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Pluripotent Stem Cells as Models of Retina Development

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Abstract

The ability of pluripotent stem cells (PSCs) to differentiate into retinal tissue has led to many attempts to direct this process to yield specific retinal cell types. The ability to do so would greatly impact both the study of normal retina development in model systems that can be precisely controlled and the generation of a homogeneous population of cells optimized for transplantation in cell replacement therapy. Thus far, many reviews have focused on the translational potential of PSC retinal studies. Here, we focus on the former by summarizing the advances and reflecting on the current limitations to using in vitro differentiation of PSCs into retinal cells and organoids to model in vivo retinal development, with a specific emphasis on photoreceptors. We discuss the versatility of PSC retinal differentiation systems in investigating specific developmental time points that are difficult to assess with classic developmental model systems as well as the potential for efficient screening of factors involved in regulating photoreceptor differentiation. PSCs can be used in conjunction with existing model systems to contribute to the understanding of retina and photoreceptor development, which in turn can enhance the success of using stem cells in translational studies.

Keywords Retina · Photoreceptor · Development · Differentiation · Pluripotent · Stem cells

Introduction

Our knowledge of retina development has traditionally been derived from numerous experiments using spontaneous genetic mutants, whole embryo manipulations, and retina explants. These classic studies have clearly demonstrated that in vivo model systems are well suited for uncovering signal pathways that play a role in early embryonic neurogenesis and retinogenesis as well as in perinatal retina development. This includes regulation of transcription factors and detailed histological characterization of the laminated retinal layers, cell populations, and expression patterns of marker genes [1–6]. However, the shortcomings of these classic approaches using in vivo derived samples are the difficulties in supply of, and access to, embryonic ocular tissue. This has made more precise time-dependent and mechanistic investigation into the role of molecular signaling pathways in regulating transcription factors involved in lineage determination of specific retinal cell types difficult.

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More recently, in vitro pluripotent stem cell (PSC) differentiation has been used to study retina development. The value of this approach has been greatly aided by the existing knowledge of cell type-specific marker genes to identify target populations at various time points during differentiation. PSCs provide a model system that can be more precisely controlled than in vivo systems and have already contributed to our understanding of retinal development.

The optic vesicle, optic cup, and eventually, the neural retina develop from a portion of the anterior neuroepithelium and are denoted by coexpression of several transcription factors including RAX, SIX3, LHX2, SIX6, OTX2, PAX6, and VSX2, which are collectively called eye field transcription factors (EFTFs). Closure and evagination of the neural tube results in bilateral outpouchings that invaginate to form the optic cup. One layer of this structure subsequently forms the retinal progenitor cells (RPCs) of the neural retina, and the other layer forms the retinal pigment epithelium (RPE). The RPE is responsible for producing some of the molecular signals that influence downstream RPC differentiation and for providing metabolic support for the photoreceptors in the mature retina. The RPCs differentiate in a temporally conserved sequence to generate the seven cell types endogenous to the retina: retinal ganglion cells, horizontal cells, amacrine cells, cone and rod photoreceptors, bipolar cells, and Muller glia [7, 8]. Rod photoreceptors comprise over 70% of the total population of rodent retinas and detect dim light [9]. Cone

photoreceptors are capable of detecting light of different wavelengths. In humans and other trichromats, three cone types respond maximally to long, medium, and short wavelengths and are geographically concentrated in the macula region and account for much of detailed vision. Rodents have a central area of the retina with high density of photoreceptors and are dichromats with cones responding to short and medium wavelengths [10]. Dogs, cats, and pigs also lack macula and instead have the cone-rich macula-like region termed the area centralis [11–14].

Loss of photoreceptors in retinal degenerative diseases such as retinitis pigmentosa and age-related macular degeneration results in the irreversible loss visual function. The potential use of PSC-derived cells to replace the cells lost in an array of degenerative diseases has driven investigation of the therapeutic implications of stem cells in disease modeling and management. This approach is especially relevant for neurodegenerative diseases because mammalian neural cells are restricted in their capacity to repair and regenerate. In addition, the retina has the additional advantage of being easily accessible both for transplantation procedures and for non-invasive monitoring of outcomes. The ocular space has immune privilege, with the subretinal space being most immune privileged, and therefore may pose less risk for transplant rejection [15, 16]. However, neuronal cell replacement in animal models of retinal degenerative diseases have resulted in variable but generally low percentages of cells, ranging from approximately 0.4 to 6%, that survive and integrate into the proper layer within the retina [17–20]. These numbers were achieved after optimizing the differentiation stage of transplanted cells to correspond to those in early postnatal mouse retina, which has been shown to improve cell survival in both PSC and tissue-derived photoreceptor precursors [18, 21] and is associated with a lower risk of teratoma formation [22]. The number of transplanted cells in the ONL is directly related to successful functional rescue measured by optomotor testing [17, 21]. Maximal functional rescue thus far is 0.1 cycle/degree of scotopic visual acuity and scotopic contrast sensitivity of 2, compared to 0.25 cycle/degree and contrast sensitivity of 10 in wild-type mice. This limited and variable posttransplant success may be improved by detailed characterization and manipulation of the mechanisms governing development of the specific cell types that are affected in disease processes.

In theory, PSCs can be differentiated to yield large quantities of readily accessible, lineage-specific cells, and the gene expression patterns of these cells can then be compared to those of native tissue in order to determine whether the differentiation process can mimic steps during in vivo development. If so, the differentiation process would provide a robust and efficient platform to study how the interactions between extrinsic signals and gene expression govern the transition from PSC into presumptive eye field and early eye structures containing multipotential RPCs which further differentiate into the seven distinct cell types of the neural retina. In doing so, developmental studies can uncover the effects of and mechanism behind molecular pathways that influence photoreceptor development so that precise temporal control of these processes can result in efficient derivation of photoreceptors with therapeutic potential.

ESCs Can Be Differentiated Along a Retinal Lineage

A variety of in vivo studies, using fly and vertebrate genetic mutants, injection of signaling effectors into *Xenopus* embryos, and tissue explants have identified factors involved in neural and retinal lineage determination that have since been applied to PSC differentiation. These in vivo models showed that the anterior identity of the neural tube is guided by the dual inhibition of Wnt and bone morphogenetic protein (BMP) signaling while the presence of fibroblast growth factor (FGF) and retinoic acid signaling drive posterior neuralization [23–25]. Similar experiments revealed that the expression of EFTFs is negatively regulated by Wnt and BMP signaling and positively regulated by the Notch and insulin-like growth factor (IGF) pathways, in part through interactions within morphogen gradients formed by the ligands secreted by surrounding tissues [26–29].

The first studies of PSC differentiation into retinal lineages used mouse embryonic stem cells (ESCs) in the early 2000s (Fig. 1). Different cocktails of pharmacologic agents and growth factors exerted specific morphogenic effects identified through in vivo experiments (Fig. 2). Work conducted with ESCs differentiated in the presence of basic fibroblast growth factor (bFGF), either with or without supporting stroma, yielded high percentages of neural cells and were capable of generating retinal cells [32, 33]. However, these studies utilized different methods in analysis of retinal lineages such as by proportion of colonies that formed eye-like structures [32] versus expression of neural stem cell markers such as NESTIN and PAX6 [33]. The choice of photoreceptor marker genes varied between multiple members of the phototransduction cascade, but both studies used the photoreceptor outer segment protein rhodopsin (RHO). The yield of RHO+ cells was up to 6% even after 15 days of coculture with perinatal retinas to mimic the native developmental environment [33]. While these studies indicated the potential for PSC retinal differentiation, direct comparison of differentiation efficiency cannot be assessed due to variations in culture technique and analysis. Later experiments used a combination of several exogenous factors, such as Wnt inhibitor Dikkopf-1 (DKK1) and members of the transforming growth factor β (TGFβ) superfamily including LeftyA and Activin A, and the percentage of differentiating colonies expressing RAX increased from 1 to 16% [34].

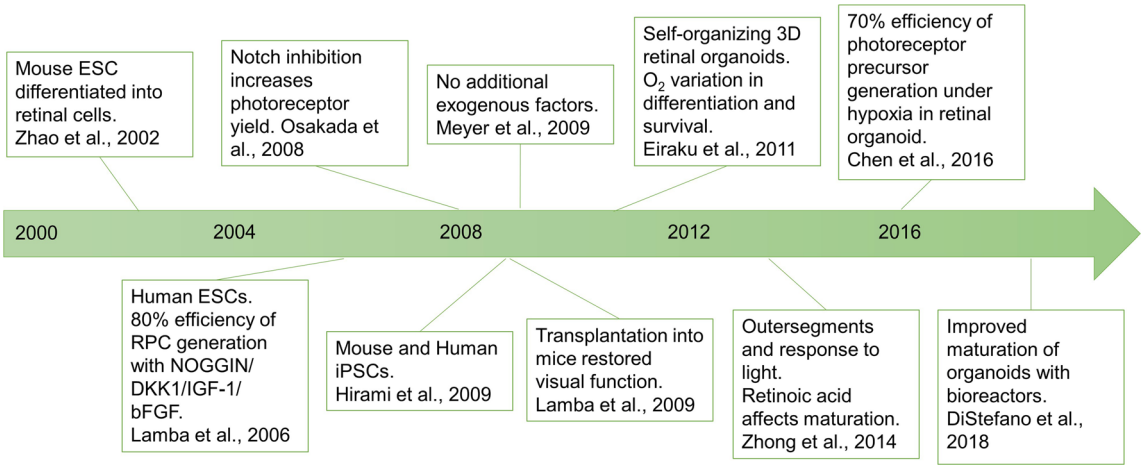

Fig. 1 Timeline of select reports of retinal differentiation. An abbreviated representation of key experiments that summarize the evolution of PSC retinal differentiation. Refer to the text for more comprehensive description and interpretation of the studies

PSC retinal differentiation protocols rely heavily on the expression of cell type-specific marker genes to estimate yield. This was at least partly due to the preservation of the developmental sequence of EFTF and cell-type specific gene expression among PSC retinal cell cultures that were derived using different in vitro differentiation protocols (Fig. 2). However, not all studies use the same marker genes or groups of marker genes to identify certain cell populations. For example, RPCs can identified using neural stem cell genes such as NESTIN and PAX6, as well as EFTFs including RAX, LHX2, SIX3, SIX6, OTX2, and VSX2. This makes direct comparison across studies difficult because the cells identified by different combinations of marker genes may represent the total population or different subpopulations of RPCs, or RPCs at different stages of maturation. Similarly, genes such as recoverin and various opsins that represent more mature photoreceptors are utilized in some studies but not in others. In addition, many of these genes also exhibit different patterns of temporal expression throughout retina development [2]. Therefore, it is important to critically assess the choice of marker genes used to report differentiation yield when comparing across studies. Ideally, histological analysis of coexpression of marker genes should be implemented to better identify subpopulations of cells that express different combinations of these genes. An example of this would be identification of RPCs via coexpression of PAX6, which is expressed in neural stem cells, RPCs, and inner retinal neurons, with VSX2, which is expressed in RPCs and retinal bipolar cells in order to more precisely describe the population identified as RPCs to be PAX6+/VSX2+ and therefore unlikely to be another cell type in which either of these genes could be expressed separately [4, 7].

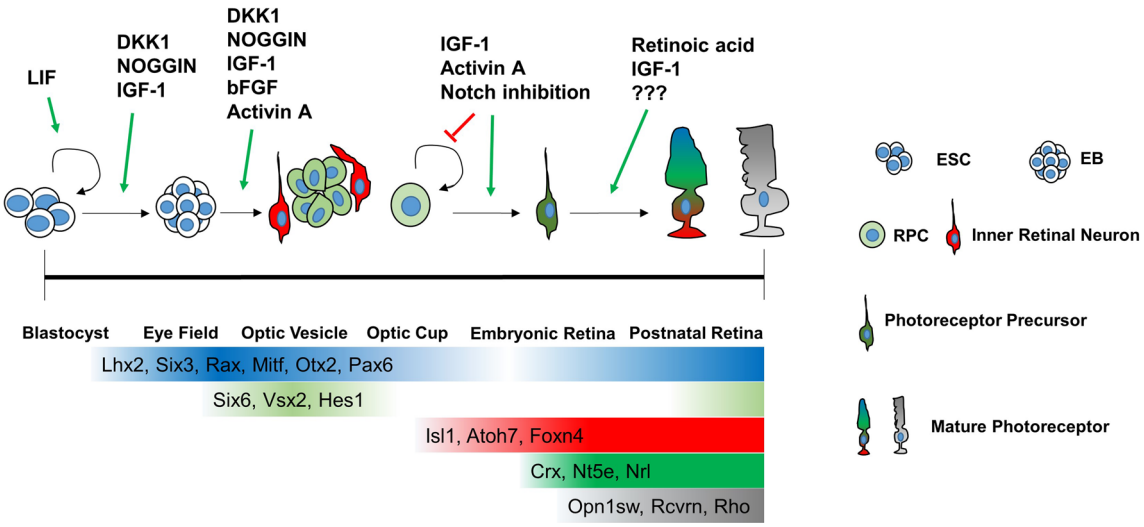

Fig. 2 Schematic of effect of select signaling molecules on specific stages of PSC photoreceptor differentiation. In vitro differentiation begins with inhibition of PSC self-renewal, then step-wise introduction of factors that activate or inhibit signaling pathways involved in retinal lineage determination. The development and correlating differentiation stages are aligned with selected stage- and cell-type specific marker genes based on summarized expression patterns from [2, 5, 20, 30]. Image adapted from [31]

The first use of human ESCs to generate retinal cells employed multiple exogenous factors—this time DKK1, bone morphogenetic protein (BMP) inhibitor NOGGIN, insulin-like growth factor 1 (IGF-1), and bFGF [35] (Fig. 1). Retinal cells were derived following embryoid body (EB) induction of ESCs followed by serum-free adherent culture of undissociated EBs on a Matrigel substrate, which contains basement membrane proteins such as integrins, entactins, and laminins as well as some growth factors. It reported a greatly increased efficacy of RPC yield, 80% as defined by coexpression of PAX6 and VSX2, compared to previous work. It also used calcium imaging techniques on ESC-derived inner retinal neurons, which represents the first testing of some neuronal functionality postretinal differentiation. This protocol has since been shown to generate retinal populations with expression patterns that mimic the temporal sequence of retinal cell type generation, as defined by expression of marker genes, present in human fetal retina and the resultant RPCs are able to migrate, form synapses, and cause some improvement in electroretinogram measures of visual function in recipient mice [36–38]. However, the yield of photoreceptor precursors expressing the marker gene CRX was ~ 12% while the proportion of cells expressing RCVRN, a protein involved in phototransduction, was only 6% even after retina coculture for 6 days. The general failure of ESC-derived RPCs to generate photoreceptor precursors and mature photoreceptors suggests that appropriate signals are absent in the protocols described thus far.

Generation of Photoreceptors from PSCs

The primitive retina can be identified and manually dissected from surrounding ocular tissue following formation of the optic cup. Experiments using primary mammalian retinal cells dissociated after dissection and retinal explants have shown that Notch, signal transducer and activator of transcription 3 (STAT3), and SHH signaling pathways maintain RPCs [39–42] while inhibition of Notch or STAT3 caused terminal differentiation [42–45]. Specifically, Notch signaling maintains proliferative RPCs and needs to be inactivated at specific points during development for neural retinal cell types to arise in their temporally conserved sequence [41, 44].

Manipulation of Notch signaling has been explored as a potential way to improve photoreceptor yield from PSC differentiation. Multiple groups using human and mouse ESCs and induced pluripotent stem cells (iPSCs) have reported that addition of Notch inhibitor DAPT can improve the yield of photoreceptors 2–3-fold by inducing exit from mitosis and competence-dependent commitment to cell fate [46–49]. Interestingly, these protocols utilizing DAPT to improve photoreceptor differentiation were performed on cultures that are enriched for RPCs—generally through dissection or sorting of cell populations that express RAX, an important EFTF. DAPT does not induce improved photoreceptor production in unsorted populations, suggesting that cells must be at a stage capable of yielding photoreceptors or that off-target effects of DAPT on non-RPC populations can negatively impact photoreceptor differentiation [46]. Taken together, the series of reports using DAPT are promising proof-of-concept works that demonstrate how extrinsic signals involved in photoreceptor development behave similarly in PSC retinal cultures and samples obtained from in vivo retina. Of note, Notch signaling interacts positively with the STAT3 pathway to maintain neural stem cell populations in other regions of the central nervous system [50, 51]. STAT3 activation decreased rhodopsin expression and inhibition of STAT3 by IGF-1 or protein kinase C resulted in increased photoreceptor maturation in native retina and explants [43, 45, 52]. This suggests that Notch could be acting through or in concert with STAT3 in PSC-derived retinal cultures and inactivation of STAT3 could be a future approach to target photoreceptor maturation in these systems. New STAT3 inhibitors are actively being developed for other applications, such as cancer research, and these novel agents could potentially be applied to PSC retinal differentiation.

Highly comparable protocols that allowed comparison of mouse and human-derived ESCs and iPSCs revealed similar yields of cells expressing EFTFs and photoreceptor markers [46, 53]. However, iPSCs generally required longer culture times in order to progress to comparable stages of differentiation. For example, mouse ESC differentiation yielded 15% RAX+ cells after 9 days and RHO expression was detected in the RAX+ sorted cells beginning on day 28 while mouse iPSC differentiation had no detectable RAX expression at day 9, 22% RAX+ at day 15, and rhodopsin expression was first described at day 45. This delay may be due to intrinsic differences in these cell lines or variability in the culture medium needed to maintain the pluripotency of the undifferentiated culture. For reasons that are thus far unexplained, human PSCs yield higher proportions of CRX+ photoreceptors compared to mouse PSCs (12% in mouse, 25–35% in human) [46]. This may be due to the differing emphasis on cones, which make up the majority of human central vision, in comparison to the rod-dominant rodent retina and the demonstration of a cone-default pathway in photoreceptor determination in the absence of rod-specific cues [54]. Another important detail in cell line comparisons is that while retinal induction of mouse PSCs generally requires 20–35 days, human PSCs require approximately 150–200 days to achieve comparable developmental stages. This, in addition to the observation that expression of photoreceptor markers is reproducibly dependent upon prolonged time in culture [33, 46, 48], puts emphasis on growth conditions and factors that improve differentiation and survival outside the in vivo environment.

Impact of Exogenous Factors and Intrinsic Cell-Line Properties on PSC Retinal Differentiation

Previous studies using in vivo samples and the PSC retinal work discussed thus far show that extrinsic signaling is important for the proper formation of the retina [55–58]. However, several landmark studies were able to achieve retinal differentiation using different types of PSCs without the addition of exogenous factors. These studies generally initiated differentiation using EBs and then grow the cultures on Matrigel in medium containing serum-free supplements. Following induction, the areas displaying optic cup-like morphology were manually isolated. These isolated regions displayed remarkable architectural similarity to in vivo embryonic retinas with invaginated structures, distinct RPE and neural retina regions, apical RPCs, and differentiation of inner retinal neurons [59]. The yield of CRX+ photoreceptor precursors from human ESCs and iPSCs after dissecting the ~20% of the overall culture that was VSX2+/MITF+ and displaying retinal morphologies was 65%, with 50% of these progressing to express opsin proteins associated with maturing photoreceptors [60]. Despite the lack of additional exogenous factors in the culture medium during the initiation of retinal induction, the differentiating cells in these studies endogenously expressed *DKK1*, *NOGGIN*, and *FGF* soon after induction [48, 60, 61]. Furthermore, activation of BMP and Wnt pathways in these cultures caused drastic reduction in expression of EFTFs, indicating that the same signaling mechanisms were present in cultures that did not have exogenously added ligands. As well, Matrigel contains additional factors that may mimic the interactions with extraocular tissue in addition to the adherent basement membrane properties because purified entactins and laminins alone were unable to induce *Rax* expression to the level of Matrigel alone, unless NOGGIN was added to culture [62]. Serum-free supplements used with or in place of serum in these culture conditions included progesterone, insulin, and other additives that might interact with signaling pathways involved in retina development. Thus, though these culture approaches did not supplement with signaling molecules, they were not factor-free.

There is growing evidence that variation in eye induction efficiencies observed among cell lines, in one study ranging between 30 and 70% RAX+ [62], may be due to intrinsic differences at the transcriptomic and epigenetic levels. While PSCs cluster together at both these levels and differ drastically compared to differentiated somatic cells and cancer-derived cells, there are distinct variations between gene expression and methylation signatures between different PSC lines [63–65]. These variations, such as maintenance of hypermethylation at differentiation loci in certain ESC lines but not others, as well as dissociation of methylation versus expression levels, have been implicated in the propensity of distinct PSC lines to favor differentiation along certain lineages. For example, while ESCs tended to have uniform DNA methylation patterns and gene expression levels for genes associated with an undifferentiated state such as PSC marker SOX2, the expression levels of genes involved in development such as PAX6 varied. Further, some lineage-specific genes such as myeloid surface receptor CD14 exhibited both variations in methylation and expression [63]. In addition, expression levels of mediators in several signaling families, such as Wnt, TGFβ, and IGF-1, varied over 10-fold among different human ESC lines [64]. These intrinsic differences may be due to differences in PSC derivation, maintenance, storage, and tissue source. This is especially important to consider in iPSC experiments due to potentially large variations in donors, starting tissue source, and reprogramming protocol which may impact the endogenous expression levels of components of signaling pathways and differentiation loci. For example, iPSCs that were derived from photoreceptors exhibit higher efficiency of retinal differentiation compared to those derived from fibroblasts by virtue of epigenetic memory [66, 67].

Ultimately, caveats to "factor-free" approaches suggest that while they are useful for studying differences in intrinsic programs, differentiation protocols using exogenous factors may better reproduce efficiencies between cell lines by at least partially negating the effects of intrinsic differences so that novel roles of extrinsic pathways can be better investigated. It is also important to consider the propensity of the PSC line to differentiate along a retinal lineage and verify results in multiple cell lines.

Role of Spatial Configuration of the Culturing System on Differentiation

There is growing appreciation that three-dimensional (3D) organization of cultures can have profound effects on differentiation. Organoid cultures are 3D systems that allow for growth of multiple cells and cell types in clusters suspended in either liquid medium or in semi-liquid matrix and are thought to mimic some in vivo tissue architecture. Organoid cultures obtained by 3D reaggregation of dissociated tissue-derived retinal cells helped trace proliferation of RPCs and the spontaneous spatial organization of their daughter cells in the 70s–80s [68–70]. These studies were important in identifying that rod photoreceptors and Muller glial maturation rely on cell interactions and that molecular signals, likely from the RPE, play a role in correct laminar organization. More recently, organoid cultures have been generated from PSCs to model development of multiple organ systems. This novel application of the traditional culture system was pioneered by research in differentiation of PSCs into cortical and retinal lineages [62, 71, 72]. Within these organoids, multiple loci of

early ocular tissue can be identified as tightly packed ribbon-like structures that express EFTFs such as RAX (Fig. 3). Of these new retinal organoid studies, the serum-free culture of EBs with quick reaggregation (SFEBq) protocol that involves entirely 3D culture in a Matrigel matrix, commonly using a *Rax*-expressing transgenic reporter ESC line, has been further optimized by early culture in hypoxic conditions to better mimic in vivo environments during retinal specification followed by culture in hyperoxia to improve long-term survival [18, 62, 74]. While the original SFEBq approach with dissection of morphologically identified curling ribbons of optically clear neural retina can yield retinal structures, most of the regions within EBs that express EFTFs are discarded after dissection and extracellular signals from discarded areas may be lost. Therefore, retinal induction following SFEBq has been performed without dissection by visual inspection but instead subjected to trisection of the organoid without selection for a specific morphological area, and oxygen levels were then adjusted to allow sufficient contact with matrix components and nutrients for development and survival [47]. Cells obtained from these modified protocols retained their ability to respond to exogenous cues such as Notch inhibition and together. These optimizations have improved the generation of photoreceptors arising from retinal regions of 3D organoids from 35 to over 70%. Injection of the immature photoreceptors produced by this method demonstrated that the cells can integrate into recipient retinas and respond to electrophysiologic stimuli such as calcium efflux, patch clamping, and light stimuli [18, 19, 47, 62, 74–76].

Despite these advances in yield of photoreceptors from retinal organoids, these 3D cultures generally involve more laborious mechanical isolation and specialized tissue culture setups involving rotators, bioreactors, and oxygen systems to ensure sufficient nutrient exchange for long-term survival [77, 78]. Quantification of photoreceptors within these structures actually do not show marked improvement over the maximal reported proportions of photoreceptors from neural rosette structures in adherent cultures (~ 60–70%), which can also yield cells capable of integration into recipient retinas [37, 61, 79]. The advantage of 3D cultures arguably lies not in their ability to yield RPCs or photoreceptor precursors. Instead, these systems are excellent for studying dynamics of retinal folding and the later stages of photoreceptor development involved in functional maturation. The exciting capacity of PSC retinal differentiation to produce functional photoreceptors that respond to light stimuli, as opposed to electrophysiological or pharmacological stimuli, in a system completely lacking in vivo derived cells or tissue, which were used in retina explant coculture and posttransplantation studies, was first demonstrated using a 3D organoid system [75]. That report and others have shown that prolonged 3D culture with manipulations to improve survival, such as hypoxia and

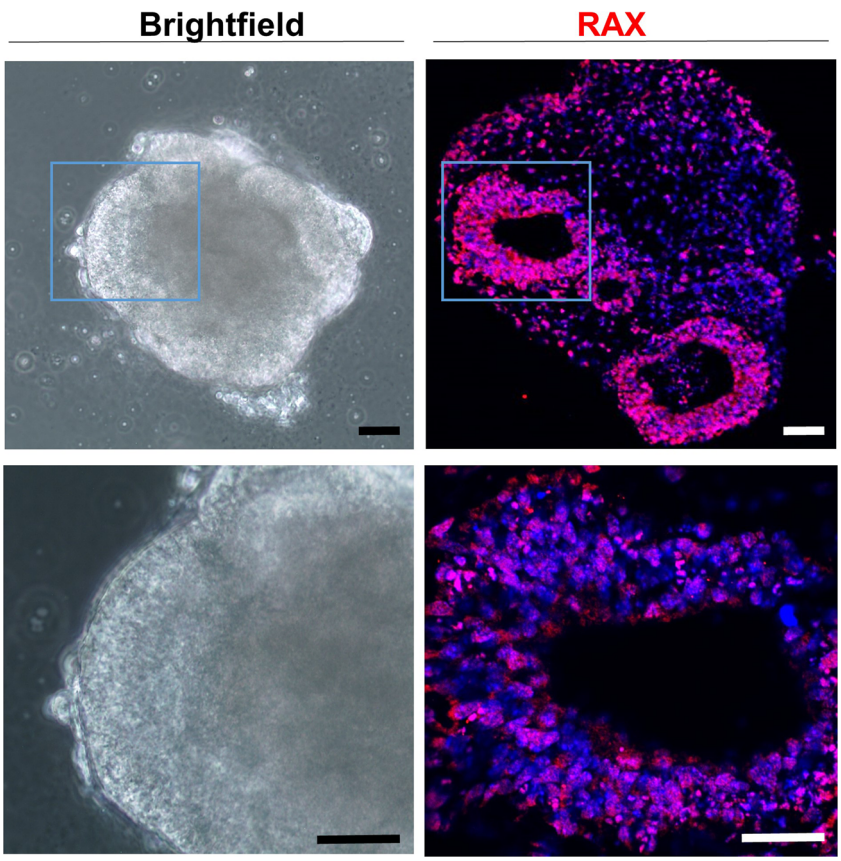

Fig. 3 Three-dimensional organoids contain specific regions that express EFTFs. Representative images of three-dimensional organoids grown to 9 days in vitro following the protocol detailed in [73]. Organoids were either imaged live under brightfield microscopy (left column) or fixed for immunofluorescent detection of RAX (red, right column, Abcam ab86210). Insets are shown in higher magnification in bottom row. Scale bar, 50 μm

retinoic acid, result in multiple layers of photoreceptors within the presumptive outer nuclear layer (ONL) containing inner and outer segments with emerging cilia and small discs [75, 76, 80]. The electron microscopy images of these primitive outer segment discs that are small, few in number, and not stacked resemble those of early postnatal rodent retinas before the peak of functional maturation and eyes opening [81, 82]. Experiments on *Xenopus laevis* have revealed that proper outer segment development requires factors secreted by the pigment epithelium such as polysaccharides and pigment epithelium derived factor [83–85]. The same factors may be required in PSC-derived photoreceptor maturation. While the ability of PSC-derived photoreceptors to morphologically and functionally mimic early photoreceptors highlights the achievements of in vitro retinal differentiation thus far, it also reveals our gap in knowledge regarding the process of photoreceptor maturation.

PSC Retinal Differentiation to Investigate Mechanisms Regulating Development

The PSC retinal differentiation system offers an easily accessible way to control signaling events during culture times that correspond to specific in vivo developmental stages. This allows more detailed and individualized studies of specific factors that are difficult to do in an intact animal or tissue. Furthermore, this system allows investigation into the mechanism behind the phenotypic effects of these signaling pathways at multiple temporal stages. The work thus far has proven that all approaches to in vitro retinal differentiation have roughly conserved the sequence of EFTF expression and retinal cell type appearance that occurs in vivo and has demonstrated that existing knowledge of signaling garnered from in vivo studies can be used to improve in vitro differentiation efficiency [20, 30, 35, 47, 72]. However, there have been fewer studies that take advantage of the potential of PSC differentiation to robustly screen for and investigate regulatory mechanisms.

Several groups have utilized of the accessibility and ease of precise control of PSC differentiation to elucidate the interactions between ligands of the TGFβ, FGF, Wnt, and IGF-1 signaling pathways during eye field formation and generation of RPCs [86–88]. These experiments demonstrated the importance of specific temporal activation or inhibition of pathways, such as IGF-1 and BMP, respectively, at this early stage of development that would determine the proportion of cells that would form the eye field. Recent work has also demonstrated the utility of PSC differentiation in identifying the role of SoxC transcription factors in promoting differentiation into retinal ganglion cells [89]. Nevertheless, a large portion of these data have already been reliably generated from classic developmental biology model systems using in vivo samples [5, 23, 26]. An advantage of PSC differentiation models lays in the time frames during development that are covered relatively poorly by previous model systems. Very early human retinal development is especially challenging to investigate and utilizes postmortem fetal samples that span a relatively long gestational period [90, 91]. Early embryonic stages leading up to formation of early embryonic ocular structures has generally been studied in non-mammalian whole embryos while maturation of specific cell types can be studied in postnatal mammalian eyes. Therefore, PSC differentiation seems to be appropriate for studying the periods ranging from the formation of the retina to just prior to birth, which is approximately embryonic day 12 (E12) to E18 in mice. This corresponds to the periods when RPCs become committed to the photoreceptor lineage.

To illustrate this concept, our reports of the effect of Activin A on promoting generation of photoreceptor precursors from ESCs fill an important gap in knowledge regarding the transition from proliferating RPCs to postmitotic photoreceptor precursors [31, 73]. Investigation into the mechanism of Activin signaling revealed that it acts through SMAD2/3 effectors which can directly bind to regulatory regions of key cell-type determining genes [31]. Further, Activin A treatment decreased the number of PCNA+ cells and resulted in a 3-fold increase in photoreceptor precursor yield without fluorescent sorting of transgenic reporter-tagged cell lines, implying that manipulation of Activin signaling might have translational use (Fig. 4).

Current State of PSC Retinal Differentiation

The PSC experiments described previously have shed light on the importance of understanding the interactions between intrinsic factors and extrinsic cues to guiding the differentiation process into a progressively restricted eye, then retinal, then photoreceptor fate. They also reveal several potential bottlenecks to this process: (1) the assumption of early eye identity, as measured by expression of EFTFs; (2) the production of RPCs from EFTF-expressing regions; (3) the transition from RPCs into photoreceptor precursors; and (4) the maturation of precursors into functional photoreceptors. The first 2 points have been well-characterized by applying knowledge from early transplantation and gene manipulation work performed in vivo. For example, PSC retinal differentiation systems have been able to recapitulate the developmental role of IGF-1 signaling in promoting eye formation [88, 92]. They have also confirmed that modulation of WNT and BMP signaling by endogenously expressed DKK1 and BMP are necessary for efficient induction even in protocols that describe lack of exogenous application of these factors [35, 60, 62]. Other studies have demonstrated the utility of using the ESC induction model to test interactions of multiple signaling pathways at

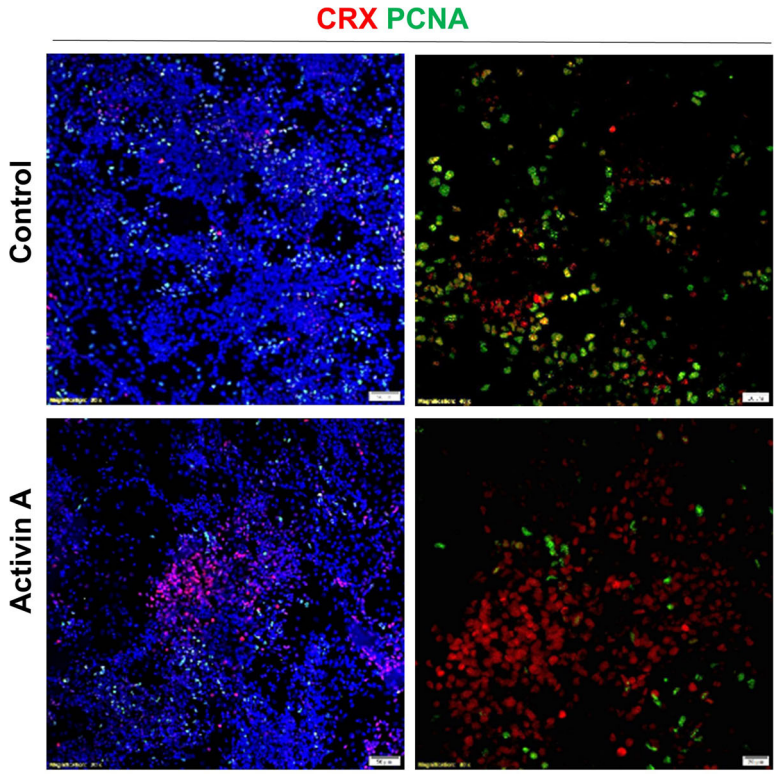

Fig. 4 Time frame-specific effect of Activin signaling on proportions of proliferating RPCs and photoreceptor precursors. Activin A treatment on ESC-derived RPCs at a time corresponding to early embryonic retinogenesis results in decreased PCNA+ RPCs (green) and 3-fold increase in proportion of CRX+ photoreceptor precursors (red). Image adapted from [31]

restricted time windows during neural and eye induction and one group uncovered a novel role of the Activin signaling pathway in promoting eye field and neural retina despite the traditional view of Activin being largely involved in RPE formation [86, 87]. Overall, these studies have shown a time-specific effect of multiple signaling pathways to increase the efficiency of photoreceptor generation. The key steps in this process appear to be the inhibition of Wnt and BMP pathways in order to induce anterior neural identity, then activation of IGF-1 and Activin pathways to increase specification of ocular lineage, followed by further patterning by FGF and retinoic acid, and eventually Activin A, IGF-1, and retinoic acid for photoreceptor differentiation. These experiments prove that the response of PSC-derived retinal cultures to these signals is similar to that of retinal tissue and that the differentiation system may be used as a tool for screening novel factors that regulate retinal development.

Discussion

The expression of the ligands and receptors of signaling pathways change with the developmental age of the retina [52, 93]. This suggests that there is an intimate relationship between the intrinsic expression of certain genes within the retina during specific temporal windows and the extrinsic factors that the cells are exposed to in the final decisions regarding cell fate. Photoreceptor lineage determination and maturation are controlled by a complex gene regulatory network that involves a large array of transcription factors whose activities are dependent upon intrinsic cues and external signals that are not fully understood. Careful dissection of the processes that control photoreceptor lineage determination and subsequent alteration of these processes can improve yield of photoreceptor-lineage committed precursors from PSC retinal differentiation. Due to the nature of the starting cells and the complex interactions required, it may not be possible to achieve 100% yield of photoreceptors from this approach. However, attempting to achieve a differentiation efficiency so that the proportion of photoreceptors is similar to in vivo retina would be worthwhile for understanding the cell biology mechanisms and molecular environment required for correct in vivo development. This would also improve the efficacy and safety of any downstream therapeutic applications.

PSC retinal differentiation offers an easily accessible way to control signaling events during culture times that correspond to specific in vivo developmental stages. This allows more detailed and individualized study of specific factors that is difficult to do in an intact animal or tissue. The main advantages of this approach are as follows: (1) the ability to follow precisely timed manipulations longitudinally in the same model system, (2) the time frame of PSC differentiation spans mid-embryonic periods of development that are difficult to efficiently assess using classic model systems, and (3) the

relative ease of investigating the mechanism behind phenotypic effects of these signaling pathways at multiple time intervals through genome editing or changes in culture environment afforded by the in vitro experimental setup.

Experiments can be designed to effectively utilize PSC retinal differentiation in modeling development if the advantages and limitations of the system are carefully factored. Work thus far has shown that PSC differentiation is a robust tool amenable to screening for factors that affect retina development. Novel advancements made using ex vivo models in uncovering molecular factors that regulate retina and photoreceptor development can be rapidly applied to PSC retinal models to test whether these phenotypic changes are retained in a differentiation model and if so, considered for translational studies. Examples of these potential novel agents include adenine nucleotides, micro-RNAs, and epigenetic modulators [94–96]. Figure 5 illustrates how these developmental studies fit into the pipeline of PSCs in regenerative strategies. First, the starting PSC type should be considered. Because the seeming advantage of PSC retinal systems is the ability to study mid-embryonic stages of retinal development during which RPCs commit to specific lineages, a potential approach would be to reliably and efficiently generate a largely homogenous population of RPCs to test downstream transition to photoreceptor precursors (Fig. 5a). However, the works citing high (~ 70%) yield of photoreceptor precursors largely originate from quantifying yield within subpopulations, for example, dissected neural retinal morphologies or laminated, viable regions of retinal organoids. Moreover, the studies demonstrating expression of mature photoreceptor markers, such as those proteins involved in phototransduction, require long periods in culture. Both the limited yield and long culture time restrict our ability to quickly generate large quantities of photoreceptor precursors to use in translational studies and to screen for novel factors involved in photoreceptor maturation that have not been previously identified using retina explants and primary retinal cultures. Therefore, a robust and reproducible differentiation protocol for screening factors that promote photoreceptor lineage commitment is a limiting factor in wider use of PSC retinal differentiation in basic and translational science endeavors. Due to practical considerations such as longer time needed for human PSC differentiation, technical demand, and cost to derive and maintain iPSCs, initial screening for exogenous factors that can alter the expression of lineage-specific genes as a measure of targeted differentiation are likely to first be conducted in mouse ESCs. Following identification of factors that screened positive, validation can be performed on retinal tissue or in vivo studies, which are generally more work intensive for the early developmental stages studied and therefore may be unsuitable for screening processes. A recent example of this is the use of COCO, a Cerberus/Dan family member, which increased cone photoreceptor yield in ESC differentiation, and these effects were recapitulated using retinal tissue-derived cells [79, 97].

Importantly, even among mouse ESCs, different cell lines have are varying propensities for differentiation to certain lineages [63–65]. We have noted differences in temporal expression of ligands and receptors of signaling pathways implicated in retinal development among two different mouse ESC lines, which impacted the temporal effect of Activin A treatment on these cell lines [31]. This is relevant when considering therapy using patient iPSCs because epigenetic memory based on the tissue of origin can affect the differentiation efficiency [67, 98, 99]. Together, these highlight the importance of interpreting

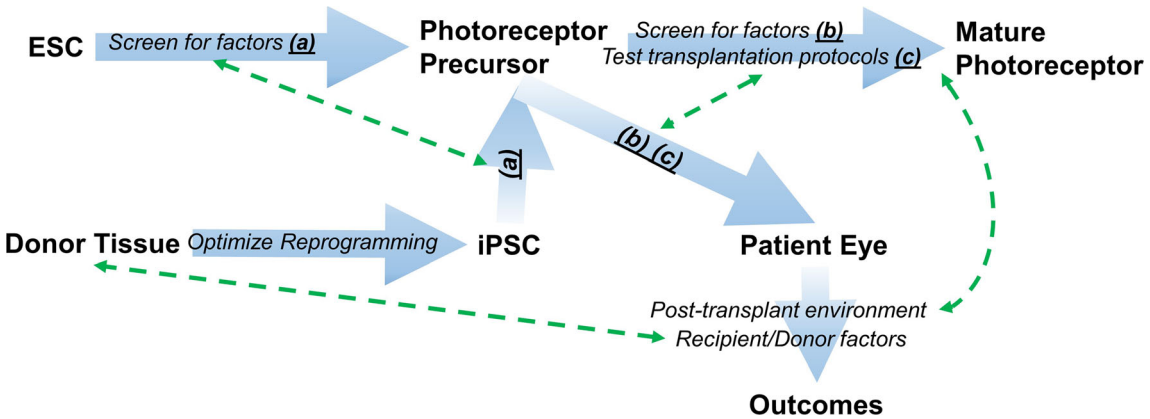

Fig. 5 Proposed pipeline for regenerative approaches using PSC-derived photoreceptor precursors. ESCs should be utilized for initial experiments due to relative ease to obtain and lower time and cost of culture. Screening for factors that increase photoreceptor precursor yield can thus be robustly performed in a precisely controlled environment (**a**). The mechanism behind these factors can be investigated to understand development and to improve precision of the effect of manipulating their downstream targets. The resulting photoreceptor precursors can be used to further screen for molecules that drive their maturation (**b**), which would affect considerations for optimizing the recipient environment. These precursors can also be applied to testing of various transplantation protocols (**c**). After the derivation of photoreceptor lineage-committed precursors and/or the transplantation protocol have been improved, iPSCs can be used to verify the process. Discrepancies between the results of iPSC and ESC experiments as well as between different donor tissues and genetic backgrounds are suitable for further investigation using an in vitro differentiation system (dotted green arrows)

the effects of signaling pathway manipulation in the context of the expression levels and patterns of the pathway components during the time of treatment as well as in comparing the relative expression of these components between different cell lines. Specifically, temporal and absolute comparisons of expression of the receptor of any exogenously added ligand or inhibitor should be carefully characterized so that the agent is added at a biologically meaningful time point, which is not necessarily preserved among cell lines. These variations should be accounted for during the design and adaptation of differentiation protocols between different PSC lines.

Further, due to the multiple steps of trained handling and specialized tissue culture setups associated with 3D culture and the reports that both 3D and adherent cultures are able to generate high yields of RPCs, adherent cultures are probably a more suitable system for initial purposes. Following the optimization of the derivation of photoreceptor precursors from RPCs, the cell population of interest can be isolated through multiple methods. Protocols with highly efficient generation of RPCs through the use of transgenic cell lines carrying retina-specific fluorescent reporters that facilitate cell sorting will be invaluable for further investigations in photoreceptor development using PSC retinal differentiation method. However, they are likely not relevant for human therapy due to ethical concerns and risk of genome integration as well as packaging of genetic material that are encountered in clinical translation of gene therapy approaches. Other protocols such as those using stepwise addition of a cocktail of exogenous factors [35], or manual isolation of populations through technically rigorous identification and dissection, have more general applicability to both experimental and clinical studies. Another alternative is to use cell surface markers to circumvent the need for transgene reporters. Several recent studies have shown the feasibility of a combination of photoreceptor markers in identifying and isolating cells of the photoreceptor lineage to examine factors involved in photoreceptor maturation (Fig. 5b) or to test transplantation techniques as well as effects of recipient environment on transplant outcomes (Fig. 5c) [30, 100, 101].

While PSCs are a robust source for cell replacement therapy, there is risk for teratoma formation in both ESC and iPSC-derived grafts due to persistence of mitotically active, undifferentiated cells after transplant into various tissues [102]. This is also concerning in retinal cell replacement, with up to 55% of transplants exhibiting tumor formation when the injected cells were at a stage corresponding to those in perinatal retina [22]. However, tumorigenesis after ocular transplantation decreased significantly following prolonged in vitro differentiation [22, 103]. Pre-clinical and clinical trials have further shown that RPE derived from multiple stem cell sources is capable of long term survival posttransplant without teratoma formation [104, 105]. Differences in the differentiation protocol and starting cell lines may contribute to varying tumorigenicity. Establishing efficient methods to address this risk is important to safe therapeutic application of PSC-derived retinal cells. The utility of PSC models can be seen in treating differentiating cells with Activin A, or other approaches to manipulate SMAD2/3 or its interacting factors, to manufacture postmitotic cells committed to the photoreceptor lineage without additional genetic manipulation in order to yield donor cells with decreased risk of tumor formation.

From a therapeutic perspective, the retina is host to an array of pathologic conditions resulting from dysfunction of the RPE and photoreceptors that lead to death of retinal neurons. Multiple laboratory experiments have demonstrated the capacity of PSCs to generate RPE that not only morphologically resemble tissue-derived RPE but is also able to phagocytose materials that contain rhodopsin [60, 106]. In general, the ability of transplanted RPE to delay disease progression is thought to stem from either RPE-specific functions such as phagocytosis or from secretion of factors that promote growth and survival. However, this would be insufficient to reverse existing damage due to neuronal cell loss. Studies in animal models have proven that PSC-derived photoreceptors can successfully integrate into the host retina, form synaptic connections, and ultimately restore some visual function in animal models of photoreceptor degeneration [18, 19, 37, 107–109]. Recent work has shown that purified PSC-derived cone photoreceptors can also integrate into recipient retinas [20]. Multiple research groups have carefully reviewed the therapeutic implications of experiments using either RPCs or photoreceptors derived from various stem cell sources [110–114] as well as PSCs in particular [115–117] in animal models of inherited retinal degeneration resulting from mutations in rod or cone-specific genes involved phototransduction. Despite the positive outcomes from these studies, the percentage of PSC-derived cells that successfully migrate into host ONL is only ~ 5% with many injected cells clumping in the subretinal space [18, 20]. This suggests that improvements in some or all aspects of the transplantation protocol, including optimization of the cells being transplanted, the injection or grafting technique, and the host environment are warranted.

This then prompts the question of what the target cell population for developmental and therapeutic studies should be. It is clear that cell–cell interactions including factors secreted by surrounding tissue, synapse formation, and spatial orientation are required for optimal recapitulation of retina development. Therefore, the argument can be made that a mixed population with proportions of specific cell types that closely resemble those of in vitro retina grown in a system that allows for gradient and substrate interactions would be ideal for further understanding how photoreceptors mature past time frames corresponding to the early postnatal period. This would likely involve techniques to improve nutrient delivery such as

bioreactors, size-controlling the organoids using specific microwell matrices, and interaction through a biofilm or scaffold with functional RPE [78, 118]. Conversely, the optimal end-product of PSC differentiation for therapy may depend on whether treatment is targeted at neuroprotection to slow the progression of disease or for cell replacement. In terms of neuroprotection, phase 1/2 clinical trials have investigated the use of human ESC-derived RPE in macular degeneration and while this work has demonstrated the safety and survivability of the graft, the long-term effect on vision is not yet proven [105, 119]. Other studies have focused on neuroprotection via injection of other biologics, including autologous RPCs, and these therapeutic trials have been recently reviewed in detail [117]. Therapy aimed at cell replacement is likely dependent upon the specific disease or disease state. For example, if the non-photoreceptor cell types within the recipient retina are largely intact and functional, then injection of a purified photoreceptor population may be ideal to maximize the number of transplanted photoreceptors that can potentially integrate. This number in turn is correlated to the degree of functional rescue [17, 120]. However, if the disease has progressed such that the ONL structure or other cell types are compromised, it may be suitable to transplant a retinal sheet. These retinal sheets are able to form synapses with host retinas, in some cases even at end stage retinal degeneration, and evoke cortical responses [121–123]. Recent works have further demonstrated integration, light reactivity, and optomoter responses after transplantation of mouse iPSC and human ESC-derived retinal organoid sheets [107, 124, 125]. Another factor to investigate is whether scar tissue in the setting of end-stage degeneration should be digested or disrupted in order for successful transplantation. In addition, while mature photoreceptors are capable of integrating into recipient retinas, they do so with decreased efficiency compared to cells equivalent to early postnatal stages [19, 126]. Screening of agents necessary for photoreceptor lineage commitment and maturation of photoreceptor precursors may help define environmental requirements for obtaining functional photoreceptors so that large populations of transplantable precursor cells can be obtained for transplant and survive and function in the recipient retina.

In summary, PSC retinal differentiation is a model system that can be used to study both retinal development and success of transplantation. One potentially effectively approach is to apply the knowledge of regulation of photoreceptor development obtained from screening with ESC retinal differentiation protocols to human and disease-specific model systems, such as human-derived iPSCs. Following this validation step, the screened agents can be used to efficiently generate large quantities of photoreceptors suitable for transplantation experiments. In this way, lessons from developmental biology and molecular signaling can facilitate efforts towards regenerative therapy.

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