2.8-fold increase in surviving RPE numbers: 2100 ± 400 vs 750 ± 160 (**p<0.01, Figure 3.6B). Donor photoreceptors were found on the outer edge of the remaining inner nuclear layer (Figure 3.6C). Notwithstanding their low abundance overall, co-transplantation resulted in a 2.1-fold increase in photoreceptor survival: 210 ± 20 vs 100 ± 30 (**p<0.01, Figure 3.6D).
Figure 3.6. Co-transplantation leads to increased survival for both RPE and photoreceptors. (A) Representative immunohistochemistry image of donor RPE cells in a co-transplanted animal, stained for human-specific STEM121. INL, inner nuclear layer. Scale bar is 10 µm. (B) Quantification of surviving donor RPE cells in co-transplanted animals, compared to animals transplanted with RPE alone (mean ± SEM, n=12 for RPE, n=11 for Photo+RPE). Statistical comparison was performed by a 2-tailed, Mann Whitney test. **p<0.01. (C) Representative immunohistochemistry image of donor photoreceptors in a co-transplanted animal, stained for GFP. INL, inner nuclear layer. Scale bar is 10 µm. (D) Quantification of surviving donor photoreceptors in co-transplanted animals, compared to animals transplanted with photoreceptors alone (mean ± SEM, n=7 for Photo, n=12 for Photo+RPE). Statistical comparison was performed by a 2-tailed, unpaired t-test. **p<0.01.
3.5 Discussion

Advanced stages of retinal degeneration are characterized by pathological changes in RPE and photoreceptors. It has been previously demonstrated that either RPE or photoreceptor transplantation alone holds some promise for restoring vision in models of early retinal degeneration that exhibit defects in RPE or photoreceptors, respectively [2,3]; however, these genetic models don’t reflect the pathology observed in human disease where degeneration of RPE and photoreceptors leads to blindness. Using a mouse model where both RPE and photoreceptors die, we demonstrate the novel approach of transplanting RPE together with photoreceptors, in an injectable biomaterial. For the first time, to the best of our knowledge, we achieve some visual recovery in an animal model of complete blindness by dissociated cell co-transplantation. Unlike approaches where retinal sheets are transplanted [73,223], our technique does not require the use of custom-made surgery tools and can be performed through a very fine, 34-gauge needle, minimizing the invasiveness of the surgery. Furthermore, dissociated cell transplantation allays concerns related to graft orientation or rosette formation, which has been extensively observed in retinal sheet transplants and may limit integration into the host tissue [224,225].

The lack of genetic animal models that faithfully reproduce advanced retinal disease has hindered studies into clinically relevant approaches to reverse it. We employed the NaIO₃ mouse model, which the mechanism of RPE cell death after treatment has been studied [226]; yet, the temporal onset of blindness at the behavioral level after NaIO₃ injection has only been studied in a few studies [210,227,228]. We found that the loss of vision after NaIO₃ treatment followed a biphasic profile, where there was an acute decrease in visual acuity after injection, which recovered to baseline levels before the onset of a second, slower phase of decreasing visual acuity, ultimately resulting in blindness after 35 days. This pattern has been observed before [227-229], but its underlying cause remains elusive; we hypothesize that necrotic RPE death, which is known to happen soon after NaIO₃ administration [230], leads to accumulation of debris in the subretinal space, which impairs vision. Clearance of the debris may allow vision to resume temporarily, until the loss of RPE triggers photoreceptor death and then functional blindness. The data suggest that the cell death kinetics of RPE and photoreceptors after NaIO₃ injection can be observed at the behavioral level. At the time of cell transplantation, 2 months after NaIO₃ treatment, virtually all the host RPE and most host photoreceptors were absent.
We employed hES-derived RPE and primary mouse photoreceptors for transplantation. Clinical studies using pluripotent stem cell-derived RPE, currently underway in the United States, the United Kingdom and Japan, underline their clinical relevance [12]; notwithstanding the excitement associated with these clinical trials, given the symbiotic relationship between RPE and photoreceptors and their degeneration in diseases like AMD and retinitis pigmentosa, vision repair in advanced stages of these diseases, as emulated herein, requires co-transplantation of both cell types.

Despite recent advances in deriving photoreceptors from human pluripotent stem cell populations [84,225,231], more research is needed to identify an optimal developmental stage for transplantation of these cells, as has been demonstrated for early postnatal mouse photoreceptors [80,81]. Efforts to identify surface markers that will enable the purification of such integration-capable photoreceptors from human stem cell sources are currently underway [85,86].

We observed visual recovery only in co-transplanted animals using three different outcome measures: OKT, light avoidance and full field ERG. Co-transplanted animals recovered approximately 10.4% of their visual function by OKT, which is a quantitative assay that requires higher order vision to observe a bigger change [214,215,232]. Even though extrapolating such data to humans is difficult [233], small improvements in visual acuity can lead to significant changes in a patient’s quality of life, enabling visually-guided behaviors such as object localization and obstacle avoidance [234].

Co-transplanted animals performed similarly to wild type controls in the light avoidance assay, which measures gross mouse behavior (time spent in compartments and activity) and may be more qualitative, requiring a lower threshold of visual perception to trigger the complete magnitude of the response. Similar tests, depending on rudimentary light perception, have demonstrated complete recovery of blind mice by therapeutic interventions in other settings [232,235]. Light aversion testing has been widely employed in assessing mouse anxiety levels [218]. To ensure that anxiety was not a contributing factor to the observed differences, we demonstrate that neither the number of entrances in the lit chamber nor the fecal boli produced were different among the groups.

Interestingly, behavioral recovery was observed in the scotopic light range, which is consistent with the physiological role of rods for night vision. This finding is compelling because it
suggests that our behavioral recovery is mediated by rod photoreceptors. Due to the small number of surviving donor rods, it is unlikely that these cells alone are sufficient to produce useful vision and suggests that surviving host photoreceptors are involved in the observed restored vision. Since cone photoreceptors die before rods and within weeks after NaIO₃ administration [236], the absence of host (or donor) cones explains the absence of behavioral responses in photopic conditions.

Our ERG data show detectable scotopic b-waves in co-transplanted animals below flash strengths of 0.5 log cd/m² that were quenched at higher light levels and under photopic conditions. These observations might reflect light-driven rod responses that rapidly saturate, perhaps due to an incomplete maturation state in both transplanted photoreceptors and RPE. Both host rod photoreceptors, whose outer segment integrity has been compromised by degeneration, and transplanted rods are morphologically impaired, which has been shown to provide limited light responsiveness [237]. Notwithstanding the small amplitude of the ERG responses, even this response is remarkable given the large number of synchronous light-evoked potential events required to generate a detectable signal [2].

Consistent with the well-established mutual relationship between RPE and photoreceptors, we found that cell survival was significantly greater for both cell types in the co-transplant group than each cell type transplanted alone. While transplanted RPE should enable any surviving host photoreceptors to function, our data demonstrate that this is insufficient for behavioral recovery. The lack of visual recovery in the NaIO₃ mouse model after RPE-only transplants is consistent with other reports where both hES-derived and adult stem cell-derived RPE were transplanted without functional repair [210,238].

The combined delivery of photoreceptors and RPE is essential to visual recovery; however, the mechanism by which the transplanted rods contribute to behavioral amelioration is unclear. On the one hand, the donor photoreceptors may promote transplanted RPE survival, which in turn enhance vision through surviving donor and host photoreceptors. This hypothesis is supported by the increased donor RPE numbers detected in the co-transplant group, compared to the RPE alone group. This suggests that, in this model, there is a lower threshold of RPE survival, below which vision cannot be achieved. On the other hand, the donor rods may secrete signals that potentiate the function of host rods. Such candidate factors include: the full-length isoform of
rod-derived cone viability factor (RdCVFL) [239], which is produced by rods; and all-trans retinol, secreted by photoreceptors as a by-product of the visual cycle. Viral delivery of RdCVFL sustained rod function in the rd10 mouse model of retinal degeneration, without affecting cones [240]. All-trans retinol was recently demonstrated to augment the light sensitivity of rods through the insulin-like growth factor 1 pathway [241]. These hypotheses are not mutually exclusive; it is possible that the donor rods exert effects on both donor RPE and host rods.

In conclusion, we developed a novel, minimally invasive approach to reverse blindness at an advanced stage of retinal degeneration. Delivering photoreceptors together with RPE improves the outcome of cell transplantation at both behavioral and tissue levels, and significantly better than delivering either cell type alone, which, until now, has been best practice. These results provide a pathway forward for future research and translation of cell transplantation approaches for blindness.

3.6 Acknowledgements

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4 Thesis Discussion

4.1 HA-based Gels Promote Rod Photoreceptor Survival in vitro

Despite significant progress made in understanding retinal biology and disease in recent years, culturing photoreceptors in vitro has remained elusive. We demonstrate, in chapter 2 of this thesis, that HA gels afford an approximately two orders of magnitude increase in photoreceptor survival in vitro. This finding provides an invaluable addition to a neuroscientist’s toolbox. The HA gels can provide a platform to study the effects of soluble factors and ECM molecules on photoreceptors as well as their interactions with other retinal cell types, without the complexity and confounding factors of the in vivo environment. In addition, these gels can be used in a disease modeling context, for example in library screening assays aiming to identify molecules that improve survival of photoreceptors harboring mutations such as those found in RP patients. Such approaches have been undertaken in multiple fields and are based on the premise that iPS cells derived from patients and differentiated into the desired cell type exhibit the disease phenotype in vitro [242-245]. Importantly, in vitro drug or genetic treatments have been demonstrated to correct disease phenotypes [245,246] and predict clinical responses [247,248].

The implementation of the HA-based hydrogels described in chapter 2 in retinal disease modeling will enable a more faithful recapitulation of the in vivo retinal niche and may improve the acquisition of a diseased phenotype, as seen elsewhere [247,249].

4.1.1 Rod Photoreceptors Do Not Respond to Stiffness Cues

Over the last fifteen years, a significant body of research has demonstrated that a plethora of cell types respond to physical cues, including stem cells [185,186] and differentiated cell types [184]. Surprisingly, rods do not seem to be affected by elastic modulus alterations in the 1.79-87.79 kPa range, and survived well in the presence of HA even on tissue culture polystyrene, whose Young’s modulus lies in the GPa range. Previous research has demonstrated that Muller glial cells modify their gene expression and morphology in response to stiffness cues [187], suggesting that these cells might constitute the stiffness sensors of the retina.
In our culture platform, photoreceptors were plated on preformed HA gels and allowed to infiltrate into the matrix. It is unclear if photoreceptors actively migrate into the gels or merely fall through surface crevices, but based on the round cell morphology observed, the latter is more likely. This suggests that the cultured photoreceptors are not completely engulfed by the ECM stimulus. Furthermore, even though our system results in a 3D culture setup of approximately 200 µm in the z-dimension, cellular distribution along the z-axis could be drastically improved by chemistries that enable cell encapsulation during gel crosslinking. Such systems are currently being developed in the Shoichet lab. HA-based gels that can be crosslinked at physiological pH and preserve cell viability have been achieved by using ketone-oxyamine or tetrazine-norbornene click chemistry. Cells encapsulated in hydrogels would be exposed to ECM on all sides; it is plausible that stiffness alterations in these environments might affect photoreceptors differently [250]. Research in other fields has demonstrated that cell encapsulation in HA hydrogels yields significantly different phenotypes and cell behavior than culture on the surface of such hydrogels.

4.1.2 HA Acts Through the RhoA, Wnt and mTOR Pathways on Photoreceptors

Being a ubiquitous ECM component, HA has been demonstrated to act through a variety of receptors and activate a plethora of signaling pathways [54]. Inspired by previous research that had demonstrated that the mTOR signaling pathway has a pro-survival role for photoreceptors [175,196], we investigated its activation in the HA gels. We found that mTOR is upregulated upon photoreceptor culture in HA and that mTOR inhibition abolishes the pro-survival effect of HA. This was not due to non-specific toxicity, since mTOR inhibition on photoreceptors in standard culture conditions did not induce further cell death. This finding demonstrates that mTOR is the main signaling target of HA in rod photoreceptors.

Given the cellular role of mTOR as a central signaling node that integrates information about cellular health, nutrient and growth factor availability, we asked what upstream mediators connect HA with mTOR. Through a biased screening of inhibitors, based on signaling pathways that have been shown to be: i) upstream of mTOR and ii) activated by HA, we identified the canonical Wnt and the RhoA pathway as signaling intermediaries. Interestingly, research on the
role of these pathways in retinal degeneration corroborates our findings [199]. These pathways represent compelling targets for both pharmacological approaches aiming to minimize photoreceptor death in retinal degenerative diseases, and bioengineered approaches aiming to improve transplanted cell survival.

Notwithstanding the elucidation of the signaling pathways acting downstream of HA in rods, the initial HA receptor that induces the cascade remains elusive. Rods do not express the main HA receptors, namely, CD44 and RHAMM. Two potential candidates are the glycoproteins SPARC and SPARCAN identified by Hollyfield and colleagues [60-62]. These proteins are found on photoreceptors, they bind HA, and their mutation leads to retinal degeneration (Christian Hamel, personal communication and [63]). Future studies will demonstrate whether they induce the signaling pathways we identified herein and whether they are the only HA receptors expressed on photoreceptors.

4.1.3 HA-based Gels Induce Maturation of Photoreceptors in vitro

Since the photoreceptors cultured in the HA-based gels were isolated at P11, which is the age when photoreceptors start developing outer segments in vivo, we asked whether the improved survival in the HA-based gels was accompanied by evidence of maturation. We found that both peripherin and ABCA4, two outer segment markers, were significantly upregulated at both the RNA and protein levels in the HA-based gels. This finding is remarkable because it demonstrates that photoreceptors can proceed along their developmental path in vitro, in the absence of other cell types or ECM cues. Furthermore, our data demonstrate that improving photoreceptor survival is not on its own sufficient to also promote maturation, since no maturation was observed in the Geltrex™ cultures despite superior survival. Instead, our data suggest that HA acts as a double trigger: it both promotes photoreceptor survival and induces their maturation. Future studies will demonstrate whether the same signaling pathways mediate survival and maturation, or whether HA acts in different ways to induce the two effects.
4.1.4 Additional Cell Types or Factors Needed to Further Improve Photoreceptor Survival/Maturation in vitro

Notwithstanding the significant increase in cell survival and maturation achieved in vitro by using HA-based gels, approximately 90% of the cells still die by day 14. Despite the upregulation of the two outer segment markers observed, no full-fledged outer segment formation was seen. Thus to optimize survival and maturation of photoreceptors in vitro, additional cell types or ECM/soluble factors are needed. The subretinal space contains a multitude of protein and carbohydrate components with significant roles in photoreceptor function [52].

Based on their role in photoreceptor development and homeostasis, the RPE and/or Muller glia would be excellent candidates for addition to the culture system. Stem cell-derived RPE and transgenic mouse lines with fluorescent reporters under the CRALBP promoter [251] could be employed as cell sources. As discussed in section 5.2.1, we attempted to co-culture photoreceptors and RPE in this system and, unexpectedly, observed pronounced photoreceptor cell death, which may be attributed to metabolic byproduct accumulation by the RPE. Muller glia is involved in ion/metabolite buffering [252] and may mitigate this issue.

In addition to HA, the subretinal space ECM contains copious amounts of CSPGs, HSPGs and vitronectin. Addition of these matrix molecules into the gels may provide a more physiological environment for the photoreceptors. This could be achieved either by mixing native proteins into the gel during the polymerization reaction, modifying the proteins (e.g. with maleimide groups) in order for them to be chemically immobilized onto the gel or employing short peptides constituting the receptor-binding domain of these proteins.

4.2 RPE and Photoreceptor co-transplantation Induces Visual Recovery in NaIO₃-treated Mice

Despite our avid understanding of the mutual relationship between RPE and photoreceptors, the prevailing approach for cell transplantation strategies to cure blindness has been transplanting either cell type separately. We demonstrate, in chapter 3 of this thesis, that co-transplanting RPE and photoreceptors leads to significant improvements at both the behavioral and tissue levels.
Previous approaches have either performed dissociated single cell type transplants in saline which are plagued by low cell survival [6,80], or retinal sheet transplants that are stymied by the invasiveness of the required procedure [94]. Furthermore, retinal sheet transplants consist of grafts of entire retinas, including the inner nuclear and ganglion cell layers, which results in host retinas with two sets of these layers. This is obviously non-physiological and can hinder graft-host connectivity. In our method, we use an injectable biomaterial to deliver the two cell types, combining the ease of dissociated cell transplants with the improved survival afforded by the material [6].

As discussed in the introduction section on cell transplantation, both suspension and graft transplants of RPE are accompanied by advantages and drawbacks. A direct comparison of our HAMC-mediated RPE delivery to RPE grafts would provide valuable information and allow conclusions to be drawn on which approach is best.

4.2.1 Cell Transplantation in the NaIO₃ Animal Model

Modeling advanced retinal degeneration in rodents is a formidable challenge [253]. Previously employed animal models for photoreceptor transplantation typically had structurally intact retinas with mutations in visual cascade proteins [6,80], or exhibited photoreceptor-specific degeneration [1,82]. Conversely, RPE transplants were usually performed in animal models early in degeneration [3,208], before blindness occurred, with the goal of preventing vision loss. To test the hypothesis that co-transplantation of RPE and photoreceptors is beneficial for visual recovery, we needed to make use of an animal model that recapitulates advanced retinal degeneration and lacks both cell types. The NaIO₃ model satisfies this criterion.

NaIO₃ has been demonstrated to induce acute necrotic cell death in the RPE [210,230], leading to photoreceptor degeneration. The mechanism behind the RPE specificity of NaIO₃ is posited to involve the high metabolic demands of RPE and toxic byproducts resulting from the reaction of NaIO₃ with melanin [254]. However, neither of these two proposed mechanisms can completely explain the RPE specificity of the toxin. Highly metabolic cells can be found in other tissues, while melanin-containing cells are abundant in the choroid and yet are not targeted by NaIO₃.
Furthermore, NaIO$_3$ induces blindness in albino animals, which questions the contribution of melanin to RPE toxicity [255].

Notwithstanding the incomplete understanding of its mechanism of action, NaIO$_3$ induces a retinal phenotype similar to that found in advanced retinal degeneration patients. Within a month, the RPE is virtually completely absent and most of the photoreceptors die. This creates a hostile environment for the transplanted cells. Unsurprisingly, our survival rates for RPE and photoreceptors were low, at approximately 8% for the RPE and 0.4% for the photoreceptors. It is difficult to compare RPE survival to what others have found, since most labs do not quantify survival of transplanted RPE. Photoreceptor survival is lower than what has been observed in other models [2,80,81], which may be a function of i) the overestimation of donor photoreceptor survival in other settings due to GFP transfer (discussed below) and ii) the more hostile environment provided by the NaIO$_3$-injured model. The limited survival of transplanted cells observed here leaves significant room for improvement by bioengineering strategies, incorporating pro-survival factors in the delivery vehicle, as discussed in section 5.2.6.

4.2.2 GFP Transfer Between Donor and Host Photoreceptors

For years, it was believed that integration rates of as high as 15% could be achieved by transplantation of early postnatal Nrl-GFP$^+$ rods into structurally intact retinas [2]. The donor photoreceptors would adopt a perfectly physiological morphology, including outer segments and synapse formation to the underlying bipolar cells. It was recently discovered that as much as 90% of that assumed integration is actually due to GFP transfer from the donor to the host photoreceptors [88-90]. This finding challenges the previously reported survival and integration rates and suggests an alternative pathway for the observed behavioral benefits of cell transplants: transfer of healthy donor proteins to substitute their counterparts in mutant host photoreceptors.

Interestingly, it appears that GFP transfer primarily functions between photoreceptor cells, and its presence is drastically decreased in animal models with severe retinal degeneration that lack most photoreceptors (Wallace lab, personal communication). Even though we cannot conclusively exclude the presence of some GFP transfer in our experiments, the low numbers of GFP$^+$ cells found combined with the fact that approximately 70% of the host photoreceptors had
degenerated at the time of cell transplantation suggests that transfer is not a main factor in our setting. Future studies employing NaIO₃-treated animals on an RFP background or DNA labeling of the donor cells (e.g. by EdU) will address this outstanding issue.

4.2.3 Mechanism of Observed Behavioral Recovery

We found that behavioral recovery was induced in previously blind NaIO₃-injured animals only when RPE and photoreceptors were transplanted together. The exact mechanism underlying the behavioral recovery remains elusive. The limited survival of donor photoreceptors suggests that surviving host photoreceptors are essential for the recovery; however, we saw no evidence for improved host photoreceptor survival. Given the essential role of the RPE in the visual cycle, we hypothesize that the RPE act by enabling host and donor photoreceptors to function. The improved RPE survival observed in the co-transplant group may afford those animals a functional benefit. If this is the only mechanism underlying the behavioral effect, improving RPE survival without administering photoreceptors should have achieved the same behavioral recovery. Since RPE transplantation alone did not achieve the same functional repair as observed for RPE and photoreceptor co-transplantation, the mechanism remains unclear.

Alternatively, donor photoreceptors may improve visual recovery in two ways: i) by directly providing synaptic input to underlying bipolar cells and ii) by secreting factors (e.g. RdCVFL and 9-cis retinal discussed in chapter 3) that potentiate the function of host photoreceptors. These mechanisms might act in concert to achieve vision repair in co-transplanted animals. Direct synaptic connectivity can be tested by immunostaining for synaptic markers such as synaptophysin or bassoon. In addition, the contribution of the donor photoreceptors to visual recovery can be assessed by employing genetic constructs that can induce cell death in a temporally controlled manner [256,257]. Should the observed visual recovery be lost after targeted killing of the donor photoreceptors, that would signify a direct contribution of these cells to light detection.
5  Conclusions

Over the course of this thesis, a culture system was developed for isolated photoreceptors in vitro, its mechanism of action was elucidated, and its bioactive component was used in transplantation experiments comparing co-delivery of hES-RPE with photoreceptors relative to delivery of each cell type alone. This validated the primary hypothesis that “Co-transplantation of stem cell-derived RPE and photoreceptors in the subretinal space of a rodent model of retinal degeneration will result in better survival of the cells and behavioral recovery of the animals than with either of those cell types alone”. HA-based hydrogels were found to drastically increase rod photoreceptor survival in vitro, and this was independent of material stiffness. Instead, rod survival was induced by the HA component of the hydrogels. Motivated by the lack of previous knowledge on HA-induced pathways on photoreceptors, we identified mTOR, Wnt and RhoA signaling as its downstream effectors.

In the second part of this thesis, a mouse model of advanced retinal degeneration was validated and used to perform transplantation experiments of hES-RPE and photoreceptors, together or separately. Based on our findings on the pro-survival role of HA on photoreceptors, an injectable HA-based hydrogel was used for cell delivery. Completely blind, NaIO₃-injured animals were able to regain some vision only when co-transplanted with RPE and photoreceptors and neither in RPE alone nor photoreceptor alone groups. These findings establish co-transplantation of RPE and photoreceptors as a valuable approach for late-stage retinal degeneration.

5.1  Achievement of Objectives

This research was motivated by the following hypothesis:

*Co-transplantation of stem cell-derived RPE and photoreceptors in the subretinal space of a rodent model of retinal degeneration will result in better survival of the cells and behavioral recovery of the animals than with either of those cell types alone.*

Achievement of the objectives originally laid out in Chapter 1 is summarized below:

1. To identify a culture platform that enables photoreceptor survival and maturation in vitro.
   - HAPEG hydrogels were found to drastically increase photoreceptor survival in vitro.
• Modifying the HAPEG hydrogel stiffness had no effect on photoreceptor survival.
• HA was necessary for the pro-survival effect.
• HA acted by activating the mTOR, Wnt and RhoA pathways.
• Photoreceptors upregulated maturation markers in HAPEG gels.

These data were presented in Chapter 2 and published in *Advanced Functional Materials* [211].

2. To evaluate the effect of co-transplantation of RPE and photoreceptors in terms of survival and restoration of visual function.
• RPE were derived from hES cells.
• The NaIO₃-induced model of retinal degeneration was validated.
• Co-transplantation of RPE and photoreceptors significantly improved visual acuity in blind NaIO₃-injured mice, while single cell type transplants did not.
• Cell survival for both donor photoreceptors and RPE was greater in the co-transplanted animals compared to the single cell type controls.

These data were presented in Chapter 3 and submitted to *Nature Biomedical Engineering*

5.2 Recommendations for Future Work

5.2.1 Enriching Culture Environment of Photoreceptors In Vitro

As described earlier, despite the improved survival and maturation of rod photoreceptors achieved in the HA-based gels, approximately 90% of the cells die by day 14. Supplementing the culture platform with additional cell types may generate a more physiological environment for the photoreceptors and further improve their survival and maturation.

In preliminary experiments, we found that when hES-RPE are co-cultured with NrlGFP⁺ rods in the HAPEG hydrogels, the survival of the rods decreases sharply. At first glance, this finding appears counterintuitive; the RPE has a well-studied supportive role for the photoreceptors, as discussed previously. However, RPE cells are contact-inhibited and will de-differentiate and proliferate to reach confluence [258]. Hence, dissociating and mixing RPE with the
photoreceptors in the HAPEG gels induces RPE proliferation which deprives photoreceptors of nutrients. On the contrary, plating the RPE as a monolayer at the bottom of the well leads to non-physiological RPE to photoreceptor cell ratios: a confluent well of a 96-well plate contains approximately 100,000 RPE, while photoreceptors are plated at a concentration of 10,000 cells per well. This translates to an RPE to photoreceptor ratio of 10 to 1, while the native ratio ranges from 1 to 20 in the central retina and 1 to 40 in the periphery [259]. The resulting abundance of highly metabolic RPE cells leads to acidification of the medium (RPE are known to be highly glycolytic, secreting lactic acid in the culture medium [260]) and photoreceptor cell death.

To address this challenge, ECM micropatterning technology developed in the Zandstra lab [261] could be employed. By patterning only a portion of the well surface, this technology enables precise control of RPE cell numbers, while maintaining the RPE locally confluent. In addition to preventing further proliferation, RPE confluence is essential to their polarization and maturation [258]. In preliminary experiments we found that, as expected, RPE cultured on patterned wells acidified the medium to a smaller extent compared to confluent RPE. This might allow photoreceptor co-cultures to be achieved.

An alternative approach could focus on implementing continuous flow in the cell culture system, to excrete metabolic byproducts such as lactic acid and provide nutrients at a constant rate. Microfluidic devices could be employed to achieve this. Literature from the organ-on-a-chip field has extensively demonstrated that flow can exert a profound effect on cell physiology and in vitro morphogenesis [262]. Furthermore, hydrogels have been previously used successfully in microfluidic devices [263], indicating that combining the HA-based materials described herein with a flow-generating system is feasible.

In addition to the RPE, Muller glia would be a compelling cell type to include in the culture system. The native role of Muller glia includes ion buffering and growth factor secretion [252], and their addition to the culture system may rectify culture medium acidity. Furthermore, the Muller glia end processes lie in the interface between the RPE and photoreceptors in vivo, contributing to the formation of the outer limiting membrane. This suggests that Muller glia may hold an active role in mediating the bilateral signaling between RPE and photoreceptors [264].
5.2.2 Employing the mTOR Pathway for In Vivo Transplants

In the work described in chapter 2 of this thesis, we identified an essential role of the mTOR pathway for rod photoreceptor survival. Others have shown that mTOR exerts a similar role in cone photoreceptors, enhancing their survival in mouse models of retinal degeneration [175,265]. This pathway could be exploited to improve the survival of transplanted photoreceptors, which we and others have shown [1,6,81] is severely limited.

Photoreceptors could be isolated from animals with genetic deletions in the phosphatase and tensin homologue (PTEN), or the tuberous sclerosis complex genes (TSC1 and TSC2). These deletions lead to aberrant activation of the mTOR pathway [266]. Since transplanted photoreceptors face a hostile environment characterized by degeneration and pro-apoptotic signals, activating a pro-survival pathway should mitigate the observed cell death. Others have used animals with conditional deletions in PTEN or TSC1/2 and demonstrated that these mutations have no negative effect on photoreceptor function [265], which suggests that the mutant photoreceptors would retain their ability to establish host connections and contribute to visual recovery.

Alternatively, inactivating mutations for PTEN and/or TSC 1/2 could be induced in vitro by using modern genetic tools [267]. This would obviate the need for genetic animal breeding to establish lines combining the Nrl-GFP transgene with the mutant PTEN or TSC 1/2 alleles. Furthermore, this approach could also be employed for stem cell-derived photoreceptors (discussed below).

5.2.3 Elucidating the Mechanism of Visual Recovery by RPE-rod co-transplants

In the work described in chapter 3 of this thesis, we demonstrated that co-transplantation of hES-RPE and NrlGFP+ rods leads to some visual recovery in previously blind NaIO3-treated animals. Deciphering the molecular mechanism mediating that recovery would constitute a significant biological finding.

As a first step, it would be helpful to repeat our study and collect tissue at multiple time points after cell transplantation. Cell survival should be monitored over time, and both photoreceptors
and RPE should be characterized in terms of maturation status. This would provide useful hints towards investigating the mechanism of visual recovery.

As described earlier, we have two hypotheses for the mechanism of recovery: i) increased RPE numbers in the co-transplant group lead to recovery, and ii) donor photoreceptors promote recovery by potentiating host photoreceptor function. To address the first hypothesis, transplants of RPE-alone could be performed, with a higher number of donor RPE injected than the 25,000 RPE used in our experiments, to achieve host RPE numbers similar to the ones achieved by co-transplantation in our experiments. If similar recovery is observed in these studies, that would suggest that increased RPE abundance in the co-transplant group mediates our findings.

Our second hypothesis would be challenging to test experimentally. One way it could be tested is by genetically inhibiting exocytosis in photoreceptor cells before transplantation, hence preventing secretion of factors that may affect host photoreceptors. This could be achieved by photoreceptor-specific knock-outs of vesicle fusion proteins, such as EXO70 [268]. If knocking-out secretion in photoreceptors does not affect their survival, the mutant photoreceptors could be co-transplanted with RPE to assess visual recovery. If visual recovery is achieved by co-transplanting the mutant photoreceptors with RPE, that would signify that factor secretion from donor photoreceptors is dispensable for recovery and in turn suggest that photoreceptors mainly act in our experiments by improving RPE survival. Alternatively, if the specific factors secreted by donor photoreceptors that affect host photoreceptors were known, expression of these factors could be inhibited genetically in donor photoreceptors. For these experiments, it would be important to ascertain that the photoreceptor manipulations do not influence their crosstalk with the RPE.

5.2.4 Using a More Clinically-Relevant Cell Source for Photoreceptors

In our experiments, we employed photoreceptors derived from the Nrl-GFP mouse developing retina. Even though these cells represent an invaluable tool for animal testing of transplantation strategies due to their relatively high survival and integration rates in genetic models [2,81], more clinically relevant cell sources should be employed to promote translation. Furthermore, it
is conceivable that human photoreceptors might interact more efficiently with human RPE, potentially further increasing the observed behavioral effect.

Various protocols could be followed to derive rod photoreceptors from human pluripotent stem cells. Early work demonstrated efficient derivation of retinal progenitors from hES cells, a portion of which would give rise to rods [269,270]. More recent protocols have achieved retinal organoid formation in vitro, containing all the retinal cell types [171,271]. In either case, isolation of photoreceptors would require a transgenic pluripotent cell line expressing a fluorescent reporter under a photoreceptor-specific promoter (i.e. CRX-GFP, IRBP-GFP) or implementation of cell surface markers such as those identified by Ader, Sowden, and colleagues [85,86]. A challenge with using pluripotent stem cell-derived photoreceptors is their ill-characterized integration potential as a function of maturation state. Such work has been performed for mouse developing photoreceptors [80,81] and may need to be repeated with human photoreceptors before an optimal stage can be chosen, in terms of their survival and integration after in vivo transplantation.

van der Kooy and colleagues have extensively demonstrated that the mammalian retina contains a rare, multipotent stem cell population that can be expanded in vitro and give rise to all the retinal cell types [272,273]. Previous research in the van der Kooy and Shoichet groups demonstrated that these cells can differentiate into rod photoreceptors with very high efficiency, and studied their integration into host retinas at different stages of maturation [6,274]. Given the relative ease of isolation of these cells from patient eyes and the potential they provide for autologous therapies, retinal stem cells (RSCs) would constitute an ideal candidate for translation. Differentiation of RSCs into RPE has recently been achieved in the van der Kooy lab (Tahani Baakdah, personal communication). Hence, RSCs constitute a compelling, clinically-relevant cell population from which to derive both RPE and photoreceptors for transplantation.

5.2.5 Using Cone Photoreceptors

A determining characteristic of AMD is that the retinal degeneration occurs in the macula, the cone-dominated area of the retina responsible for high-acuity vision. Due to their rarity (constituting 3-5% of all photoreceptors), cones are less studied than rods. However, the
development of a cellular product for AMD patients would require RPE and cone photoreceptors, since these are the cell types initially lost in the disease.

Recent work has demonstrated high-efficiency cone photoreceptor differentiation from both hES [231] and RSCs (Saeed Khalili, personal communication) by using coco, a protein inhibitor of TGFβ, BMP and Wnt signaling. These stem cell-derived cones would represent excellent cell candidates for both in vitro modeling experiments (such as the ones described in chapter 2) and in vivo transplantation experiments. Furthermore, the Nrl\textsuperscript{-/-} mouse provides a valuable source of cone-like photoreceptors for initial optimization studies [275].

Interestingly, in chapter 3 of this thesis we demonstrate that only scotopic visual recovery was achieved by RPE-rod photoreceptor co-transplantation in NaIO\textsubscript{3}-treated animals. This leaves ample room for visual improvement in photopic conditions. Unfortunately, the initiative towards transplanting cone photoreceptors has only begun recently, and there is a dearth of studies investigating cone survival and integration after transplantation [83,276]. Given this relative lack of knowledge, it is difficult to speculate as to whether donor cones will survive at all in a host environment as hostile as the NaIO\textsubscript{3}-treated mouse retina. Studies should begin with pilot experiments where animals are sacrificed at early time points to assess donor cell survival before proceeding to behavioral testing and long-term survival assays.

Given the translational focus of our laboratory, this is the research direction that I would pursue next.

5.2.6 Using Bioengineering Strategies to Improve Transplanted Cell Survival

As we and others have shown [1,6,81], photoreceptor survival after transplantation is minimal, especially in hostile degenerative environments. Notwithstanding previous work in the Shoichet lab [6] demonstrating that the HAMC hydrogel can significantly improve transplanted photoreceptor survival in the retina, the vast majority of the cells still die. In addition to directly stimulating cell survival, limiting backflow and reducing shear stress during injection, HAMC constitutes a platform that can be further modified to include pro-survival factors. These factors may act either directly on the transplanted cells to reduce cell death, or on the host environment to make it more amenable to regeneration.
Previous work in the Shoichet lab has demonstrated that HAMC can be modified with insulin-like growth factor 1 (IGF-1), which is delivered in a controlled manner by affinity release and promotes hES-RPE survival in vitro, a paper that I co-authored (Appendix A and [213]). IGF-1 has been demonstrated to promote survival in various contexts [277,278]. Interestingly, IGF-1 acts by inducing the mTOR pathway [279] which we have shown to be essential in photoreceptor survival. This would constitute a compelling system for in vivo testing. IGF-1 has been shown to induce VEGF expression, which would be undesirable in the context of AMD [280]. However, co-delivery of this factor with RPE may mitigate its pro-angiogenic effect. Healthy RPE are responsible for controlling the pro/anti-angiogenic balance by secreting PEDF and VEGF in a polarized manner [20]. PEDF is one of the most potent angiogenesis inhibitors found in vivo [281]. Given the tools already available in our lab for controlled delivery of IGF-1 and the fact that this factor has a pro-survival effect on both RPE and photoreceptors, this is the factor I would pursue first for cone-RPE transplants.

Other factors that could be considered are ciliary neurotrophic factor (CNTF) and RdCVF. CNTF has been extensively studied in animal models and in the clinic and has been found to exert a pro-survival effect on photoreceptors, by inducing factor secretion in the Muller glia [135,136,282]. RdCVF is normally secreted by rods and is essential to cone photoreceptor survival; it acts by activating a glucose transporter on cones [283]. This factor would be useful for cone transplants, since the degenerative host environment may provide limited amounts of it. Efforts to develop drug delivery systems based on HAMC modified with either CNTF or RdCVF are currently underway in the Shoichet lab.

An intriguing approach to improving donor cell survival and integration would be to modulate the local immune response. Retinal degeneration is associated with inflammation, macrophage infiltration and complement activation both in humans and animal models [284,285]. Previous research has demonstrated that regeneration in multiple tissues requires a Th2 immune response, which would limit inflammation and instead promote remodeling [286,287]. Interestingly, Deepak Lamba and colleagues [288] recently demonstrated that mesencephalic astrocyte-derived neurotrophic factor (MANF) promotes a Th2 phenotype in retinal immune cells of both mouse and fly. They proceeded to demonstrate that MANF co-delivery with Nrl-GFP⁺ photoreceptors in a mouse model of retinal degeneration enhanced integration and behavioral recovery. Even though the field of neuro-immunology is still nascent, one could draw candidate factors from
basic immunology knowledge. A breadth of cytokines have been demonstrated to modulate immune responses towards a Th2 phenotype, with interleukin-4 (IL-4) being the most well-studied [289]. IL-4, MANF and/or other Th2-modulators could be compared side by side by performing retinal transplants combined with factor delivery. Analysis of transplanted cell survival, integration, and the immune activation of retinal microglia and macrophages will provide input on which factor is the most beneficial for retinal transplants. The selected factor could then be immobilized on HAMC by employing technology currently available in the Shoichet lab to create a delivery vehicle that tailors the host micro-environment to promote regeneration. This would complement the already demonstrated anti-inflammatory properties of HA [290].

Approaches to deliver factors modulating the transplanted or host cells could be combined with activating the mTOR pathway on photoreceptors. This could be achieved by co-delivering siRNA or CRISPR/Cas9 by using material systems or viruses [291,292]. These systems could be employed for photoreceptor transplantation, by combining PTEN or TSC 1/2 -targeting siRNAs/CRISPR-Cas9 with pro-survival or pro-regenerative factors. This would constitute a truly combinatorial approach where both cells, genetic material and factors are delivered in a temporally tunable manner.
### 6 Appendix A: Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2D</td>
<td>2-dimensional</td>
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<tr>
<td>3D</td>
<td>3-dimensional</td>
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<tr>
<td>7-AAD</td>
<td>7-Aminoactinomycin D</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>ABCA4</td>
<td>ATP-binding cassette, sub-family A, member 4</td>
</tr>
<tr>
<td>AMD</td>
<td>Age-related macular degeneration</td>
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<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>cGMP</td>
<td>Cyclin guanosine monophosphate</td>
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<tr>
<td>Cho</td>
<td>Choroid</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
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<tr>
<td>CRALBP</td>
<td>Cellular retinaldehyde binding protein</td>
</tr>
<tr>
<td>CRX</td>
<td>Cone-rod homeobox protein</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>CSPG</td>
<td>Chondroitin sulphate proteoglycan</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EdU</td>
<td>5-ethynyl-2-deoxyuridine</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>ERG</td>
<td>Electroretinography</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
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<tr>
<td>GCL</td>
<td>Ganglion cell layer</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>hES</td>
<td>Human embryonic stem</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin 4</td>
</tr>
<tr>
<td>INL</td>
<td>Inner nuclear layer</td>
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<tr>
<td>iPS</td>
<td>Induced pluripotent stem</td>
</tr>
<tr>
<td>IRBP</td>
<td>Interphotoreceptor retinoid binding protein</td>
</tr>
<tr>
<td>KSR</td>
<td>Knock-out serum replacement</td>
</tr>
<tr>
<td>LCA</td>
<td>Leber’s congenital amaurosis</td>
</tr>
<tr>
<td>MANF</td>
<td>Mesencephalic astrocyte-derived neurotrophic factor</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-activated protein</td>
</tr>
<tr>
<td>MC</td>
<td>Methylcellulose</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MERTK</td>
<td>Tyrosine-protein kinase Mer</td>
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<tr>
<td>MITF</td>
<td>Microphthalmia-associated transcription factor</td>
</tr>
<tr>
<td>MMP2</td>
<td>Matrix metalloproteinase-2</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NF-200</td>
<td>Neurofilament of 200 kDa</td>
</tr>
<tr>
<td>Nrl</td>
<td>Neural retina leucine zipper</td>
</tr>
<tr>
<td>OKT</td>
<td>Optokinetic head tracking</td>
</tr>
<tr>
<td>ONL</td>
<td>Outer nuclear layer</td>
</tr>
<tr>
<td>OS</td>
<td>Outer segments</td>
</tr>
<tr>
<td>OTX2</td>
<td>Orthodenticle homeobox 2</td>
</tr>
<tr>
<td>PEDF</td>
<td>Pigment epithelium-derived factor</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGS</td>
<td>Polyglycerol sebacate</td>
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<tr>
<td>PI3</td>
<td>Phospho-inositol 3</td>
</tr>
<tr>
<td>PKC-α</td>
<td>Protein kinase C alpha</td>
</tr>
<tr>
<td>PLA</td>
<td>Polylactic acid</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly-L-glycolic acid</td>
</tr>
<tr>
<td>PMEL17</td>
<td>Premelanosome protein 17</td>
</tr>
<tr>
<td>PMMA</td>
<td>Polymethyl methacrylate</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PRPH2</td>
<td>Peripherin-2</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RCS</td>
<td>Royal college of surgeons</td>
</tr>
<tr>
<td>RdCVF</td>
<td>Rod-derived cone viability factor</td>
</tr>
<tr>
<td>RdCVFL</td>
<td>Full length isoform of rod-derived cone viability factor</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>RHAMMM</td>
<td>Receptor for hyaluronan-mediated motility</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homolog gene family, member A</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>RP</td>
<td>Retinitis pigmentosa</td>
</tr>
<tr>
<td>RPC</td>
<td>Retinal progenitor cell</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal pigment epithelium</td>
</tr>
<tr>
<td>RSC</td>
<td>Retinal stem cell</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short-interfering RNA</td>
</tr>
<tr>
<td>SPACR</td>
<td>Sialoprotein associated with cones and rods</td>
</tr>
<tr>
<td>SPACRCAN</td>
<td>Sialoprotein associated with cones and rods proteoglycan</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper cell type 2</td>
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<tr>
<td>TSC</td>
<td>Tuberus sclerosis complex</td>
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<tr>
<td>TYR</td>
<td>Tyrosinase</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zonula occludens-1</td>
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</table>
7 Appendix B: A hydrogel for simultaneous tunable growth factor delivery and enhanced viability of encapsulated cells in vitro

This appendix was published in Biomacromolecules.


*: James Parker and Nikolaos Mitrousis contributed equally to this work.

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Abstract

Poor cell survival in vitro and in vivo is one of the key challenges in tissue engineering. Pro-survival therapeutic proteins, such as insulin-like growth factor-1 (IGF-1), can promote cell viability but require controlled delivery systems due to their short half-lives and rapid clearance. Biocompatible materials are commonly used for drug-delivery platforms or to encapsulate cells for increased viability, but few materials have been used for both applications simultaneously. In this work we present a dual-use platform. A blend of hyaluronan (HA) and methylcellulose (MC), known to promote cell survival, was covalently modified with Src homology 3 (SH3)-binding peptides and demonstrated tunable, affinity-based release of the pro-survival fusion protein SH3-IGF-1. The material also significantly increased the viability of retinal pigment epithelium cells (RPE) in anchorage-independent conditions. This novel platform is applicable to a broad range of cells and protein therapeutics and is a promising drug delivery/cell transplantation strategy to increase the viability of both exogenous and endogenous cells in tissue engineering applications.
Introduction

Increasing cell viability both in vitro and in vivo is a major challenge in regenerative medicine. Poor survival rates are characteristic of both endogenous cells damaged by degenerative diseases and the exogenous cells used to replace them. Protein therapeutics are an effective method to improve cell viability, but many proteins suffer from short half-lives in vitro and in vivo, and are cleared quickly from the body, limiting their efficacy [293,294].

Implantable drug delivery systems made from a variety of materials are commonly used to prolong the effects of protein therapeutics [295-297]. This is preferably achieved with materials that provide tunable release of the therapeutic in order to maximize its efficacy and applicability [298]. A common strategy is the use of nano- or micro-particles embedded in a polymer matrix to provide a diffusive barrier that attenuates protein release [299-301]. However, these particle-based systems often suffer from poor protein loading and denaturation during processing [302,303]. Affinity release systems overcome these limitations by avoiding the use of harsh chemicals [298], and instead take advantage of the interaction between the growth factors and matrix to attenuate release, as has been shown with hyaluronan [297,304], heparin-poly(lactic-co-glycolic acid) (PLGA) [305], alginate [306], among others.

Cell transplantation has emerged as a compelling therapeutic approach for neurodegenerative diseases that are currently incurable [101]. One of the most promising targets for such regenerative therapies is the eye, due to its immunoprivileged nature and the clearly defined transplantation site. Retinal pigment epithelium cells (RPE) are found at the posterior of the retina and their death and dysfunction are associated with many degenerative diseases, including age-related macular degeneration (AMD), the most common form of irreversible blindness in the developed world [20,71,307,308]. Transplantation of new RPE cells to reverse AMD is currently being pursued in clinical trials [12]. Despite significant progress in the field, the efficacy of cell transplantation is still hindered by low cell survival, improper distribution and minimal integration into the host tissue [101]. In the vast majority of studies, cell survival after transplantation into the eye is in the range of 1-2% [101]. In addition, many of the surviving cells do not adopt the proper tissue morphology and instead form multicellular aggregates that prevent their integration into the functional circuitry of the eye. We have shown that a hydrogel comprising hyaluronan and methylcellulose (HAMC) can greatly increase the survival and
integration of retinal stem cell-derived rod photoreceptor progenitors into the host eye [6]. RPE cells suffer from poor survival on non-adhesive environments in vitro and in vivo [308-310], such as those found immediately after scaffold-free cell transplantation. Increasing the survival of RPE in both in vitro and in vivo environments is one of the major challenges of current RPE treatments [310].

Materials comparable or identical to those used in these drug delivery systems can also be used to encapsulate cells and improve their viability through cell interactions with covalently attached growth factors or the material itself [311-314]. However, despite the use of similar or identical materials in both drug delivery and pro-survival cell encapsulation platforms, few materials have been designed for simultaneous use in both applications. Such a material could potentially be used to simultaneously improve the effects of protein therapeutics, through sustained delivery, and the viability of encapsulated cells via interactions with the biomaterial and localized drug [315].

HAMC is biocompatible, fast-gelling, injectable [316,317], promotes wound healing and reduces inflammation [318]. It has been shown to increase transplanted cell survival in the retina, brain and spinal cord [6,100,314] and can be modified to allow the sustained delivery of protein therapeutics [298,304]. It is an excellent candidate for use as a dual drug-delivery and pro-survival cell-delivery material for RPE.

The choice of protein therapeutic for delivery is equally as important as that of the cell type. A factor with strong trophic effects on RPE as well as broad applicability to a wide variety of cell types would be ideal. Insulin-like growth factor-1 (IGF-1) is a potent therapeutic, which enhances the viability of many cell types [319-325] and has been shown to improve the survival, proliferation, and migration of RPE in vitro and in vivo [326-331]. There is also strong indirect evidence that IGF-1 reduces RPE apoptosis in anchorage-independent conditions, such as those found in advanced retinal degeneration [332-338]. Tunable release of IGF-1 [339] is required to maximize its efficacy in a broad range of applications, including cartilage growth [319,340,341], cardioprotection [342], and retinal cell proliferation and survival [326,343].

Here we present a HAMC hydrogel (Fig. 1) that has been covalently modified to provide tunable affinity release of IGF-1 while simultaneously increasing the viability of encapsulated RPE in non-adhesive conditions in vitro.
Materials and Methods

Materials

3-maleimidopropionic acid was purchased from TCI America (Portland, USA). Modified pET32b vector was a gift from Dr. Karen Maxwell (University of Toronto). Cloning of the SH3-FGF-2 and IGF-1 vectors was done by GenScript (Piscataway, USA). Sodium hyaluronate (1.4 x 10^6 – 1.8 x 10^6 g/mol) was purchased from Kikkoman Biochemifa (Tokyo, Japan). Methyl cellulose (3 x 10^5 g/mol) was purchased from Shin Etsu (Tokyo, Japan). Sandwich ELISA kit for human IGF-1 was purchased from Assaypro (St. Charles, USA). All buffers were made with distilled and deionized water prepared using a Millipore Milli-RO 10 plus and Milli-Q UF Plus at 18 MΩ resistance (Millipore, Bedford, USA). Artificial cerebrospinal fluid (aCSF) was

Figure 1. Schematic of hydrogel designed for combined tunable, affinity release of protein therapeutics and cell encapsulation.  A) Hyaluronan and methylcellulose hydrogel (HAMC) known to increase cell viability; B) modified HAMC with SH3-binding peptide for the affinity release of SH3-IGF-1 fusion protein; C) modified HAMC hydrogel with encapsulated cells.
prepared as previously described [344]. All other solvents and reagents were purchased from Sigma Aldrich and used as received. Human embryonic stem cells were supplied from Dr. Andras Nagy (University of Toronto).

Methods

2.2.1 Synthesis of SH3-IGF-1 gene

The IGF-1 gene was amplified from the IGF-1 gene (Genscript) by PCR using the forward primer CTGGCGGTGCACCAGATGCGCCGCCCCGAAAC and the reverse primer GTGCGGCCGAAGCTTTTATCAGGCC GATTTGCGCCG. The SH3-linker gene was amplified from the SH3-2F gene using the forward primer TTCCAGGGCCCATGGCCCGGCTGCTACCGCA and the reverse primer TTCCGGCCGCCATCGGTGCACC CCAGAG. Both genes were purified using QiaGen PCR purification kit and combined into the SH3-IGF-1 gene by PCR using the IGF-1 forward primer and the SH3 reverse primer. The SH3-IGF-1 gene and modified pET32b plasmid were digested with NcoI-HF and HindIII-HF, purified with QiaGen PCR purification kit and ligated using Clontech InFusion™ kit. The resulting vector was used to transform chemically competent E. coli BL21.

2.2.2 Expression and Purification of SH3-IGF-1

E. coli BL21 colonies were selected and grown in 20 mL LB with 100 µg/mL ampicillin for 18 h and used to inoculate large cultures in 1.8 L LB flasks containing 100 µg/mL and 10 drops of Anti-Foam 204. The large cultures were grown with air sparging at 37 °C until OD$_{600}$ = 0.8. Expression was induced using a final concentration of 0.8 mM IPTG and maintained for 24 h at 16 °C. The cultures were centrifuged at 7 000 rpm (Beckman Coulter centrifuge Avanti J-6 with rotor JLA-8.1000) for 10 min and resuspended in 20 mL of 6 M guanidine hydrochloride, 0.1 M NaH$_2$PO$_4$, 10 mM Tris, 10 mM imidazole, pH 8.0 (Buffer A). The resuspended pellets were incubated on a rotator at 4 °C for 16 h, centrifuged at 45 000 g (Beckman Coulter centrifuge Avanti J-26 with rotor JA-25.50) for 15 min and the supernatant collected. The supernatant was incubated with 2 mL of Ni-NTA agarose for 25 min washed 5 times with 20 mL of Buffer A. The protein was eluted by adding 50 mL of 6 M guanidine hydrochloride containing 0.2 M acetic acid.
Purified SH3-IGF-1 was refolded by dialyzing (8 kDa MWCO) in 50 mM Tris, 125 mM Arginine, 5 mM Cysteine for 36 h at 4 °C. The protein was then dialyzed against 50 mM Tris, 0.5 mM EDTA, pH 8.0 (tobacco etch virus protease (TEV) cleavage buffer) for 36 h and concentrated to 0.2-0.3 mg/mL. 3 mM glutathione (reduced), 0.3 mM glutathione (oxidized) and TEV at 6:100 TEV:SH3-IGF-1 ratio were added and the mixture was incubated for 24 h at room temperature to cleave the thioredoxin (Trx) domain. The resulting mixture was incubated for 30 min with 2 mL of Ni-NTA and filtered through a gravity filtration column. The eluent was collected, passed through again and collected as pure SH3-IGF-1. Pure SH3-IGF-1 was dialyzed (3.5 kDa MWCO) against 50 mM Tris, 100 mM NaCl, pH 8.0 buffer for 48 h, sterile-filtered and stored at -80 °C for future use.

2.2.3 SH3-IGF-1 Bioactivity

2 x 10^6 MCF-7 cells were seeded on a T25 flask and grown for 24 h in DMEM/F-12 with 10% FBS, 1% Penicillin/Streptomycin (P/S) and 1% insulin. Cells were trypsinized and 2 x 10^4 cells/mL (200 μL) were added per well in a 96-well plate and allowed to attach at 37 °C for 24 h. The cells were serum-starved for 24 h, after which the media was replaced with either serum-free media, serum-free media with 50 ng/mL commercial IGF-1 or serum-free media with 50 ng/ml recombinant SH3-IGF-1. The cells were cultured for 48 h and proliferation determined by CellTiter 96 AQeous One Solution Cell Proliferation (MTS) assay (Promega).

2.2.4 Preparation of MC modified with SH3-binding peptide

MC was covalently modified with SH3-binding peptide (either weak-binding peptide (WBP) GGGKPPVVKKPHYLS, K_D = 2.7 x 10^{-5} M or strong-binding peptide (SBP) GGGKKTKPTPPPPKSHLKPK, K_D = 2.7 x 10^{-7} M) as described previously [298]. Briefly, carboxylated MC was prepared using a Williamson ether synthesis and converted to thiolated MC (MC-SH) by coupling with 3,3’-dithiobis(propionic dihydrazide) (DTP) via 1-ethyl-3-[3(dimethlamino)propyl]-carbodiimide (EDC) and reducing with dithiothreitol (DTT). Michael addition was then used to couple the MC-SH to maleimide-WBP or maleimide-SBP. A schematic of the reactions follows.
2.2.5 In vitro SH3-IGF-1 Release from HAMC, HAMC-SBP and HAMC-WBP

HAMC and HAMC-peptide hydrogels were prepared by adding 310 μg/mL SH3-IGF-1 (46.8 μL, 890 pmol) and sterile-filtered artificial cerebral spinal fluid (aCSF, 46.8 μL) to: i) 1.4 mg of HA, 5 mg of MC (HAMC gel); ii) 1.4 mg of HA, 2.91 mg of MC and 2.09 mg MC-WBP (115
nmol WBP/mg MC, WBP:SH3-IGF-1 = 270:1, HAMC-WBP gel); or iii) 1.4 mg of HA, 4.49 mg of MC and 0.51 mg of MC-SBP (88 nmol SBP/mg MC, SBP:SH3-IGF-1 = 50:1, HAMC-SBP gel) in sterile, 2 mL Eppendorf tubes. The samples were dispersed using a planetary mixer, centrifuged at 16,162 g (Sigma 1-14 microcentrifuge) to remove air bubbles and dissolved overnight at 4 °C. Samples were gelled by incubation on an orbital shaker at 37 °C for 7 min, and 900 μL of sterile, 37 °C aCSF was added to simulate in vivo dilution conditions. At the specified time points (1 h, 3 h, 5 h, 8 h, 24 h, 48 h, 96 h, 168 h, 240 h), the aCSF was removed and replaced. Aliquots were frozen at -20 °C until used in a sandwich ELISA (Assaypro, Human IGF-1 ELISA Kit) to determine SH3-IGF-1 concentration. The standard curve in the ELISA was prepared using serial dilutions of the expressed SH3-IGF1.

2.2.6 Gel Degradation Study

HAMC hydrogels were prepared by adding sterile-filtered aCSF (115 μL) to: i) 1.72 mg of HA, 6.14 mg of MC (HAMC gel); ii) 1.72 mg of HA, 3.57 mg of MC and 2.57 mg MC-WBP (115 nmol WBP/mg MC, HAMC-WBP gel); or iii) 1.72 mg of HA, 5.51 mg of MC and 0.63 mg of MC-SBP (88 nmol SBP/mg MC, HAMC-SBP gel) in sterile, 2 mL Eppendorf tubes. The Eppendorf tubes were weighed individually before the beginning of the experiment. The samples were dispersed using a planetary mixer, centrifuged at 16,162 g (Sigma 1-14 microcentrifuge) to remove air bubbles and dissolved overnight at 4 °C. Samples were gelled by incubation on an orbital shaker at 37 °C for 7 min, and 1105 μL of sterile, 37 °C aCSF was added to simulate in vivo dilution conditions. The aCSF was removed immediately and the gel-containing tubes weighed, which constituted the T0 weight. The aCSF was then replenished, and the samples incubated at 37 °C on an orbital shaker. At the specified time points (1 h, 3 h, 5 h, 8 h, 24 h, 48 h, 96 h, 168 h, 240 h, 336 h), the aCSF was removed, the samples were weighed and the aCSF was replaced.

2.2.7 Effects of HAMC-SBP and HAMC-WBP with SH3-IGF-1 on RPE survival and proliferation in vitro

96-well plates were coated with poly(2-hydroxyethylmethacrylate) (poly(HEMA)) as described by Phung et al. [345], 60 μL of poly(HEMA), in 95% ethanol (5 mg/mL) was added to each well of a 96 well plate under sterile conditions. The plates were incubated at room temperature for 96
h with the lids on, 1 h with the lids off, sealed with parafilm and stored at 4 °C for 24 h before using.

RPE were derived from human embryonic stem cells as has been shown previously [346] CA1 human embryonic stem cells were cultured on Geltrex™ (Life Technologies) in mTESR medium (Stem Cell Technologies). For differentiation, cells were grown to confluence (12-14 days post plating) and the medium was then changed to DMEM/F-12 containing 13% knock-out serum replacement, 1% non-essential amino acids, 1% Glutamax, 1% Penicillin-Streptomycin and 0.1% b-mercaptoethanol (all from Life Technologies). The cells were fed every other day and pigmented clusters started appearing 3-4 weeks after the beginning of differentiation. The clusters were allowed to enlarge for another 4 weeks (in total 7-8 weeks of differentiation) and were then manually picked using a scalpel under a dissection microscope. The picked colonies were dissociated with 0.25% trypsin and plated on Geltrex™ in full media, consisting of DMEM/F-12 containing 7% knock-out serum replacement, 5% Hyclone FBS (Thermo Scientific), 1% non-essential amino acids, 1% Glutamax, 1% Penicillin-Streptomycin, 0.1% b-mercaptoethanol and 10 ng/ml basic Fibroblast Growth Factor (R&D Systems). After allowing the picked cells to grow for 2 weeks, the centers of the colonies, which exhibited uniform cobblestone morphology were re-picked using the same method. These double-picked cells formed monolayers with cobblestone morphology and uniformly expressed RPE markers at the protein and RNA levels. For immunostaining, the following antibodies were used: anti-RPE65 (Novus Biologicals, 1:200), anti-Bestrophin (Novus Biologicals, 1:200) and anti-ZO-1 (Life Technologies, 1:100).

HAMC gels (0.9% HA/0.9% MC (w/v)) were made in 1.25 mL bulk gels of HAMC alone (11.25 mg HA, 11.25 mg MC), HAMC-WBP (11.25 mg HA, 8.25 mg MC, 3 mg MC-WBP) or MC-SBP (11.25 mg HA, 10.13 mg MC, 1.12 mg MC-SBP) by adding 614 μL of serum-free DMEM/F-12 and 614 μL of 50 mM Tris, 100 mM NaCl, pH 8 (HAMC gels) or 614 μL of 53.4 μg/mL SH3-IGF-1 in 50 mM Tris, 100 mM NaCl, pH 8 (HAMC-WBP and HAMC-SBP gels). The samples were dispersed using a planetary mixer, centrifuged at 16,162 g (Sigma 1-14 microcentrifuge) to remove air bubbles and dissolved overnight at 4 °C. RPE cells (1.6 x 10⁵ cells/mL) were diluted 1:6 in serum-Free DMEM/F-12, DMEM/F-12 + 10% FBS or the appropriate 0.9% HA/0.9% (w/v) HAMC gel (final composition 0.75% HA/0.75% MC w/v). The cells in were plated on the poly(HEMA)-coated plates at 75 μL per well and incubated for
10 min at 37 °C to allow gels to form. 125 μL of serum-free media was then added (final cell concentration of 1 x 10⁴ cells/mL). Prestoblupe fluorescence was used to measure RPE viability at 0 h, 48 h and 96 h.

2.2.8 Statistical Analysis

Statistical analysis was done in Graphpad Prism version 5. Unpaired student’s t-test was used to compare 2 groups, and one-way ANOVA with Tukey’s posthoc to compare multiple groups. Graphs are annotated with p values represented as *p≤0.05, **p≤0.01 and ***p≤0.001.

Results and Discussion

The SH3-linker gene (228 base pairs (bp)) and IGF-1 gene (216 bp) were PCR-amplified from their source vectors and ligated together to create the SH3-IGF-1 gene (444 bp) as detected by agarose gel electrophoresis (Supplementary Figure 1). The gene was successfully ligated into the pET32b plasmid, which appends a thioredoxin (Trx) sequence to the N-terminus to aid with protein refolding and solubilisation, and transformed into E. coli BL21. Trx-SH3-IGF-1 was expressed at high levels in BL21 E. coli and successfully purified (Supplementary Figure 1B), after which the Trx domain was cleaved by TEV protease and removed using Ni-NTA chromatography (Supplementary Fig. 1C). The mass of the final product was confirmed by mass spectrometry (Supplementary Fig. 2).

To verify the bioactivity of our SH3-IGF-1, we compared it to commercially available IGF-1 in terms of MCF-7 cell proliferation [347,348]. SH3-IGF-1 was found to stimulate MCF-7 proliferation to the same degree as commercially available IGF-1 as determined by the MTS metabolic assay after 48 h (Fig. 3). There was a significant difference (p < 0.05) in the number of live cells between serum-free conditions and both commercially available IGF-1 and SH3-IGF-1. There was no significant difference (p > 0.05) in the formazan absorbance signal between commercial IGF-1 and SH3-IGF-1.
Figure 3. SH3-IGF-1 has equivalent bioactivity to commercially available IGF-1. MCF-7 cells were cultured on 96 well plates in DMEM/F-12 with 10% FBS and 1% P/S for 24 h, then serum-starved for 24 h. The cells were incubated in serum-free media containing SH3-IGF-1 (50 ng/mL) or commercial IGF-1 (50 ng/mL) for 48 h. Proliferation was measured using an MTS colourimetric assay. Both SH3-IGF-1 and commercial IGF-1 caused significantly different proliferation from the control, but were not significantly different from each other (n = 3, mean ± standard deviation plotted, *** indicates p < 0.001, ANOVA, Tukey’s post hoc).

In order to investigate affinity release of SH3-IGF-1, maleimide-conjugated SH3-binding peptides were successfully bound to thiolated MC via Michael addition. Two binding peptides were immobilized: either weak-binding peptide (WBP) GGGKPPVKKPHLYS, \( K_D = 2.7 \times 10^{-5} \) M, or strong-binding peptide (SBP) GGGKKTKTPPPKPSHLKPK, \( K_D = 2.7 \times 10^{-7} \) M. By amino acid analysis, substitution was determined to be on average 130 nmol for WBP/mg MC and 89 nmol for SBP/mg MC with no detectable protein adsorption on dialysis controls of maleimide-peptide with non-thiolated MC.

The release of SH3-IGF-1 from HAMC, HAMC-WBP or HAMC-SBP was compared. After 24 h, approximately 67% of SH3-IGF-1 was released from HAMC, 40% from HAMC-WBP and
19% from HAMC-SBP (Fig. 4A). HAMC and HAMC-WBP released SH3-IGF-1 for 4 days and 10 days, respectively, while HAMC-SBP gels appeared to stop releasing SH3-IGF-1 after 24 h, with only nanogram quantities of SH3-IGF-1 (0.03% of total) released over subsequent timepoints.

To ensure that the release profile was indeed due to the reversible binding of SH3 with its weak and strong binding partners and not due to changes in degradation profiles, the mass of the gels was measured over the same timeframe as that followed for release. We observed no significant change in the degradation profiles of HAMC vs. HAMC-WBP and HAMC-SBP (Fig 4B), thereby confirming the reversible binding with SH3 and its binding partners as the mechanism for the different release profiles.
Figure 4: (A) In vitro cumulative release profile of SH3-IGF-1 delivered from HAMC, HAMC-weak binding peptide hydrogels (HAMC-WBP, 270:1 molar ratio of WBP:SH3-IGF-1) and HAMC-strong binding peptide (HAMC-SBP, 50:1 molar ratio of SBP:SH3-IGF-1). SH3-binding peptides attenuate release such that different release profiles are achieved (n = 3, mean ± standard deviation plotted). (B) To ensure that the release profile is unaffected by degradation, the mass loss of HAMC vs. HAMC-WBP and HAMC-SBP were compared: conjugation of these SH3-binding peptides to MC did not alter the
degradation profile of the HAMC gels (n=3, mean ± standard deviation plotted, p>0.05, ANOVA, Tukey’s post-hoc).

The rates of SH3-IGF-1 release from HAMC, HAMC-WBP and HAMC-SBP were significantly different. Release rates were compared using a plot of cumulative fractional protein release against the square root of time (Fig. 5). This was done by assuming unidirectional diffusion from a plane sheet as has been previously done for this affinity system [298,304], using the equation

\[
\frac{M_t}{M_\infty} = k t^{1/2}
\]

where \(M_t\) is the mass of drug released at time \(t\), \(M_\infty\) is the mass of drug released as time approaches infinity and \(k\) is the diffusion constant and slope of the line in the graph. The linear fit of the data is indicative of Fickian diffusion (Fig. 5A), and the slopes of the curves are proportional to the protein diffusivity within the gel [349]. All \(k\) values were significantly different between HAMC (\(k = 2.59 \times 10^{-3}\)), HAMC-WBP (\(k = 1.35 \times 10^{-3}\)), and HAMC-SBP (\(k = 7.92 \times 10^{-3}\)) (p < 0.001 between HAMC and HAMC-WBP and HAMC-SBP, p < 0.001 between HAMC-WBP and HAMC-SBP) (Figure 5B). HAMC and HAMC-SBP showed Fickian diffusion release (applicable for the first 60% of protein release) for the first 8 h, while HAMC-WBP showed extended Fickian diffusion to 48 h. (Please note that these values are expressed as square root of time in seconds in Figure 5.)
Figure 5. Cumulative release of SH3-IGF-1 from HAMC, HAMC-WBP (270:1 molar ratio WBP:SH3-IGF-1 and HAMC-SBP (50:1 molar ratio SBP:SH3-IGF-1) against the square root of time. (A) The slope of SH3-IGF-1 release from HAMC, HAMC-WBP and HAMC-SBP hydrogels against the square root of time. The slope is representative of Fickian diffusion coefficient, k, for each gel. (B) Graphical representation of the slopes of SH3-IGF-1 release from each hydrogel and with significance indicated (** indicates p < 0.01, *** indicates p < 0.001, by ANOVA, Tukey’s post hoc; n = 3, mean ± standard deviation plotted).

Interestingly, HAMC-WBP gels were able to release up to 25% more IGF-1 than a similar, non-tunable IGF-1 affinity release system over a similar time-scale [341]. Notably, comparable amounts of total protein were released from the HAMC and HAMC-WBP systems, an effect which is not commonly seen in affinity release systems as slower-releasing platforms often release lower amounts of detectable protein [304,341,350,351].

The plateau in release from HAMC-SBP gels had not been previously observed at this early timepoint with this system [298,304]. It may be a result of aggregation and possible denaturation from high local protein concentration, as well as an artifact of freeze-thawing of samples rendering some fraction of the release samples undetectable by ELISA. The apparent reduction in released quantities of proteins from this affinity system due to sample freezing and thawing has been observed before and is much more significant in release samples with low protein concentration, such as those from HAMC-SBP after 24 h [298,352]. This phenomenon can potentially disguise a slow release rate, giving the appearance of a plateau.

RPE, derived from human embryonic stem (hES) cells, were pigmented and exhibited typical morphology and marker expression (Fig. 6A), in agreement with what has been reported by others [353]. RPE viability in HAMC gels was then tested on non-adhesive poly(HEMA)-coated plates. RPE encapsulated in HAMC-SBP/SH3-IGF-1 showed increased viability at day 4 in comparison to RPE in HAMC alone (p < 0.01) or serum free media (SFM) (p < 0.001, Fig. 6B). Interestingly, the addition of soluble SH3-IGF-1 to SFM led to increased RPE viability on days 2 and 4 (p<0.001) compared to SFM alone (Fig. 6C). Cells in all conditions either maintained or experienced decreased viability after day 4, possibly due to depleted nutrients in the same SFM. There were no significant differences in viability between RPE in HAMC-SBP and HAMC-
WBP with or without SH3-IGF-1 at any timepoint (p>0.05, Fig. 6A, Fig. 6B). In addition, RPE in HAMC exhibited similar survival to RPE in HAMC-WBP or HAMC-SBP in the absence of SH3-IGF-1 (p>0.05, Fig. 6B).

Despite, or possibly because of, the plateau phenomenon in HAMC-SBP gels, these hydrogels demonstrated the most potent effects on RPE viability, with 2-fold and 4-fold greater cell viability than cells in HAMC alone or SFM alone, respectively, based on the Prestoblue signal. When SFM was supplemented with SH3-IGF1, the 4-fold increase in RPE survival was observed on day 2; earlier than in the HAMC-SBP/SH3-IGF-1 hydrogels, and maintained until day 4.

This could indicate increased availability of IGF-1 in SFM in the earlier time points compared to HAMC-SBP, potentially because of the delayed release of IGF-1 in the gels. RPE viability in the HAMC-WBP/SH3-IGF-1 gels was significantly (3-fold) greater than SFM alone, however it was not statistically different from HAMC alone. This may be attributed to the quicker release of SH3-IGF-1 from HAMC-WBP, from the gels into the media, where it becomes unavailable to the cells. Thus, the tunable, controlled-release IGF-1 hydrogel is coupled with RPE cell viability in anchorage-independent conditions.

To confirm that the effects of RPE proliferation can indeed be attributed to SH3-IGF-1, we ran a series of controls (Fig 6C), where only SFM supplemented with SH3-IGF-1 had a 4-fold increase in RPE survival on days 2 and 4. None of the other vehicle controls, where SH3-IGF-1 was absent, showed any cell proliferation. This series of controls was important as HAMC itself can promote cell survival and proliferation [354,355]. The CD44 receptor, which binds to HA and promotes cell survival [356], is upregulated in proliferating RPE [357]. The proliferative effects of IGF-1 on RPE may increase the pro-survival and proliferative effects of CD44 on RPE, increasing the beneficial effects of the material. However, since we did not observe a significant difference in survival between RPE in HAMC alone and SFM conditions, we attribute the difference in number of viable cells to IGF-1. We acknowledge that our hES-derived RPE are more representative of fetal human RPE rather than primary adult human RPE. The latter may respond differently to our gels and/or IGF-1.
Figure 6. (A) RPE derived from hES cells exhibited typical RPE morphology, were pigmented, and uniformly expressed the tight junction marker ZO-1 and the RPE markers Bestrophin-1 and RPE65. The scale bar is 30 um. (B) HAMC-SBP with SH3-IGF-1 significantly increased the proliferation of RPE in vitro at day 4 relative to HAMC (p<0.01) and SFM (no HAMC, p<0.001). RPE were suspended in serum-free DMEM/F-12 or encapsulated in (0.75%/0.75% (w/v)) HAMC, HAMC-WBP with 10 ug/ml SH3-IGF-1 (270:1 molar ratio WBP:SH3-IGF-1) or HAMC-SBP with 10 ug/ml SH3-IGF-1 (50:1 molar ratio SBP:SH3-IGF-1) hydrogels. (C) SH3-IGF-1 alone significantly increased the proliferation of RPE in vitro. RPE were suspended in serum-free DMEM/F-12 with or without 10 ug/ml SH3-IGF-1 or encapsulated in HAMC, HAMC-WBP or HAMC-SBP.
The cells were plated on poly(HEMA)-coated non-adhesive 96-well plates and incubated for 0, 2, and 4 days. Cell viability was measured by the Prestoblu metabolic assay. RPE in serum free media with SH3-IGF-1 showed significantly increased viability compared to RPE in serum free media alone (p<0.001) at day 2 and day 4 (n = 6 for HAMC and No HAMC conditions, n=3 for all other conditions, mean + standard deviation plotted; ** indicates p < 0.01, *** indicates p < 0.001, one-way ANOVA, Tukey’s post hoc).

Conclusions

HAMC hydrogels modified with SH3-binding peptides were able to attenuate the release of SH3-IGF-1 fusion protein over several days in comparison to HAMC gels alone. The rate of release of SH3-IGF-1 is slower from HAMC-SBP gels than HAMC-WBP gels, thereby reflecting the difference in equilibrium binding between the strong and weak binding peptides and SH3. This system was able to release a greater proportion of IGF-1 than other IGF-1 affinity systems on a similar timescale. Encapsulation of RPE within the HAMC-IGF-1 release system was shown to increase cell viability in anchorage-independent conditions in vitro in comparison to serum-free media or HAMC alone. This work shows that tunable protein delivery improves cell viability and that IGF-1 promotes RPE cell viability in anchorage-independent conditions. This new type of multi-functional material is efficacious in vitro to simultaneously extend the benefit of protein therapeutics on cells through sustained delivery, while increasing the viability of encapsulated cells via interactions with the biomaterial and localized biomolecule. This system lays the foundation for future in vivo studies where we anticipate that this combined cell and biomolecule delivery system will enhance cell survival after transplantation, thereby overcoming one of the key barriers to success.
Supplementary Figures

Figure S1. SH3-IGF-1 gene synthesis, expression and purification. (A) Agarose gel showing ligation of SH3-linker gene with IGF-1 gene: Lane 1: SH3-linker gene; Lane 2: IGF-1 gene; Lane 3: SH3-linker-IGF-1 fusion gene. (B) Agarose gel showing purified Trx-SH3-IGF-1. (C) Agarose gel showing purified SH3-IGF-1 after Trx cleavage: Lane 1: Trx protein (17,313 g/mol); Lane 2: purified SH3-IGF-1 (16,318 g/mol).
Figure S2. Purified SH3-IGF-1 mass spectrometry trace shows 16,317.5 g/mol which matches the theoretical mass = 16,318 g/mol.

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Appendix C: Post-translational modifications of histones in vertebrate neurogenesis

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Abstract

The process of neurogenesis, through which the entire nervous system of an organism is formed, has attracted immense scientific attention for decades. How can a single neural stem cell give rise to astrocytes, oligodendrocytes and neurons? Furthermore, how is a neuron led to choose between the hundreds of different neuronal subtypes that the vertebrate CNS contains? Traditionally, niche signals and transcription factors have been on the spotlight. Recent research is increasingly demonstrating that the answer may partially lie in epigenetic regulation of gene expression. In this article, we comprehensively review the role of posttranslational histone modifications in neurogenesis in both the embryonic and adult CNS.

Introduction

Histone post-translational modifications (PTMs) have been implicated in a multitude of developmental processes and diseases [358-361]. Throughout the last decade, interest into the role of histone PTMs in neurogenesis has risen. The goal of this review is to provide an overview of the current evidence for histone PTM involvement in neurogenesis, the known mechanisms that initiate these processes, as well as investigate the contribution of histone PTMs to the complex and interconnected network of epigenetic modifications. We begin with a brief introduction into neurogenesis and histone PTMs, then move on to discuss the major histone PTMs (i.e., acetylation and methylation) that have been investigated in the context of neurogenesis, highlighting a few studies that exemplify the main conceptual insight. Lastly, we discuss how these processes are initiated and how specificity is achieved.
1.1 Neurogenesis

Neurogenesis in vertebrates begins after ectodermal cells acquire a neuroepithelial identity through the process of induction and subsequent morphological transformation to become radial “glial” cells with stem cell properties [362,363]. Neuroepithelial cells, followed by radial glial cells, undergo a period of population expansion before radial glial cells differentiate into committed transit amplifying progenitors (Figure 1) that eventually give rise to mature cells of the central nervous system (CNS). Through a cell intrinsic clock, these neural stem cells initially differentiate into neurons and switch their competence toward generating astrocytes [364], as depicted in Figure 1. Oligodendrocytes are the last fate to arise and are typically generated during postembryonic stages. Interestingly, isolated neural stem cells from the embryonic cerebral cortex follow the same order of differentiation in vitro, initially giving rise to neurons and then astrocytes [365]. The mechanism by which this intrinsic clock is established, which is common among most developing neural tissues, has been a long lasting question in developmental neurobiology.

Neurogenesis persists in adulthood with notable species variation, but is typically limited to specific neurogenic regions that can be associated with functional plasticity and regeneration [366-368]. Although adult neural stem cells share most characteristics with their embryonic counterparts, such as marker expression and multipotentiality in vitro [369,370], they are typically restricted under physiological conditions producing primarily neurons in vivo [371-373]. Thus, while the process of neurogenesis is fundamentally conserved among brain regions, ontogeny and putatively throughout vertebrate evolution, neurogenesis is nonetheless shaped by significant adaptations to distinct physiological states and life histories [374].

Factors that influence the extent of neurogenesis include proliferation and survival of neural stem and progenitor cells (NSPCs), their efficiency of differentiation into neurons and glial cells, and the survival and function of the differentiated progeny. The term NSPCs is used when neural stem cell identity cannot be readily distinguished from that of a more committed progenitor cell identity.

1.2 Histone post-translational modifications
The nucleosome, a fundamental unit of chromatin, consists of 146-147 bp of DNA that is wrapped around 1 histone octamer, which includes 2 molecules of each of the core histones H2A, H2B, H3 and H4 [375]. In the last two decades it has become increasingly evident that nucleosomes have a broader role than just facilitating the packaging of DNA into the tiny space that is a cell nucleus. Histones participate in the regulation of gene expression and are the target of a plethora of transcription factors or associated proteins. The histone N-termini that protrude from the tightly packed octamer, are somewhat less structured [375,376]. They are free to interact with DNA, and are also exposed to modification enzymes. These enzymes modify histone N-termini by, for instance acetylation, methylation, phosphorylation, SUMOylation, ubiquitination, citrullination, or ribosylation [377]. Each of these modifications has an effect on gene expression through 2 potential mechanisms. The first one is based on electrostatic interactions [378]. Specifically, DNA is negatively charged and histone N-termini are positively charged. Depending on the modification, the positive charge of the N-terminus may be concealed or exposed. For example, acetylation of a lysine residue will mask its positive charge and prevent strong attraction to the DNA. This will lead to a more relaxed chromatin state, and when this occurs in a promoter, transcription factors have more room to bind DNA and exert their functions. The converse would happen in the case of histone deacetylation at a lysine residue. The second major mechanism of action of chromatin modifications is through creating binding sites for transcription factors and adaptor proteins that recognize specifically modified histone residues [376,378].

While histone PTM is a major contributor to epigenetic regulation of gene expression, it is important to keep in mind that it represents only one aspect of an ever-expanding network of epigenetic regulators [360]. Epigenetics can be loosely defined as changes in gene expression, or the phenotype, that are not induced by changes in the DNA sequence [379]. In addition to histone PTMs, a long list of regulatory mechanisms of gene expression fit under this term including DNA methylation, microRNAs, long non-coding RNAs and methyl-DNA binding proteins. In reality, the various epigenetic mechanisms co-operate and form complexes or groups of enzymes in which there is a cascade of signals linking an extracellular trigger to a gene expression event [380].
Figure 1. Embryonic neuroepithelial (NE) cells of the neural tube give rise to radial glial cells, which through an intrinsic clock differentiate first into neurons and then into astrocytes. The neurogenesis “window” extends approximately from E11 to E18. nIPC, neurogenic intermediate progenitor cell; oIPC, oligodendrogenic intermediate progenitor cell. Note that no oligodendrocytes are generated before birth. The figure represents embryonic neurogenesis in the mouse.

2. Histone PTMs in Neurogenesis

2.1 Histone acetylation

Histone acetylation was the first histone PTM to be discovered as well as associated with gene expression [381,382]. Histones are acetylated at lysine residues by histone acetyl-transferases (HATs) and are deacetylated by histone deacetylases (HDACs) [376]. The involvement of histone acetylation in neurogenesis is widespread and ranges from embryonic neurogenesis, to adult neuronal survival and differentiation. Below we describe, using selected examples, how histone acetylation could influence the process of neurogenesis.
One of the initial culprits to be identified was c-AMP responsive element binding protein (CREB) [383-385]. CREB is a generic transcriptional activator that is involved in a plethora of developmental processes as well as cancer, and it functions by recruiting CREB-binding protein (CBP or CREBBP), which possesses HAT activity [386]. CREB function has been implicated in neuronal plasticity, hippocampal learning and memory [387], partially by regulating the secretion of the growth factor BDNF [388]. Various reports have also shown that CREB is involved in both embryonic and adult neurogenesis [389,390]. CREB knockout mice [391] analyzed at E14.5 display severe deformities in both their embryonic brain and retina, sometimes completely lacking an olfactory bulb. Additionally, embryonic NSPCs from CREB−/− mice show decreased survival in vitro, but normal differentiation [391]. Investigating the mechanism, the authors identified that the transcripts for the anti-apoptotic protein bcl-2 and the growth factors BDNF, NGF and PACAP were decreased in CREB−/− NSPC cultures. Interestingly, analyzing CREB−/− mice at E18.5, Rudolph et al. [392] reported only subtle morphological alterations of the brain structure. This indicates that CREB loss of function could delay the neurogenesis “window” so that obvious deformities exist at early stages but might be compensated for at later stages in development. An alternative explanation for these seemingly conflicting results could be based on the cAMP response element regulatory protein (CREM). CREM belongs to the CREB family of transcription factors and is known to compensate for the lack of CREB [393,394]. In the report by Rudolph et al., there was a strong upregulation of CREM upon CREB knockout at E18.5, which was not observed by Dworkin et al. at E14.5. This, along with observations in very early CREB−/− embryos [395] may suggest that the complementation of CREB function by CREM may only occur at time points later than E14.5. In order to overcome this functional redundancy issue, Mandamadiotis et al. [393] generated conditional CREB knockout mice on a CREM−/− background. When the deletion was driven by Nestin-Cre, therefore occurring in NSPCs, there was a decrease in brain size with differences becoming obvious from E16.5 onwards, which coincided with the onset of strong CREB deletion. The cortex and the hippocampus were the most strongly affected areas, and mice died perinatally. The phenotype was attributed to increased apoptosis in the affected areas. Interestingly, when CREB deletion was driven by the promoter of calcium/calmodulin-dependent protein kinase II-α (CamkIilα) gene to drive expression in mature neurons postnatally, a progressive neuronal degeneration over
the course of 6 months was observed. The areas mostly affected were the hippocampus and the striatum and the animals exhibited a behavioral phenotype reminiscent of models of neurodegenerative disease [396-400]. Therefore, these studies demonstrated that histone acetylation triggered by CREB is necessary for neurogenesis both at the NSPC and the mature neuron level.

Notably, histone acetylation has also been implicated in neurodevelopmental disorders. Rubinstein-Taybi syndrome is characterized by cognitive dysfunction, and caused by a haploinsufficiency in the cebp (encoding for CBP) gene [401]. Cbp<sup>−/+</sup> mice show cognitive impairment in adulthood, which gave rise to the hypothesis that the effect is due to defective neural circuits [402]. Wang et al. [403] demonstrated that cebp<sup>−/+</sup> mice also show deficits in embryonic neurogenesis and gliogenesis. Cbp<sup>−/+</sup> pups displayed an altered frequency, duration and number of ultra-sonic vocalizations (USV) after separation from their mothers. USV changes are believed to reflect cognitive and social behavior impairments [404]. Embryonic neural precursors differentiated into all three lineages (neurons, astrocytes, oligodendrocytes) with less efficiency both in vitro and in vivo, when CBP was knocked down. The HAT activity of CBP was necessary for that effect, since an HDAC inhibitor could rescue the effects of the CBP knockdown. CBP was shown to directly bind and acetylate the H3K9/14 at promoters of genes involved in neural (βIII-tubulin), astrocytic (GFAP, S100β) and oligodendrocytic (MBP, PLP2) development, thus up-regulating their expression. Moreover, the accessibility of HDACs and CBP to gene promoters involved in NSPC specification in the zebrafish hindbrain is regulated by the interaction of Meis and Pbx transcription factors [405]. This indicates that transcription factors can act as gatekeepers, or conversely molecular beacons, for HDAC/HAT recruitment in a region specific manner. Lastly, the phosphorylation of CBP by atypical protein kinase C ζ (aPKCζ) was shown to be necessary for promoting the differentiation of neural precursors, and has been linked to spatial memory formation [403,406]. The latter provides a functional link between CBP function and extracellular signals that are known to be involved in neurogenesis, such as fibroblast growth factors (FGFs) and neurotrophins [407,408], and may pave the way to new approaches for treatment of various neurological disorders.

2.1.2 Histone acetylation and neuronal differentiation: the case of orexin neurons
Histone acetylation appears to also be involved in the differentiation of specific neuronal subtypes. Hayakawa et al. [409] showed that the differentiation of orexin neurons from mouse embryonic stem (ES) cells depends on DNA methylation and histone acetylation. Orexin neurons are localized in the hypothalamus and are involved in the sleep/wake cycles as well as feeding behavior. Orexin neuron differentiation requires the expression of hypocretin (orexin) neuropeptide precursor (Hcrt), which contains two proximal regulatory regions that are CpG island-rich. The authors demonstrated that mES cells, as well as neurons derived from mES cells under standard differentiation conditions had high DNA methylation at these regions, as well as low histone acetylation at various lysine residues, which translates to low gene expression. On the contrary, treatment with N-acetyl-D-mannosamine (ManNAc) induced an increase in histone acetylation at all of the assessed sites. The authors proceeded to pinpoint that Sirt1, a class III HDAC, inhibited orexin neuron differentiation. They finally demonstrated that meningioma-expressed antigen 5 (Mgea5) was necessary for the effect of ManNAc into orexin neuron differentiation. Mgea5 has a dual enzymatic activity: it is a HAT and also serves as an O-GlcNAcase, which removes O-linked N-acetyl glycosamine (O-GlcNAc) residues from proteins including histones. Interestingly, both enzymatic activities were required for orexin neuron differentiation. This observation led to a model (Figure 2), whereby in non-orexin neurons, the Hcrt regulatory regions have deacetylated histones and are O-GlcNAc rich. In orexin neurons, Mgea5 expression leads to removal of the O-GlcNAc residues and acetylation of core histones, which induces the expression of Hcrt. The substrate of the O-GlcNAcylation remains to be defined, even though core histones are a probable candidate [410-412].
Figure 2. Epigenetic regulation of orexin neuron differentiation. In embryonic stem cells as well as non-orexin neurons, SIRT1 deacetylates the histones at the regulatory region of the HCRT gene, leading to repression of its expression. These histones also contain O-GlcNAc residues. Treatment with ManNAc activates Mgea5, which acetylates the histones of the HCRT regulatory region, and leads to removal of the GlcNAc residues. This activates the expression of the HCRT gene, and the cells turn into orexin neurons.

2.1.3 Region and cell-type specific differences in neurogenesis

The histone code of neurogenesis is not universal. Different areas of the central nervous system can be differentially affected by the same histone modification. Shaked et al. [413] demonstrated that HDACs of classes I and II are necessary for neurogenesis in the embryonic ganglionic eminence, but inhibit neurogenesis in the embryonic cortex. The results held true both in vitro and in vivo. The authors used Trichostatin A (TSA), a chemical inhibitor of class I and II
HDACs [414]. The mechanism of the observed effect was shown to involve the BMP2/4 pathway, which induces neurogenesis in the cortex but promotes astrogliogenesis in other areas of the brain [415-417]. In studies assessing the effects of HDAC inhibition on mature neuronal survival, Forgione et al. [418,419] found that treatment of cells isolated from the embryonic mouse ventral midbrain with the HDAC inhibitors TSA, valproic acid (VPA) or sodium butyrate (SB) induced cell death in neurons but not astrocytes and the cell death was largely caspase-independent. Intriguingly, the same treatment did not result in neuronal cell death in cultures from the embryonic cortex. These studies underline the complexity of the epigenetic regulatory network of neurogenesis and indicate that a one-size-fits-all model might not be entirely correct. We will describe and discuss some of these aspects in the following sections.

2.1.4 Lessons learned from HDAC inhibitor experiments

Chemical inhibitors of global HDAC function such as TSA, VPA and SB have been used to investigate the role of histone acetylation in neurogenesis and yielded somewhat conflicting results.

Hsieh et al. assessed the levels of acetylated histones H3 and H4 during adult rat hippocampal NSPC differentiation into neurons, astrocytes and oligodendrocytes and found that neuronal differentiation was associated with a greater maintenance of acetyl-H3 and acetyl-H4 than differentiation into the other 2 lineages [420]. They proceeded to show that treatment with VPA increased neuronal differentiation of these NSPCs at the expense of glial differentiation, and confirmed the results using the HDAC inhibitors TSA and SB. The effect was attributed to induction of NeuroD1, a known transcription factor involved in neurogenesis [421], by the VPA treatment. Intriguingly, VPA treatment led to 3 times as much cell death as in control cultures, which implies that selective survival of neuronal progenitor cells could also be involved. VPA treatment in vivo also decreased BrdU incorporation and increased neuronal differentiation in the hippocampus of adult rats, which supports the in vitro observations. Corroborating the study of Hsieh et al., Yu et al., [422] also observed decreased BrdU incorporation in hippocampal neural stem cells after VPA treatment, and increased differentiation both in vitro and in vivo.
On the contrary, Hao et al. [423] showed that VPA treatment of adult mice led to an increase in BrdU incorporation in the hippocampus. Furthermore, Kim et al. [424] found that administration of the HDAC inhibitors SB or TSA after ischemic hypoxia increased the BrdU incorporation and the Ki67 staining in the forebrain neurogenic zones of adult rats. In addition, PSA-NCAM staining was augmented with the treatments, which indicates that HDAC inhibition resulted in an increase in the presence of neuronal progenitors and/or immature neurons. An increase in the number of BrdU+ cells typically indicates increased proliferation of the NSPCs. However, the state of the cell cycle of NSPCs is an important consideration when interpreting BrdU incorporation data. For example, an in vitro study by Zhou et al. [425] demonstrated that neural stem cells from the forebrain subependymal zone of adult mice exhibited a G1-to-S cell cycle arrest upon treatment with SB or suberoylanilide hydroxamic acid (SAHA), another HDAC inhibitor. If cells treated with HDAC inhibitors are gradually prolonging their cell cycle towards eventual cell cycle exit, then there could be a period of increased numbers of BrdU+ cells before they undergo differentiation or cell death. The study by Zhou et al (68) showed that neuronal specification genes such as NeuroD1, Neurogenin 1 and cell cycle inhibitors such as p21 and p27 were up-regulated with the treatments while progenitor associated genes, such as Sox2 and the Notch effectors Hes1 and Hes5 were down-regulated, suggesting that these cells prematurely differentiated into neurons.

In general, these studies suggest that chemically inhibiting HDACs may alter the behavior of NSPCs in the adult brain. In some cases this could lead to a prolonging of the cell cycle prior to exit or it could lead to premature cell cycle exit and this could depend on the type NSPC or other factors. Ultimately, HDAC inhibition seems to facilitate neuronal differentiation from progenitor cells, but also cause cell death if the inhibition is prolonged after neurons have differentiated. One caveat is the potential for off-target effects and toxicity when using chemical inhibitors [418,426-428]. Additionally, HDACs are enzymes of broad specificity that often target and remove acetyl-groups from transcription factors and other non-histone proteins. Therefore, the observed phenotype could be a result of these non-histone effects, which makes the data interpretation difficult.
Another explanation may be cell-specific expression of HDACs. Spatial and temporal variations in levels of expression of individual HDACs in different cell types and/or different stages of development should affect the genome wide occupancy of the HDACs. A pan-inhibition of several types of HDACs could therefore have different and even opposite effects in progenitors from different regions and at different stages of maturation. It is therefore important to elucidate roles for specific HDACs in vivo and in vitro.

2.1.5 Lessons learned from experiments using knockdowns of specific HDACs

Genetic knockout studies have enabled us to further decipher the role of specific HDACs, as well as interactions between different HDACs, in regulating neurogenesis. Conditional knockouts of HDAC1 and HDAC2 driven by GFAP-cre, which is expressed in neural stem cells and astrocytes, revealed that their functions are redundant [429]. When either HDAC1 or HDAC2 were missing, no obvious phenotype was observed. The double knockout mice, in contrast, showed severe structural abnormalities in the cortex, hippocampus and cerebellum and died within the first postnatal week. When E14.5 cortical NSPCs were cultured in vitro, the double knockout cells exhibited an almost complete blockade of neuronal differentiation, while the astrocytic differentiation was unaffected. The phenotype was attributed to increased apoptosis of neuronal progenitors both in vitro and in vivo, but could also be explained by a lack of maintenance of the differentiated neuronal state in the absence of HDAC1/2 function consistent with the effects of HDAC inhibitors on relatively mature neurons in vitro [418]. Therefore, this report unequivocally demonstrated that HDAC1 and HDAC2 are necessary for proper embryonic neurogenesis, and is in agreement with results obtained using dominant negative HDAC1 or HDAC2 [430]. In addition, specific roles for HDAC2 in oligodendrocyte differentiation and HDAC3 in neuronal differentiation linked to acetylation of lysine 14 visavi 9 on histone H3 (H3K14, H3K9) were suggested based on RNA knockdown studies in rat embryonic cortical progenitors in vitro [431]. An important lesson from all of these studies is that global inhibition of HDACs can mask the contributions of individual HDACs to neurogenesis leading to conflicting observations especially when comparing different experimental contexts. Nonetheless, it is equally apparent that the regulation of histone deacetylation occurs at multiple levels and includes the specific subtype of HDAC, the specific cell type involved, as well as the region of the brain in which the deacetylation is occurring. The intersections between these
complex levels of regulation no doubt enable the fine-tuning of neurogenesis, yet the details of these processes remain obscure.

2.2 Histone Methylation

In addition to histone acetylation, another PTM that was discovered in the 1960’s is histone methylation [382]. Histone methylation used to be considered an irreversible mark, in stark opposition to the dynamic nature of histone acetylation, but the discovery of histone demethylases has altered that view [376,432,433]. Histone methylation can occur at lysine or arginine residues. Lysine methylation is catalyzed by histone methyl-transferases (HMT), and lysines can be mono-, di- or tri-methylated. The number of methyl-groups added can alter the resulting phenotype. Arginine methylation is catalyzed by protein arginine methyl-transferases (PRMT), and can lead to mono-methylated, symmetrically di-methylated or asymmetrically di-methylated arginines. Unlike histone acetylation, the effects that methylation has on gene expression depend on the specific residue that gets modified. For example, trimethylation of the lysine 4 residue of histone 3 (H3K4) is considered a mark of transcriptional activation [434], while trimethylation of H3K27 is a mark of transcriptional repression [434]. There is substantial evidence for the involvement of histone methylation in neurogenesis.

2.2.1 The action of methyltransferases on embryonic neurogenesis

As mentioned in the introduction, during brain development neural stem cells initially give rise to neurons, and at about E18 in the mouse there occurs a switch in differentiation competence toward astrogliogenesis. Probing the mechanism of this intrinsic switch, Tan et al. [435] demonstrated that ESET, an H3K9 methyltransferase previously known to be required for the maintenance of the pluripotent state in ES cells [436-438], is pivotal for the regulation of the fate competence of NSPCs during development. Conditional knockout of ESET in the embryonic forebrain unveiled an intriguing finding: ESET seemed to be required for the generation of the deep cortical cell lineages that arise first, but the effects on later superficial cortical cell lineages was less pronounced. Additionally, the knockout of ESET led to precocious astrogliogenesis, demonstrated by markedly increased GFAP staining both in vivo and in NSPCs in vitro [435]. The converse experiment of over-expressing ESET by in utero electroporation showed reduced astrocytic differentiation. In assessing the mechanism of action of ESET, the authors demonstrated by ChIP that it directly binds the promoters of GFAP and Sox9, associated with
astrogliogenesis, repressing their expression by H3K9 tri-methylation. In the absence of ESET, these genes exhibit less of the H3K9me3 mark, and are over-expressed. On the contrary, many genes associated with neurogenesis were down regulated in the ESET knockout brains, hinting towards an indirect regulation by ESET. Therefore, the methyltransferase ESET is involved in the cell fate regulation of the embryonic NSPCs, by actively suppressing astrogliogenesis.

The H3K9 methyltransferase G9a and the related molecule GLP, appear to function as a constitutive heteromeric complex to promote H3K9 dimethylation in euchromatic regions leading to transcriptional repression [439]. During the development of the mammalian retina, G9a has been shown to repress progenitor gene expression, which facilitates the terminal differentiation of these cells [440]. Indeed, postnatal conditional loss of function of the G9a/GLP complex causes the de-repression of neural progenitor cell genes, as well as non-neural genes, in mature neurons resulting in severe behavioral defects in these animals [441]. It remains to be determined how the G9a/GLP complex is recruited to specific loci to promote transcriptional repression, but such a mechanism would likely be active during the transition from a proliferating progenitor cell to a post-mitotic differentiated neuron or glial cell, and may even persist to ensure the maintenance of repression marks on these progenitor genes in mature cells.

2.2.2 Polycomb family members

Additional evidence for the importance of histone methylation on the onset of neurogenesis arises from seminal work on Bmi-1, associated with the Polycomb family of H3K27 methyltransferases [434]. Bmi-1+/− mice exhibit a variety of neurological defects starting from an early age [442]. Molofsky et al. [443] showed that Bmi-1−/− mice demonstrate decreased self-renewal and proliferation in both embryonic and postnatal forebrain NSCs. Bmi-1 enhanced NSC self-renewal and proliferation partly by blocking p16Ink4a and p19Arf, 2 inhibitors of cyclin-dependent kinases [443,444]. Interestingly, Bmi-1 was required for the proliferation of NSCs, but not for that of committed neurogenic or gliogenic progenitors, revealing a regulatory role upstream in the hierarchy of neurogenesis.

In a different report Fasano et al. [445] utilized a lentivirus-delivered shRNA approach to knockdown the expression of Bmi-1 in embryonic, perinatal or adult NSCs and their results were slightly different. Even though they did observe a decrease in NSC proliferation and self-renewal, the effects of the Bmi-1 knockdown were more pronounced as the age of the mice was
increasing. Therefore, adult NSCs seemed to be more dependent on Bmi-1 than embryonic NSCs. The authors attributed their effects to p21 up-regulation upon Bmi-1 knockdown, and when p21 was also knocked down the phenotype was rescued. Unlike Molofsky et al., Fasano et al. did not observe a p16\textsuperscript{Ink4a} or p19\textsuperscript{Arf} up-regulation after Bmi-1 knockdown, and simultaneous knockdown of Bmi-1, p16\textsuperscript{Ink4a} and p19\textsuperscript{Arf} failed to rescue the Bmi-1 phenotype.

The differences between the 2 studies can be attributed to the fact that the knockout mice used by Molofsky et al. might have had compensatory mechanisms in place since they had to develop without Bmi-1, while the shRNA approach provides a more acute decrease in Bmi-1. It is also plausible that the decreased levels of Bmi-1 produced by an shRNA knockdown cannot recapitulate the true null phenotype of a Bmi\textsuperscript{-/-} mouse, and the presence of a remaining minimal amount of protein is sufficient to alter the observed effects. Additionally, the different mouse backgrounds used in the 2 studies could have also contributed to the observed differences.

In a recent report by Gargiulo et al. [446], Bmi-1 was found by ChIP/RNA-seq to target p16\textsuperscript{Ink4a}, p19\textsuperscript{Arf} and p21, along with an array of additional cdk inhibitors. Gargiulo et al. also found that Bmi-1 down-regulates multiple members of the BMP and TGF-β pathways, which are long established to be involved in inducing differentiation of NSCs into neurons or astrocytes [447,448]. Therefore, it appears that Bmi-1 enhances the NSC proliferation and renewal by 2 mechanisms: i) it suppresses cdk inhibitors, thus allowing the NSCs to continue cycling and ii) it suppresses neurogenic pathways, thus delaying the onset of differentiation. A schematic demonstration of this model is shown in figure 3.
Figure 3. The role of the Polycomb protein Bmi-1 in neural stem cell self-renewal. The Polycomb complex trimethylates the regulatory regions of the cdk inhibitors p16$^{\text{Ink4a}}$, p19$^{\text{Arf}}$ and p21 at the lysine 27 residue of H3. This represses their expression, which allows cell cycle to proceed. In the absence of Bmi-1, the cdk inhibitors are expressed and block the cell cycle. This decreases the self renewal capability of the neural stem cells.

The converse experiment, namely, over-expression of Bmi-1, resulted in an increase in the self-renewal and proliferation of both embryonic and adult NSCs [449]. In addition, the authors showed that FoxG1, a forebrain-specific transcription factor [450] was necessary for the Bmi-1 – induced increase in NSC self renewal. This raises the possibility that FoxG1 is a downstream effector of Bmi-1, even though over-expression of FoxG1 was less effective at promoting an increase in NSC self-renewal compared to Bmi-1 over-expression. Therefore, either Bmi-1 utilizes additional downstream effectors, or Bmi-1 and FoxG1 operate in different pathways. Intriguingly, NSC self renewal was unchanged when Bmi-1 was over-expressed in embryonic
spinal cord NSCs, which constitutes additional evidence for the notion that the epigenetic regulation of neurogenesis utilizes divergent pathways in a region-specific manner.

2.2.2 Trithorax family members

In addition to the Polycomb family of transcriptional repressors, there exists the Trithorax family of transcriptional activators that operate by methylating H3K4 residues [434]. The Trithorax family member Mll1 (Mixed lineage leukemia 1), an H3K4 methyltransferase, was reported to be involved in postnatal neurogenesis in a very interesting manner [451]. Mll1 seemed to be necessary for the induction of Dlx2, an indispensable transcription factor for olfactory bulb neuron specification and migration [452]. NSPCs isolated from the forebrain of postnatal mice in which Mll1 had been conditionally knocked out survived and proliferated normally, but gave rise to significantly less neurons than controls. Gliogenesis was not affected. Similar phenotypes were observed in vivo. Therefore, Mll1 is required specifically for neuronal differentiation of forebrain NSPCs, unlike other epigenetic regulators that we reviewed above that are required for differentiation into all 3 lineages. Surprisingly, Mll1 did not act by catalyzing an H3K4 methylation event, but rather by recruiting an undefined H3K27 demethylase at the Dlx2 promoter. Since H3K27 methylation is inhibitory for gene expression, such H3K27 demethylase would activate transcription. Therefore, just as has been observed in other systems [434], it appears that members of the conserved Polycomb and Trithorax families of methyl-transferases co-operate to regulate neurogenesis. The Polycomb member Bmi-1 ensures that the NSCs remain in cycle for as long as needed to generate enough progeny and then passes the baton to the Trithorax member Mll1 which overviews neuronal differentiation. How this strict temporal pattern of activity of these epigenetic factors is controlled remains to be elucidated.

2.2.3 Histone demethylases

An independent report by Jepsen et al. [453] showed that JMJD3/KDM6B, a putative histone demethylase, steers interneuron differentiation in NSPCs from the mouse embryonic cortex. JMJD3/KDM6B contains a Jumonji C domain, which is responsible for its histone demethylase activity [454]. A mutant JMJD3/KDM6B lacking this domain was unable to induce interneuron differentiation. Through an in vitro demethylation assay, it was shown that JMJD3/KDM6B specifically demethylates H3K27me3, therefore causing a de-repression of the target genes, many of which appear to be neuronal. Treatment of NSCs with retinoic acid, a known inducer of
neuronal differentiation [455], induced binding of JMJD3/KDM6B to the Dlx5 promoter and reduction in the H3K27me3 levels as evidenced by ChIP. Dlx5 is expressed in differentiated interneurons [456]. JMJD3/KDM6B is normally suppressed by Nuclear Co-repressor 2 (NcoR2 or SMRT), a protein involved in a multitude of developmental processes [457]. Knocking out SMRT resulted in significant alterations in the forebrain of mouse embryos as well as decreased proliferation and increased differentiation of embryonic cortex NSPCs into neurons and astrocytes. RA signaling was necessary for the increased neuronal differentiation of the SMRT−/− cells and JMJD3/KDM6B was significantly up regulated upon the knockout. More recently, it has been demonstrated that JMJD3/KDM6B is actually required for proper neurogenesis [458]. Whether JMJD3/KDM6B is the same H3K27 demethylase that is recruited by Mll1 in the study of Lim et al. is unknown and will require further studies, but it has been shown in other cellular contexts that JMJD3/KDM6B and Mll1 indeed interact [459].

In addition to JMJD3/KDM6B, recent evidence indicates that histone demethylases JMJD2A/KDM4A and JMJD2C/KDM4C are important PTM enzymes that contribute to neurogenesis [460]. Cascante et al. demonstrated that these enzymes are required for demethylating BDNF regulatory regions at H3K9 in response to the HDAC inhibitor VPA, thus inducing its expression. At the same time, these enzymes are required for demethylation of GFAP exonic regions at H3K36, leading to a blockade of astrogliogenesis. How this differential specificity for methylated lysine is achieved remains to be identified and may involve different cofactors for each promoter. The authors showed that knockdown of JMJD2A/KDM4A and JMJD2C/KDM4C by siRNA in embryonic cortical NSPCs led to precocious astrogliogenesis and decreased neurogenesis associated with increased cell death. These data support previous results from analyses of a different histone demethylase, namely, JHDM1D. Huang et al. [461] demonstrated that JHDM1D is necessary for neural specification of ES cells, by demethylating regulatory regions of FGF4. Similar to JMJD2A/C, this enzyme appears to be bi-specific: it removes methyl residues from both H3K9 and H3K27.

In conclusion, it appears that the neuronal specification of an NSPC in addition to methyl transferase activities requires the presence of multiple histone demethylases that de-repress neuronal commitment genes that were silent in the progenitors, while at the same time suppressing alternative fates.
2.2.4 Histone arginine methylation

Apart from histone methylation on lysine residues, histone methylation on arginine residues has also been implicated in the regulation of neurogenesis. In general, symmetric dimethylation (symbolized as me2s) of an arginine residue is associated with transcriptional repression of the respective gene, while asymmetric dimethylation (me2a) is associated with transcriptional activation [462-464]. Chittka et al. [464] reported that the transcription factor Schwann cell factor 1 (SC1, also known as PRDM4) controls the onset of neurogenesis from embryonic NSPCs by recruiting the histone arginine methyltransferase PRMT5. In embryonic NSPCs cultured in vitro, SC1 was only expressed in non-terminally differentiated mitotically active progenitors, but not in TuJ1+ or GFAP+ cells. The levels of SC1 staining decreased over time in culture, along with the increase in differentiation. Silencing of SC1 by siRNA induced precocious neuronal differentiation, and decreased BrdU incorporation, while over-expression of SC1 increased the proportion of Nestin positive cells in the embryonic NSPC cultures. Through a series of immunoprecipitation experiments, the authors showed that SC1 interacts with the methyltransferase PRMT5 through its N-terminal domain. The presence of the N-terminus of SC1 was necessary to achieve the increase in Nestin+ cells after over-expression in neural stem cell cultures, indicating that PRMT5 may be necessary for the effect. Additionally, both embryonic NSCs and sections of E10.5 cortex, showed high levels of expression of both PRMT5 and SC1 and very frequent H4R3me2s modifications. The latter is the symmetrical di-methylation catalyzed by PRMT5. The authors also showed that over-expression of SC1 containing the N-terminus, led to a down regulation in the expression of the promitotic genes Bub1b and cyclinB. Conversely, siRNA knockdown of SC1 led to an up-regulation of Bub1b and cyclinB. High levels of promitotic genes have been shown to be required for proper asymmetric division [465], raising the possibility that SC1 delays the onset of neurogenesis by impeding asymmetric division of neural stem cells which would give rise to committed progenitors. Nevertheless, more research is needed to directly demonstrate that PRMT5 is necessary for these effects. Interestingly, differentiated neurons correlated with H4R3me2a marks, while undifferentiated precursors correlated with H4R3me2s marks [466]. Therefore, the methylation status of H4R3 appears to function as a binary switch that changes its position along with neuronal differentiation. Whether the epigenetic switch has a causative role in neuronal differentiation or it is a consequence of that differentiation remains to be elucidated.
2.2.5 Elucidating a putative histone methylation code of neurogenesis

Despite the plethora of convincing evidence demonstrating that histone methylation is involved in neurogenesis, correlating specific methylation marks with gene expression or repression is still difficult. This complexity was demonstrated in a report by Popova et al. [467]. The authors monitored the presence of H3K4me2 and H3K27me3 during pre- and postnatal retinal development and attempted to correlate that with the expression level of individual genes. No simple correlation was identified as for some genes increases in expression correlated with increased H3K4me2 and decreased H3K27me3, while for others they did not. This demonstrates that the particular epigenetic marks cannot be universally used as predictors of gene expression or repression, and that the specific histone modifications that regulate expression vary between genes. Interestingly, the genes with similar correlations between expression and presence of the 2 epigenetic marks could be grouped with gene ontology. Additionally, the authors were able to identify a specific epigenetic signature that could predict the identity of a gene as photoreceptor-specific. Thus, specific gene families may share common epigenetic mechanisms of expression regulation, and the identification of these mechanisms could generate novel, sensitive genetic tools for screening of genes associated with the development of specific systems.

2.2.6 Combining histone acetylation and methylation: the example of CtBPs

We have reviewed histone acetylation and histone methylation as distinct processes; however, there are examples of proteins that combine these 2 activities. C-terminal binding proteins (CtBPs) repress transcription by recruiting both HDAC and H3K9 methyltransferase activities to the promoters [468,469]. Dias et. al. [470] demonstrated that CtBPs are indispensable for repressing neurogenesis in the roof plate (RP), a BMP-secreting structure which is responsible for dorsoventral patterning of neural cells within the neural tube [471-473]. The authors demonstrated that there is an oxygen gradient within the neural tube, with the RP receiving higher oxygen levels than the neurogenic regions. This leads to higher concentrations of NADH, the reduced form of NAD⁺, within the RP region. NADH interacts with CtBPs [474], increasing their transcriptional repressive function. This leads to CtBPs repressing the expression of Math1, a proneural transcription factor, specifically in the higher oxygen environment of the RP, so as to prevent this region from generating neural tissue. Interestingly, CtBPs also bound promoter elements of Hes1, which inhibits neurogenesis, following an inverse correlation with oxygen
levels: CtBPs repressed Hes1 expression in the lower oxygen environment of the neural tube. At higher oxygen levels, CtBPs are released from the Hes1 promoter, which enables a blockade of neurogenesis in the RP.

CtBPs have also been shown to be involved in neurogenesis by inhibiting Notch signaling targets in the absence of Notch ligand [475]. Data from Xenopus laevis and mammalian cell lines have shown that the transcriptional repressor SHARP is required for proper inactivation of Notch signaling [475-477]. It has been proposed that SHARP blocks transcription by two independent mechanisms, one of which is sensitive to HDAC inhibitors [477]. It was eventually discovered that SHARP interacts with CtBPs and that this interaction is necessary for its ability to suppress Notch signaling and promote differentiation [475]. Therefore, CtBP complexes are multimodal histone modifiers that appear to be involved in neurogenesis through diverse pathways.

3. Specific Considerations

3.1 Initiation of chromatin modifications in the context of neurogenesis

What is it that triggers the initiation of chromatin modifications associated with neurogenesis? It appears that known signals that induce neurogenesis under certain contexts, such as FGFs [408], neurotrophins [407], bone morphogenic proteins (BMPs) [447,478] and other members of the transforming growth factor β (TGFβ) [448] family also trigger the initiation of histone PTMs. In the example of CREB that was reviewed above, activation is controlled by phosphorylation of serine-133. CREB can be activated by a variety of cellular pathways, including the cAMP pathway, the PI3 kinase pathway, the MAP kinase (MAPK) pathway, or even in response to increased intracellular Ca²⁺ [383]. Therefore, an array of signaling molecules involved in neurogenesis also leads to CREB activation. Nerve growth factor (NGF), platelet-derived growth factor (PDGF) and FGFs are just some examples of factors that activate the PI3 kinase pathway [479,480]. Similarly, MAPK signaling is activated by FGFs [481] and insulin-like growth factor 1 (IGF-1) [482] among others. Thus, a functional link between secreted signals involved in neurogenesis and CREB activation can be established. Additionally, the fact that CREB can be activated in response to elevated intracellular Ca²⁺ and cAMP provides a link between mature neuronal activity and CREB recruitment to chromatin. Interestingly, CREB activation by Ca²⁺
and not the other pathways) leads to the phosphorylation of 2 additional serine residues, which may alter its target specificity [483]. In an alternate example, Estaras et. al. [484] showed that Smad3 recruits JMJD3/KDM6B to neuronal promoters in NSCs. This establishes a connection between JMJD3/KDM6B H3K27 demethylase activity and the TGFβ pathway.

It appears therefore, that known exogenous neurogenic signals are sufficient to induce post-translational modifications of transcription factors, which leads to recruitment of histone modifying factors to the chromatin, and thereby changes in chromatin charge and conformation associated with increased gene expression of neurogenic factors. This functions as an additional argument for the notion that chromatin modifications are an essential component of the mechanisms regulating neurogenesis.

### 3.2 Tissue specific effects of global regulators

Most histone PTM regulators may function globally in the genome. An obvious question that arises from that observation is how a master regulator can perform a tissue specific function. There are several ways that such neurogenesis-specific function of factors with assumed general functions can be achieved. As described in the example above, exogenous signals can influence ubiquitously expressed transcription factors in specific ways resulting in signaling-context-specific recruitment of histone modifying factors to localized regions of the chromatin. In addition, the signature of the pathway that activates a global regulator can be “stamped” on it via specific modifications, which can affect the selection of the downstream targets.

Exogenous signals may also affect the levels of histone modifying factors in the cell. For example, JMJD3/KDM6B expression is highly regulated by retinoic acid, and thus factors with general function may not always be expressed evenly in different cell types, including progenitors. In fact, many factors with general function, including CBP, HDACs, HDMs etc, may not always be ubiquitously expressed and tissue- and cell-specific expression of variants of chromatin-modifying factors have been reported (for example neuro-specific BAFs [485]).

It is further necessary to consider the combination of factors expressed in a certain cell, as factors with similar function in different cell types may utilize cell-specific adaptors and/or chaperones. Such strategies are utilized by, for example, the arginine methyltransferase PRMT5 (reviewed above), that has been involved in various systems in addition to neurogenesis, such as primordial
germ cells, embryonic stem cells and erythrocyte progenitors [462,486-488], always having the function of maintaining cells in an undifferentiated state. In this case, the global gene expression regulator gets recruited within specific cell types by different adaptor proteins. For neural stem cells, the adaptor protein seems to be the transcription factor SC1, while for primordial germ cells the adaptor protein is known to be Blimp1 [486], as shown in Figure 4.
Figure 4. Neural specific roles of universally expressed histone modulators as exemplified by the arginine methyltransferase PRMT5. In germ cells, PRMT5 forms a complex with the transcription factor Blimp1 and symmetrically dimethylates regulatory regions of somatic genes, which inhibits their expression. This ensures maintenance of the germ cell fate. In neural stem cells, PRMT5 forms a complex with the transcription factor SC1 and suppresses the expression of neuronal genes, which delays the onset of differentiation and maintains the neural stem cell state.

While these examples appear straightforward, it is also important to keep in mind that there is cross-talk among the histone modifications themselves. As the modifications alter chromatin charge and conformation, they can facilitate or hinder secondary and even tertiary histone modifications and thereby directly influence the recruitment of factors to promote neurogenesis.

3.3 Chromatin modification complexes encompassing "write", "read", and "erase" functions, that have a role in neurogenesis

Considering each histone modification enzyme in isolation obscures the true picture of how these proteins are regulated. Usually, chromatin regulation operates through complexes that integrate the many aspects of epigenetics, in addition to histone PTMs.

In the context of neurogenesis, one of the most well established complexes is the one that converges on element-1 silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF) [380,489]. REST is a transcription factor that recognizes the repressor element 1 (RE1) and was long believed to only function as a transcriptional repressor of neuronal genes in non-neural tissues. REST knockouts are embryonic lethal with a range of neural and non-neural malformations. REST can be induced by a variety of signals and has in addition been suggested to induce genes involved in neurogenesis and participate in neuronal differentiation and maturation [489]. Similar to other zinc finger transcription factors, REST functions as a central node that recruits a massive array of epigenetic modifiers. An interesting key to the mechanisms underlying the many functions of REST came with the discovery of an interacting regulatory protein, Co-REST [490]. Co-REST is associated with the CtBP complex (see above) and is therefore a primary example of the plethora of enzymes with various functions recruited to DNA
and thus chromatin by single transcription factors. These proteins include “writers” such as histone methyltransferases, “erasers” such as histone deacetylases, and “readers” such as methyl-DNA binding proteins. Additionally, REST recruits non-coding RNAs, components of the SWI/SNF remodeling complex and various adaptor proteins with a wide range of activities [491]. Therefore, REST forms signal- and cell-context specific complexes that make use of many of the known epigenetic mechanisms.

Additional examples of such multifunctional epigenetic complexes are the ones formed by the H3K27 methyltransferases of the Polycomb group. We reviewed the role of Bmi-1 in neurogenesis above. Bmi-1 does not operate in isolation, but rather as part of a multi-protein complex called PRC1 (Polycomb Repressive group Complex 1). The complex exists in many alternative forms that contain H3K27me3 binding proteins (“readers”), histone ubiquitin ligases (“writers”), and additional histone binding proteins as well as activity regulators and proteins of unknown function [492,493]. This enzyme complex can either operate in isolation, or together with a different complex of the Polycomb family, PRC2. PRC2 contains the H3K27 methyltransferase enhancer of zeste 1 or 2 (EZH1 or EZH2, “writers”), along with H3K27me3 binding proteins, additional histone and DNA binding proteins (“readers”) and enzymatic regulators [494,495]. PRC2 also has a pivotal role in neurogenesis, as has been demonstrated by studies on EZH2 and other components of the complex [496-498]. In addition to the core components of the complexes, their recruitment to chromatin is dependent on DNA methylation and non-coding RNAs [380,493,494]. These complexes therefore constitute another example of integration of multiple epigenetic mechanisms within one pathway.

It is becoming increasingly evident that the interactions between different epigenetic modifications play key roles in cellular differentiation, and that the variable composition of many poly-enzymatic complexes is a way of conferring tissue specificity. Additional research in this field will improve our understanding of the epigenetic component of neurogenesis and may reveal novel mechanisms of initiation of chromatin remodeling. Furthermore, how these histone PTMs are sustained for a certain developmental interval without being removed prematurely remains elusive.

3.4 Histone dynamics: an unchartered territory for understanding chromatin regulation of neurogenesis
Even though a great deal of research has focused on the effects of histone PTMs, an interesting direction would be looking into the impact of histone abundance and turnover on neurogenesis. Histones are relatively stable proteins, and their half life in non-dividing brain cells was found to average 159 days [499]. The amount of histones within a cell is tightly regulated, since a histone insufficiency would impede the efficient packaging of DNA and lead to cell death, while an excess of histones can cause chromatin aggregation due to their highly positive charge [500-503]. The dynamics of histone synthesis differ between diving and non-diving cells, since dividing cells have to couple histone with DNA synthesis at the S-phase [500]. Thus, non-terminally differentiated cells such as NSPCs will synthesize histones at a faster rate than terminally differentiated neurons. Accordingly, a cathepsin-dependent mechanism for histone H3 turnover has been described in differentiating ES cells [504]. Additionally, there is evidence that histone turnover differs between actively transcribed and silent regions of the genome, as well as that epigenetic modification can alter histone turnover [505,506]. Most of the effects of histone PTMs in the literature have been assessed within the conceptual framework of stable nucleosomes and do not take into account potential effects on histone dynamics. Indeed, the latter could prove to be a major mechanism of action of epigenetic modifications, especially in light of recent advances in techniques to measure histone turnover [506] and findings of aberrant histone turnover in patients with the neurodevelopmental disorder Rett syndrome [507]. Whether the histone abundance in and of itself plays a role in cellular differentiation is a consideration that has not been addressed in existing research. It would be interesting to investigate the consequences of enhanced or suppressed histone synthesis on NSPC behaviour.

4. Conclusion

Histone PTMs are an essential component of both embryonic and adult neurogenesis. Some of them are induced by neurogenic signals and appear to be under the control of specific signaling pathways that are involved in neurogenesis in diverse ways. More research is needed to decipher the exact pathways that trigger histone modification by other enzymes, as well as the mechanisms that lead to the tissue-specific effects of global modulators. It is becoming clear that many of these answers lie in the intricate albeit specific cross-talk between different epigenetic modifiers within and among enzymatic complexes. Future research into how the specific
signaling networks influence the chromatin modifiers and their interactions in specific aspects of NSPC differentiation and other cellular events will increase our understanding of the process of neurogenesis and the pathologies underlying neurodevelopmental and psychiatric disorders.
References


(31) Bandyopadhyay M, Rohrer B. Photoreceptor structure and function is maintained in organotypic cultures of mouse retinas. Mol Vis 2010 Jun 26;16:1178-1185.


(92) Turner JE, Blair JR. Newborn rat retinal cells transplanted into a retinal lesion site in adult host eyes. Brain Res 1986 Apr;391(1):91-104.


(142) Mruthyunjaya P, Stinnett SS, Toth CA. Change in visual function after macular translocation with 360 degrees retinectomy for neovascular age-related macular degeneration. Ophthalmology 2004 Sep;111(9):1715-1724.


(274) Ballios BG, Clarke L, Coles BL, Shoichet MS, Van Der Kooy D. The adult retinal stem cell is a rare cell in the ciliary epithelium whose progeny can differentiate into photoreceptors. Biol Open 2012 Mar 15;1(3):237-246.


(381) PHILLIPS DM. The presence of acetyl groups of histones. Biochem J 1963 May;87:258-263.

(382) ALLFREY VG, FAULKNER R, MIRSKY AE. Acetylation and Methylation of Histones and their Possible Role in the Regulation of Rna Synthesis. Proc Natl Acad Sci U S A 1964 May;51:786-794.


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