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Hyaluronic Acid-Based Hydrogels Enable Rod Photoreceptor Survival and Maturation In Vitro through Activation of the mTOR Pathway

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The culture of isolated photoreceptors in vitro has remained elusive in neuroscience. By using defined hyaluronic acid (HA) hydrogels, photoreceptor survival and maturation in vitro is dramatically increased, 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, it is shown 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, mTOR activation is identified as the molecular mechanism underlying the pro-survival effect, and it is demonstrated 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.

1. 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.[1,2]

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.[3–5] 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,[6] when photoreceptors are isolated and cultured in vitro they undergo morphological changes and profound apoptosis.[3,4] The few surviving cells do not resemble photoreceptors as they lose their outer segments and downregulate the expression of visual cycle proteins.

3D constructs have enabled the culture of cells not normally possible[7] and were instrumental to the derivation of retinal tissue from mouse and human embryonic stem cells.[8,9] 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.[10] 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.[11] While HA itself does not form a gel, HA-furan can be chemically cross-linked with poly(ethylene glycol)-bismaleimide by a Diels–Alder click reaction, resulting in a cross-linked HAPEG[12] (**Figure 1**).

The chemical and mechanical properties of HAPEG can be tuned to create a permissive environment for cell culture.[12] 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[13] such as RhoA, PI3 kinase, and canonical Wnt in

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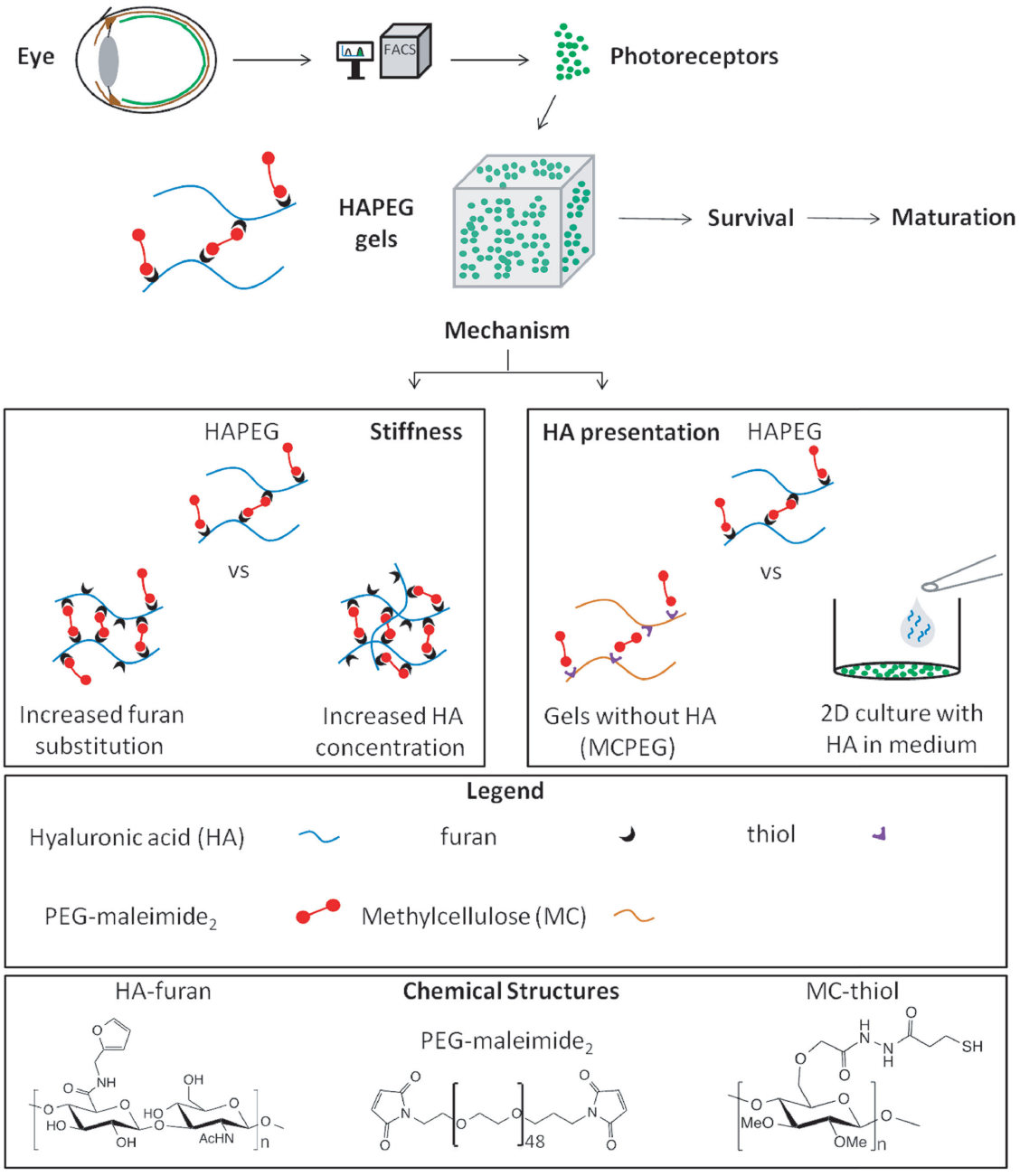

Figure 1. Schematic overview of the experiments performed in this work. Photoreceptors were isolated by FACS from the eyes of NrlGFP mice expressing GFP only in rod photoreceptors. The sorted rods were cultured in HAPEG gels and analyzed for their survival and maturation. The HAPEG gels were formed by cross-linking 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.

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.[14] 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 NrlGFP 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.

2. Results

2.1. Hydrogel Culture Drastically Improves Photoreceptor Survival

The culture of sorted NrlGFP rod photoreceptors in HAPEG gels resulted in a profound increase in their survival over a period of at least 14 d (**Figure 2**a,b). Interestingly, the absolute number of photoreceptors per well drastically decreased in the standard 2D culture from day 3 onward, with only a few clusters of cells remaining (a schematic of each well is depicted in Figure 2c and quantification is shown in Figure 2d). Unlike 2D cultures, photoreceptors cultured in hydrogels remained distributed throughout the wells. In order to compare our HAPEG gels with a commonly used, albeit 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 comprises numerous extracellular matrix proteins and growth factors[15] whereas HAPEG is well-defined and consists of only 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.

2.2. Gel Stiffness Does Not Mediate the Pro-Survival Effect

Extracellular matrix stiffness is a well-established parameter influencing cell survival and fate.[16] The stiffness of native retinal tissue has been measured between 1 and 15 kPa,[17] which is similar to the 5.95 kPa stiffness of the 1% HAPEG gels that we used (**Figure 3**a), 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 3a). 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

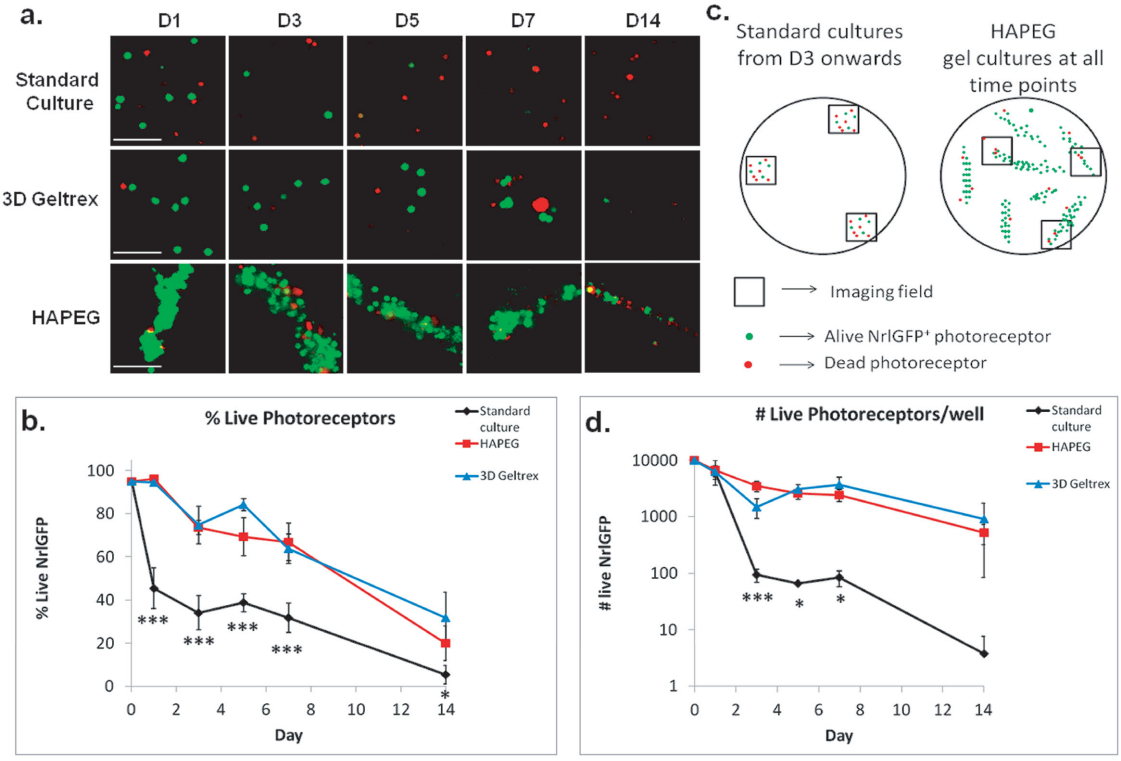

Figure 2. Culture of NrlGFP$^+$ photoreceptors in HAPEG or Geltrex hydrogels drastically improves their survival. a) Representative imaging fields over 14 d of culture in standard conditions, Geltrex or HAPEG gels. Green shows the NrlGFP fluorescence and red shows ethidium homodimer 1, which is taken up by dead cells. Scale bar is 50 µm. Split channel images and overlay with Hoechst are available in Figure S1 (Supporting Information). b) Quantification of cell survival as a percentage of live cells over total cells per well. A two-way ANOVA identified treatment and time as significant main effects, $p < 0.001$, and a significant time × 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 versus their relatively homogeneous distribution throughout the HAPEG-containing wells. d) Quantification of the absolute number of photoreceptors per well in the three different culture conditions. A two-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.)

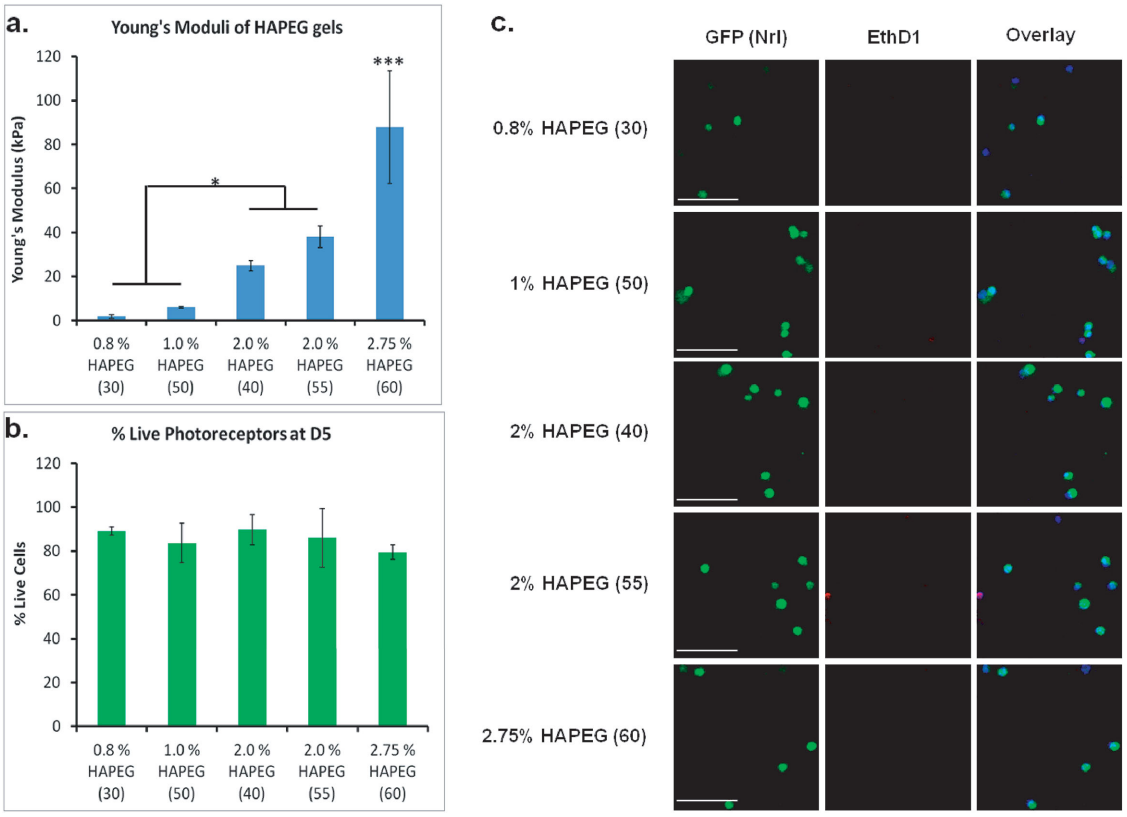

Figure 3. Gel stiffness does not mediate the pro-survival effect of the HAPEG gels. a) Young's moduli of the various HAPEG gels. Gels are labeled as the percentage of HA in HAPEG with percent furan substitution on the HA in parentheses. ($n = 4$ gels per group, mean ± standard deviation; *$p < 0.05$; ***$p < 0.001$, one-way ANOVA, Newman–Keuls post hoc analysis.) b) Quantification of cell survival of NrlGFP$^+$ photoreceptors after 5 d 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 µm.

photoreceptor cell survival; cell survival was largely consistent in all hydrogels at ≈80%–85% on day 5, irrespective of modulus (Figure 3b,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.

2.3. 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 on 2D polystyrene—the standard culture condition in which we had observed poor survival; and (2) we cultured the rod photoreceptors in a 3D, chemically defined hydrogel that lacks HA. A 3D hydrogel of similar stiffness to the 1% HAPEG gels was synthesized by cross-linking the same PEG-bismaleimide with methylcellulose-thiol, resulting in MCPEG cross-linked gels.[18]

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 4**a), suggesting that HA is key to cell survival. The importance of HA to rod photoreceptor 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 d in culture in the presence versus absence of HA in standard 2D culture conditions, and similar survival to that observed on HAPEG gels (Figure 4b,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 4a,b). This suggests that 3D culture itself may have a pro-survival 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.

2.4. 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 5**a). On day

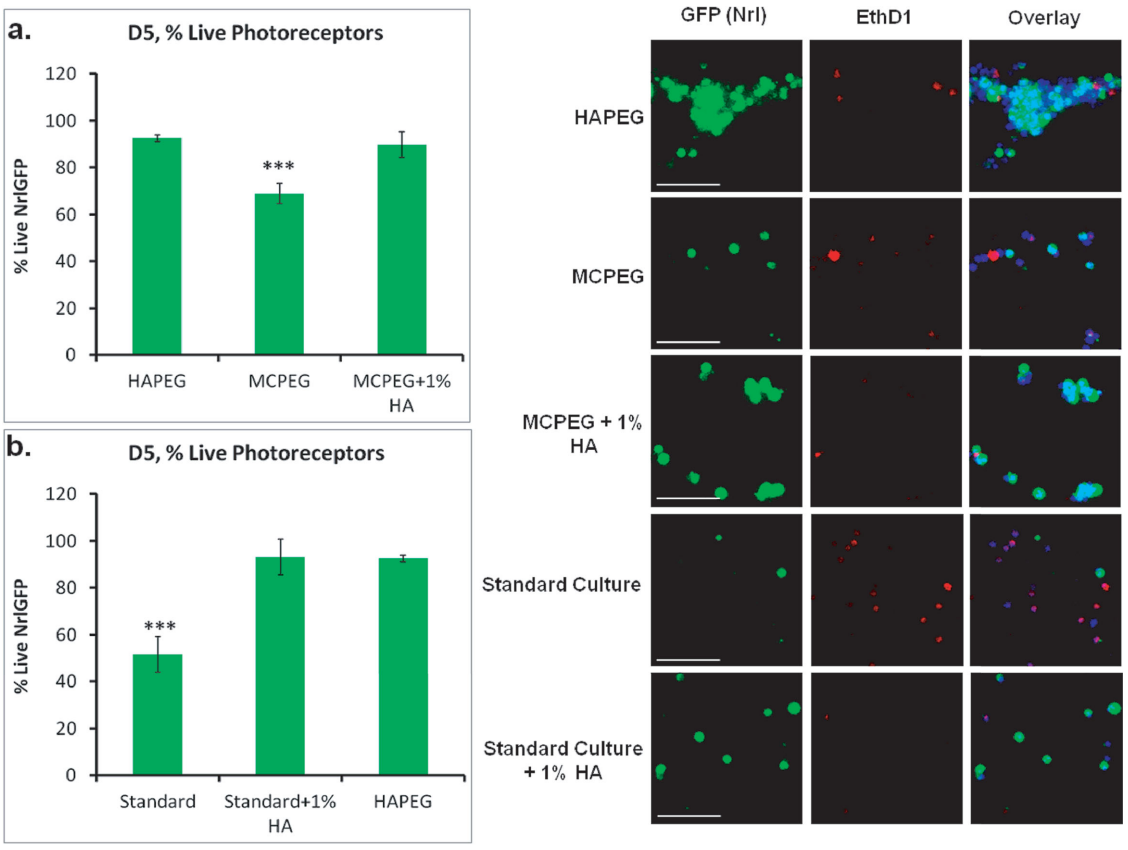

Figure 4. HA mediates the pro-survival effect of the HAPEG gels. a) Quantification of photoreceptor survival after 5 d of culture in HAPEG gels, standard culture conditions or standard culture with 1% HA in the medium shows that the addition of HA has a profound effect on survival. b) Quantification of photoreceptor survival after 5 d 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 µm. ($n = 4$ mice per group, mean ± standard deviation, ***$p < 0.001$, one-way ANOVA, Tukey's post hoc analysis.)

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 5a,b). Surprisingly, the increased activation of mTOR persisted to day 5, with 11.6% of the rods staining positive for phospho-mTOR in HAPEG versus 0.62% in the control culture (Figure 5a,c). To further test whether HA was acting through the mTOR pathway, rapamycin, a well-established inhibitor of mTOR signaling[19] was added to HAPEG cultures and abolished the pro-survival effect of HAPEG (Figure 5d). 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 S2, Supporting Information).

2.5. HA Induces mTOR through Combined Wnt and RhoA Activation

Given that the mTOR pathway is a downstream target of many signaling cascades,[20] 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 5e,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 S2, Supporting Information). Neither Y27632 nor IWR1e is as effective as rapamycin in decreasing the pro-survival effect of HA in HAPEG (Figure 5e,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 5e,f). Furthermore, combining Y27632 and IWR1e abolished the pro-survival effect of the HAPEG gels to the same extent as rapamycin (Figure 5e,f), suggesting that canonical Wnt and RhoA activation are the upstream mediators of mTOR activation in the rod photoreceptors.

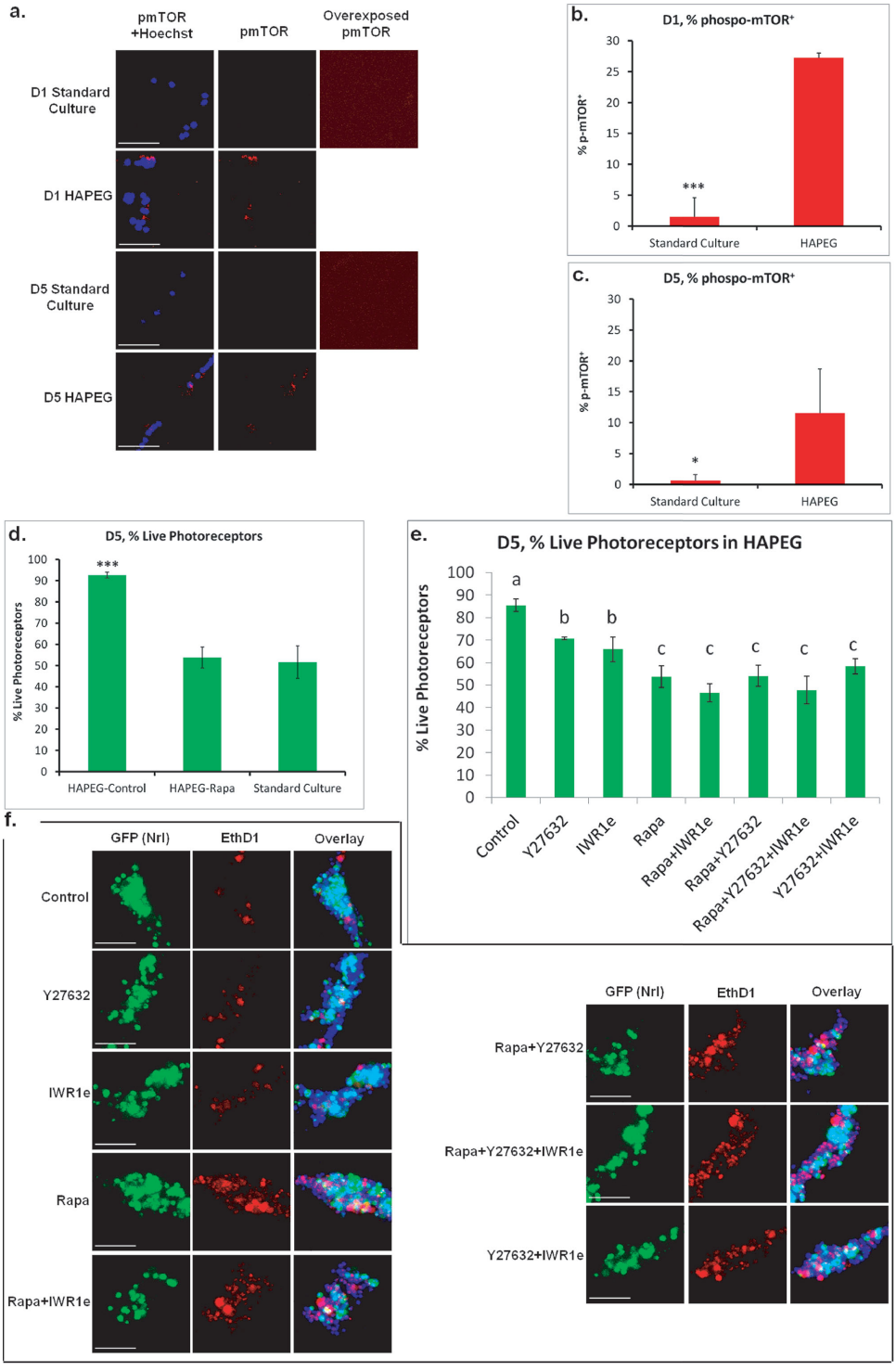

Figure 5. HA acts by inducing the mTOR pathway through the canonical Wnt and RhoA pathways. a) Activation of the mTOR pathway as evidenced by phospho-mTOR immunostaining in rod photoreceptors cultured in HAPEG for 1 and 5 d. Scale bar = 50 μm. The phospho-mTOR is significantly greater when rod photoreceptors are cultured on HAPEG versus 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, two-tailed.) d) Quantification of rod photoreceptor survival after 5 d of culture in HAPEG gels with and without rapamycin versus 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 versus b, $p < 0.01$; a versus c, $p < 0.001$; b versus 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 μm.

2.6. Culture in HAPEG Gels 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, ≈10% of cells expressed ABCA4 and 15% expressed peripherin over the course of 14 d (**Figure 6**a,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 d of culture (40% expression), which was significantly higher than the control cultures (Figure 6a). 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 to 35% on day 7 in HAPEG cultures (Figure 6b); however, by day 14, both ABCA4 and peripherin had dropped to baseline levels (of ≈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 polymerase chain reaction (PCR) for *abca4*, which encodes ABCA4, and *prph2* (Figure 6c–f), which encodes peripherin, corroborated the immunostaining data. At day 5, we observed a significant increase in *abca4* expression (Figure 6c) over controls in the HAPEG gels (8.75-fold), while *prph2* showed no differences in expression (Figure 6d). 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 6e,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 6g). Such processes were very rarely observed in the Geltrex cultures (an example is shown on day 3 for Geltrex in Figure 6g) and were never found in the standard culture.

3. Discussion

We demonstrate a novel and efficient way to maintain isolated mouse rod photoreceptors in vitro. There is robust survival for 7 d, and while there is only 20% survival after 14 d 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 d, 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,[21] neural stem cells,[22] or mesenchymal stromal cells,[23] 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 two 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.[24] 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,[25] and photoreceptor transplantation research.[26,27] While mouse retinal stem cell-derived rod photoreceptors were shown to interact with HA via CD44,[27] the receptor for HA on native rod photoreceptors remains largely elusive. The main HA receptors, CD44 and RHAMM, have not been detected in photoreceptors,[28–30] and were also undetectable in our P11 rods (data not shown). There is evidence for the existence of two glycoproteins, SPACR and SPACRCAN,[31–33] 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.[14] 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[34] that are important for the maintenance of cones. In retinitis pigmentosa, rod cell death results in the demise of cone photoreceptors,[35] 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.[36] As further evidence for the importance of the mTOR pathway on survival, Tsang et al.[37] 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 subconjuctival sirolimus, an mTOR inhibitor, for geographic atrophy reported deterioration of visual acuity in treated eyes.[38] Whether sirolimus had an effect on rod photoreceptor viability remains to be elucidated.

Consistent with the literature on the molecular cascades induced by HA,[13] we too found that HA induced multiple

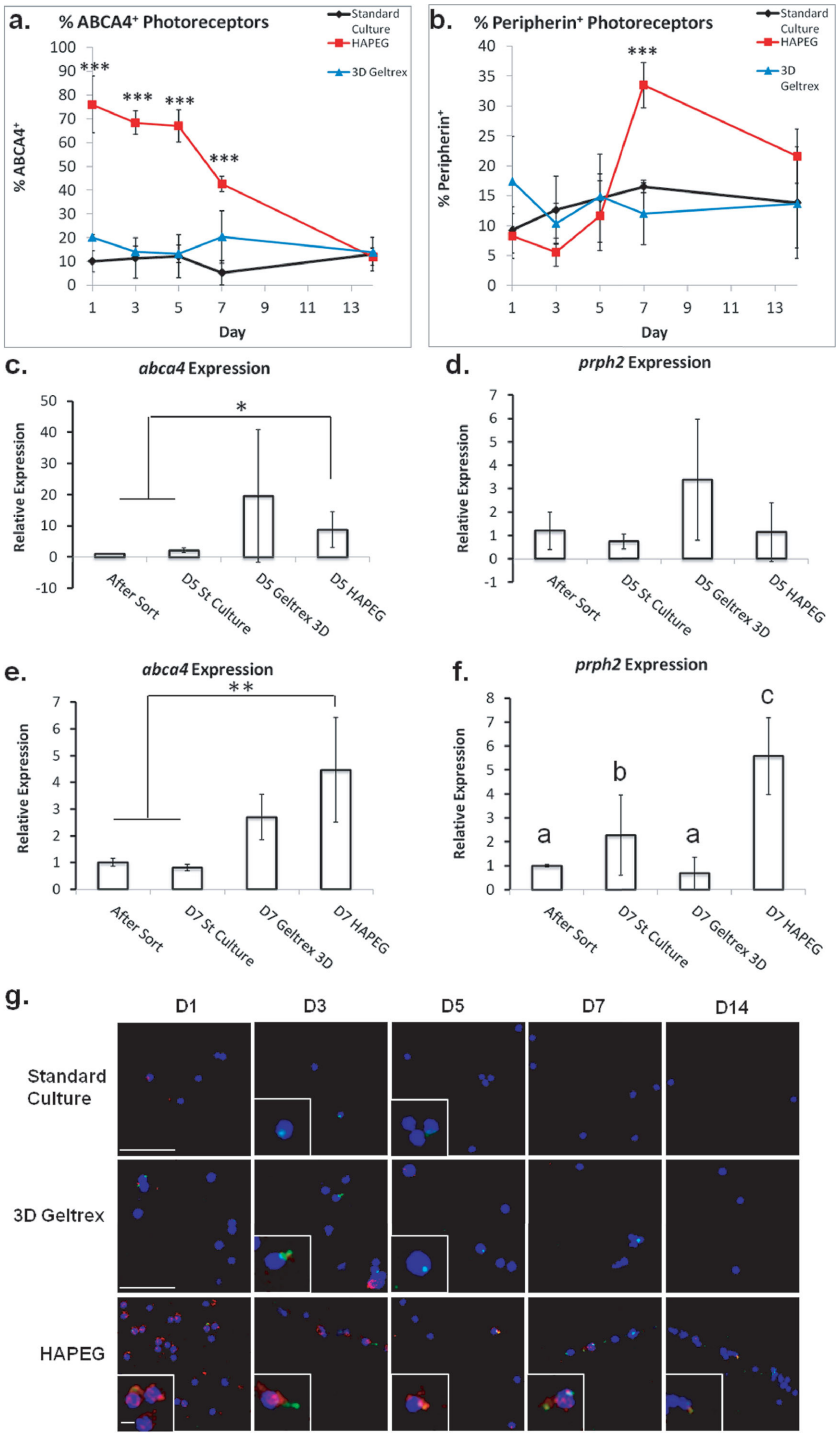

Figure 6. NrlGFP⁺ photoreceptors undergo partial maturation when cultured in HAPEG gels. a,b) Quantification of immunostaining for ABCA4 and peripherin by manual counting of immunostained positive cells over 14 d of culture in HAPEG, Geltrex gels or standard culture. A two-way ANOVA identified treatment and time as significant main effects, $p < 0.001$ and a significant time × 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 d 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, two-tailed.) e,f) qPCR for the expression of abca4 and prph2 transcripts after 7 d of culture in the indicated conditions. There is a significant increase in abca4 expression in HAPEG gels versus standard culture and between "after sort" versus 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 versus c, $p < 0.001$; b versus c, $p < 0.05$; a versus 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), and peripherin (green). Scale bar is 50 µm for the images and 5 µm for the higher magnification images. Split field images are available in Figure S3 (Supporting Information).

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[39] and has been implicated in mammalian photoreceptor degeneration.[40,41] 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.[42] For example, ROCK inhibition protected retinal ganglion cells from cell death in an optic nerve crush model[43,44] and a bovine retinal explant model of hypoxia.[44] 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.[45] 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,[46] 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 S4, Supporting Information). As ABCA4 is a membrane protein,[47] which may be damaged during retinal dissociation and cell sorting, the significantly greater expression on day 1 in HAPEG gels versus both Geltrex and standard 2D, suggests maintenance of pre-existing ABCA4 proteins. Interestingly, we observed an increase in protein expression of peripherin after 7 d 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 *abca4* and *prph2* transcripts during in vitro culture in HAPEG versus 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.

4. Experimental Section

Mouse Strain: The Nrl-GFP[48] 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.

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 cilliary 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 $GFP^+/7\text{-}AAD^-$ 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 four littermates were dissected, sorted, and cultured separately.

The sorted $Nrl\text{-}GFP^+$ 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^{-1} bFGF (R&D Systems), 5 µg mL^{-1} 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 µg mL^{-1} for 3 h at 37 °C. Inhibitors: Rapamycin (Sigma Aldrich) was used at 100×10^{-9} M, Y27632 (Sigma Aldrich) at 20×10^{-6} M, and IWR1e (Millipore) at 10×10^{-6} M. 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.

It was initially wanted to optimize the culture medium for FACS-sorted Nrl-GFP rods. Based on previous literature on photoreceptor culture in vitro,[3–5] the cultures were supplemented with bFGF and heparin, as well as the neural cell supplements B27 and N2. Small increases in cell survival were found upon bFGF addition, while the addition of serum led to a significant increase in cell survival by day 7 (Figure S5, Supporting Information). 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 and Figure S5, Supporting Information).

HAPEG Gels: HAPEG hydrogels were prepared as previously described.[12] 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 0.1 M 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 in **Figure 7**.

To prepare the gels, HA-furan (50% furan degree substitution, 40 mg mL^{-1} unless otherwise stated) and bis-maleimide PEG (Rapp Polymere, 3 kDa, 81.9 mg mL^{-1}) were dissolved separately in sterile 0.1 M MES buffer (pH 5.5). To prepare 100 µL of 1% HA gel with PEG-bismaleimide cross-linker (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) three times, PBS three times, followed by DMEM/F-12 media three times. Unless otherwise indicated, all HAPEG gels used here had 1% HA-furan/PEG-bismaleimide ($PEG\text{-}MI_2$), with a 50% furan substitution of the HA.

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 three 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 µg mL^{-1}) for nuclear staining.

Live/Dead staining was done in live cultures, using 4×10^{-6} M ethidium homodimer-1 (Life Technologies) as a dead stain and 2 µg mL^{-1} Hoechst 33342 as a nuclear stain. Live photoreceptors constitutively express GFP because of the NrlGFP 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

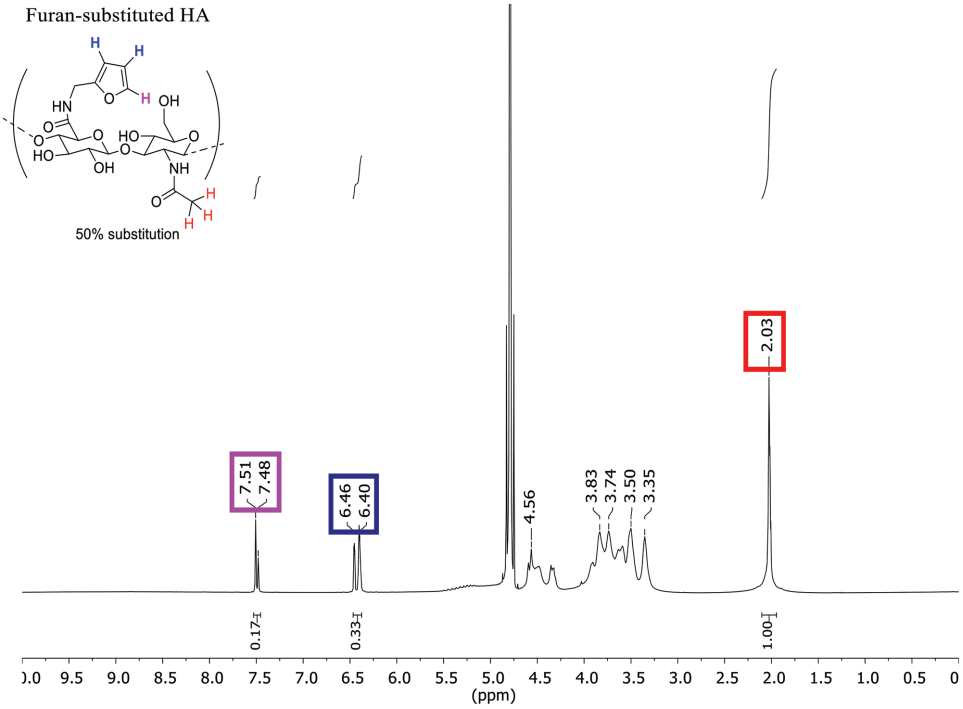

Figure 7. ^{1}H NMR of 50% furan-substituted hyaluronan (289 kDa) in deuterium oxide. Substitution of hyaluronan is calculated from the ratio of furan integrals at 6.46 and 7.51 ppm to the *N*-acetyl signal at 2.03 ppm of native hyaluronan. In this instance, $\frac{(0.17+0.33)}{1.00} * 100\% = 50\%$ substitution. Spectra obtained using a 500 MHz ^{1}H NMR spectrometer, 256 scans.

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 ≈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. The 3D reconstruction image in Figure S6 (Supporting Information) was made using Imaris software.

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 six cycles of 30 s homogenization followed by 1 min incubation 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 TCGGGACTGG-TTTGAGATTC, *gapdh* FP AGGTCGGTGTGAACGGATTTG, *gapdh* RP TGTAGACCATGTAGTTGAGGTCA. Quantification was done using the ΔΔCt method[49] and GAPDH was used as a housekeeping gene. The relative expression shown is $2^{-\Delta\Delta Ct}$.

Mechanical Compression Testing: The Young's moduli were determined for all HAPEG hydrogels as described previously.[12] Briefly, the hydrogels were pre-swollen in PBS for a day and formed into cylindrical samples with a diameter of 5 mm. 100 µl of hydrogel were used for each test, which, depending on the swelling of the hydrogels, ranged from 2200 to 2800 µm 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^{-1} 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.

Statistical Analysis: All data are presented as the mean of an $n \geq 4$ 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 two groups, and one-way ANOVA with Tukey's post hoc 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 $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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