Modeling early retinal development with human embryonic and induced pluripotent stem cells

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Human pluripotent stem cells have the potential to provide comprehensive model systems for the earliest stages of human ontogenesis. To serve in this capacity, these cells must undergo a targeted, stepwise differentiation process that follows a normal developmental timeline. Here we demonstrate the ability of both human embryonic stem cells (hESCs) and induced pluripotent stem (iPS) cells to meet these requirements for human retinogenesis. Upon differentiation, hESCs initially yielded a highly enriched population of early eye field cells. Thereafter, a subset of cells acquired features of advancing retinal differentiation in a sequence and time course that mimicked in vivo human retinal development. Application of this culture method to a human iPS cell line also generated retina-specific cell types at comparable times in vitro. Lastly, altering endogenous signaling during differentiation affected lineage-specific gene expression in a manner consistent with established mechanisms of early neural and retinal cell fate determination. These findings should aid in the investigation of the molecular events governing retinal specification from human pluripotent stem cells.

The study of human development is limited by a lack of model systems that can reproduce the precise sequence and timing of cellular and molecular events that occur during human embryogenesis, organogenesis, and tissue differentiation. However, the advent of human pluripotent stem cell technology affords a unique opportunity to follow the full course of lineage-specific cell production in vitro (1, 2). The retina provides an optimal system to investigate this potential due to its well-defined and conserved developmental program and the availability of markers to distinguish each major stage of early retinogenesis. In addition, human embryonic stem cells (hESCs) display a propensity to produce cells with retinal characteristics (3, 4). One criterion for assessing hESC-based developmental model systems is the capacity to recapitulate the normal maturation sequence present in the embryo in a controlled, stepwise fashion (1, 2). Preferably, such systems should also provide the opportunity to test the effects of developmental stimuli and enrich for early cell populations, thereby reducing contamination from undesired and/or unidentified cell lineages.

To date, hESC studies have focused on the derivation of subsets of retinal cell populations, with emphasis on the production of either retinal progenitors (5, 6) or more mature cells such as retinal pigment epithelium (RPE) (3, 4) or photoreceptors (7). Many of these studies used various exogenous factors to increase the percentage of early retinal cell types present within the mixed population of differentiating hESCs. However, despite these recent advances, the ability of hESCs to produce a highly enriched population of cells at the earliest stage of retinal specification that can progress through each of the key developmental stages of the retina has yet to be demonstrated. Moreover, the timing of onset of selected stages in retinal development has varied widely among published human pluripotent stem cell differentiation protocols, none of which approximated the timeline of normal human retinogenesis (5–9).

We addressed these issues first by examining each major step in the development of definitive retinal cell populations from hESCs. In doing so, we demonstrated that cell fate specification and maturation follows a sequence and time course highly reminiscent of normal retinal development. Furthermore, the process of retinal differentiation could be selectively altered via manipulation of endogenous developmental signaling pathways. We then investigated whether the same culture method was capable of generating an identical cohort of developing retinal cell types from human induced pluripotent stem cells, a recently described source of pluripotent stem cells derived from skin fibroblasts (10, 11). Cell populations expressing morphologic features and/or markers of the eye field, retinal pigment epithelium, neural retinal progenitors, photoreceptor precursors, and photoreceptors were observed in differentiating human iPS cell cultures at time points predicted by results using hESCs. These findings support a role for human pluripotent stem cells as in vitro model systems to investigate mechanisms involved in retinal specification and differentiation of individual retinal cell types.

Results

Eye Field Specification from Human Embryonic Stem Cells. The appearance of eye field cells within primitive anterior neuroepithelium is the first phase in the stepwise production of a retinal phenotype from an undifferentiated pluripotent stem cell (12, 13) (Fig. 1*A*). Previous reports have demonstrated that hESC-derived neuroectodermal cells will adopt anterior neuroepithelial characteristics in the absence of exogenous signaling molecules (14, 15). In the current study (Fig. 1*B*), hESCs were differentiated as free-floating hESC aggregates and prompted to adhere to laminin-coated culture dishes to permit neural rosette formation. After 16 days of differentiation, rosette-containing colonies were mechanically removed to grow as neurospheres. During this process, hESCs rapidly lost expression of the pluripotency genes Oct4 and Nanog and acquired expression of transcription factors associated with eye field specification (Rx, Six3, Six6, Lhx2, Tll), anterior neural specification (Otx2) and general neural induction (Pax6, Sox1, Sox2) (Fig. 1*C*). In RT-PCR experiments, Pax6 was present as a doublet band, reflecting the expression of both the Pax6(−5a) and Pax6(+5a) isoforms. The appropriate staging and lineage of this early cell population was further supported by the absence of the photoreceptor precursor-specific transcription factor Crx and the spinal cord-associated transcription factor HoxB4, as well as markers of other germ layers such as brachyury (mesoderm) and alpha-fetoprotein (endoderm). Immunocytochemistry showed that nearly all cells within these

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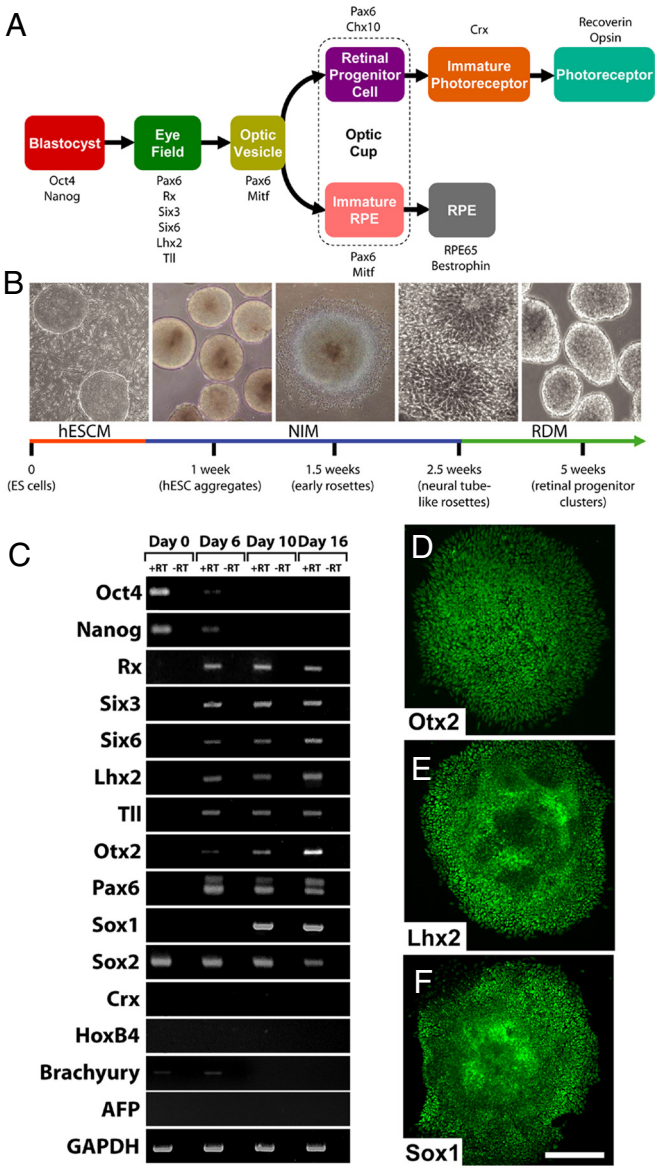

Fig. 1. Commitment toward a retinal lineage occurs as a stepwise process, beginning with the establishment of the eye field within the anterior neuroepithelium. (*A*) Each major stage in retinogenesis can be distinguished in part by the expression of various transcription factors. (*B*) Schematic of the differentiation protocol used to generate cells of a retinal lineage. (*C*) RT-PCR analysis of the changes in gene expression toward an eye field fate through the first 16 days of differentiation. (*D–F*) Immunocytochemistry of typical hESC aggregates 10 days after differentiation, demonstrating the expression of the anterior neural transcription factor Otx2 (*D*), the eye field transcription factor Lhx2 (*E*), and the definitive neural transcription factor Sox1 (*F*). (Scale bar, 200 μm.)

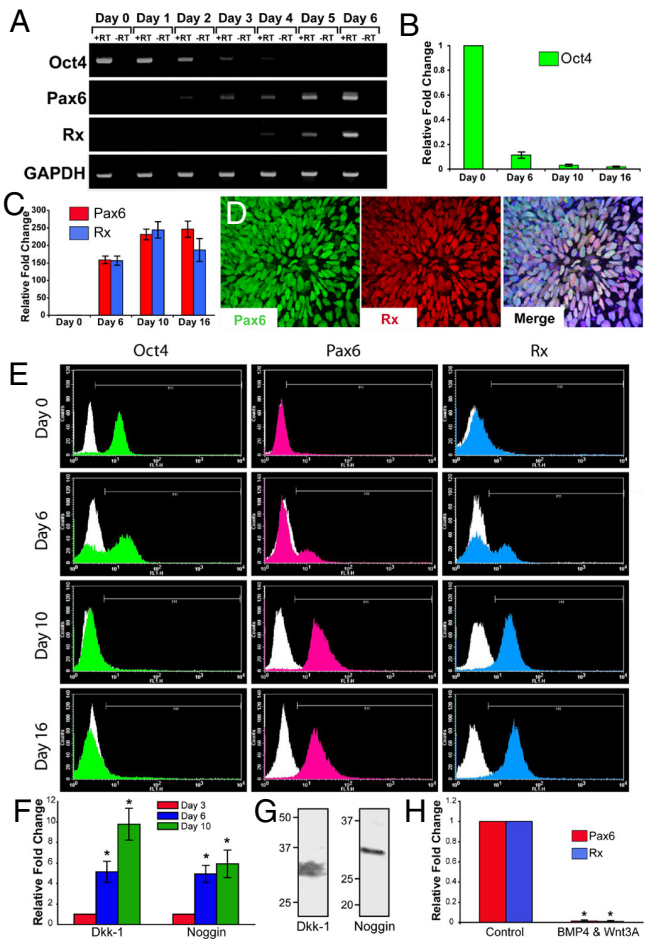

Fig. 2. Highly efficient derivation of eye field phenotypes from hESCs. (*A*) RT-PCR analysis showing the onset of Pax6 and Rx gene expression and concomitant loss of Oct4. (*B* and *C*) qPCR analysis of Oct4 gene expression (*B*) and Pax6 and Rx gene expression (*C*). Values were expressed as fold change relative to undifferentiated hESCs. (*D*) Immunocytochemical analysis of cells at day 10 showing uniform coexpression of Pax6 and Rx (merged image includes ToPro-3 nuclear stain). (*E*) FACS analysis confirming the rapid loss of Oct4 expression and the onset of both Pax6 and Rx protein expression. Negative controls for FACS analyses are indicated by the white histograms. (*F* and *G*) qPCR (*F*) and Western analysis (*G*) demonstrating the endogenous expression of the BMP and Wnt antagonists Noggin and Dkk-1. (*H*) qPCR showing the near complete loss of Pax6 and Rx gene expression in cells treated with BMP4 and Wnt3A. (Scale bar, 40 μm.)

colonies expressed Lhx2 (Fig. 1*D*), Otx2 (Fig. 1*E*), and Sox1 (Fig. 1*F*) by day 10 of differentiation.

Eye field cells are often characterized by the coexpression of Pax6 and Rx (7, 16). Therefore, the gene and protein expression of these two transcription factors was examined in further detail. RT-PCR and quantitative PCR (qPCR) analyses revealed the onset of expression of both Pax6 and Rx within the first few days of differentiation (Fig. 2 *A–C*), which was closely correlated with loss of Oct4 expression. On the protein level, nearly all cells coexpressed both Pax6 and Rx within 10 days of differentiation as determined by immunocytochemistry (Fig. 2*D*). Cell populations were then analyzed by FACS over the first 16 days of differentiation (Fig. 2*E*).

The onset of Pax6 and Rx expression was detected by day 6, when approximately 25% of all cells expressed these factors. Expression of Pax6 and Rx surpassed 90% of cells by day 10 of differentiation and increased to greater than 95% by day 16. Conversely, protein expression of Oct4 decreased to an undetectable level by day 10 of differentiation. The generation of a high percentage of cells with eye field characteristics in the absence of exogenous Wnt and BMP antagonists prompted further investigation into the endogenous expression of Dkk-1 and Noggin in differentiating hESC cultures. Both genes were up-regulated during eye field specification (Fig. 2*F*) as determined by qPCR. Furthermore, Western analysis detected protein expression of Dkk-1 and Noggin at day 10 of differentiation (Fig. 2*G*). Addition of Wnt3A and BMP4 to cultures over the first 10 days of differentiation abolished both the expression of Pax6 and Rx (Fig. 2*H*) and the appearance of neuroepithelial colonies (Fig. S1). Endogenous FGF signaling was also involved in the acquisition of early eye field features, since the addition of SU5402, a potent and specific inhibitor of the FGFR1 receptor, led to a complete loss of both Pax6 and Rx expression at day 10 of differentiation (Fig. S2).

Acquisition of Optic Vesicle and Optic Cup Cell Phenotypes. The next phase in retinal specification in vivo occurs with the formation of the optic vesicles from the paired eye fields. At this stage, all cells that will give rise to either the neural retina or the RPE express the transcription factor Mitf (17). The subset of Mitf+ cells destined to become neural retina subsequently down-regulate Mitf in response to the onset of Chx10 (also called Vsx2) expression (18, 19). When eye field rosettes were lifted and grown as neurospheres, near uniform expression of Mitf protein was observed within 14.7 ± 2.1% of all spheres by day 30 of differentiation (Fig. 3*A*). qPCR analysis further demonstrated that gene expression of Mitf increased from day 16 to day 37 of differentiation (Fig. 3*B*). Next, the relationship between Mitf and Chx10 protein expression was examined over time. Chx10 expression was only rarely observed at day 30 (Fig. 3*C*). Coexpression of Mitf and Chx10 was prevalent by day 40 (Fig. 3*D*), followed by mutually exclusive expression of Chx10 and Mitf by day 50 as Mitf expression diminished within Chx10+ neurospheres (Fig. 3*E*). qPCR analysis confirmed that Chx10 gene expression was delayed relative to Mitf (Fig. 3*F*). Similar to Mitf, Chx10 protein was eventually detected in nearly all cells of the subset of neurospheres in which it was expressed (Fig. 3*G*). Quantification of Chx10 protein expression demonstrated that 18.0 ± 3.3% of all neurospheres contained Chx10+ cells by day 40 of differentiation (Fig. S3*A*), and within these Chx10-expressing spheres, 90.7% ± 5.2% of cells expressed Chx10 by day 50 (Fig. S3*B*). By FACS, 26% of the entire cell culture population expressed Chx10 at day 40 (Fig. 3*H*). The remaining Chx10-negative neurospheres derived from the early eye field cell population maintained a neural fate as indicated by expression of Sox1 and βIII-tubulin (Fig. S4 *A–F*, and *J*). Non-retinal neurospheres also expressed forebrain markers, including Otx2 (Fig. S4 *G–I*, and *J*), but did not express endoderm (alpha-fetoprotein), mesoderm (brachyury), hindbrain (HoxB4), or midbrain (En-1) markers (Fig. S4*J*).

Among those individual cells that expressed Chx10, greater than 99% maintained expression of Pax6, which is a requirement of early retinal progenitor cells (20) (Fig. 3*I*). Furthermore, many of the Chx10+ clusters were arranged in rosettes with cells oriented radially away from a core that was positive for the tight junction protein ZO-1 (Fig. 3*J*), a feature associated with progenitor populations (14). While clusters that contained Chx10+ cells included a small number of βIII tubulin+ neurons (Fig. 3*K*), these cells rarely coexpressed Chx10. Thus, Chx10 expression was associated with a neural cell type that had not yet acquired a mature neuronal phenotype.

Given the potential role of FGF signaling in the specification of the neural retina (21), we next examined the effect of the specific FGF inhibitor SU5402, on Mitf and Chx10 gene expression. The addition of SU5402 to adherent hESC cultures during the optic vesicle and optic cup stages of differentiation (days 16–40) resulted in an 11.8-fold increase in Mitf gene expression at day 40, as measured by qPCR (Fig. 3*L*). By contrast, Chx10 expression was reduced 15.9-fold as a result of this treatment.

Differentiation of Retinal Cell Types from hESC-Derived Retinal Progenitors. The RPE is the first differentiated retinal cell type to appear during retinogenesis, arising from a population of Mitf+ and Pax6+ cells present in the outer layer of the early optic cup (21). When Pax6+/Rx+ eye field rosettes were maintained as adherent cultures, distinct patches of polygonal, pigmented cells were initially observed at approximately day 30 of hESC differentiation (Fig. 4*A*). These cells maintained expression of the transcription factor Mitf, while also expressing the RPE-associated tight junction protein ZO-1 (Fig. 4*B*). At day 40 of differentiation, FACS analysis revealed that 25% of all adherent cells expressed Mitf, and 77% of all cells expressed Pax6 (Fig. 4*C*). RT-PCR analysis demonstrated maintained expression of Pax6 in this cell population over time, as well as the acquisition of more mature RPE-associated markers such as RPE65 and bestrophin (Fig. 4*D*).

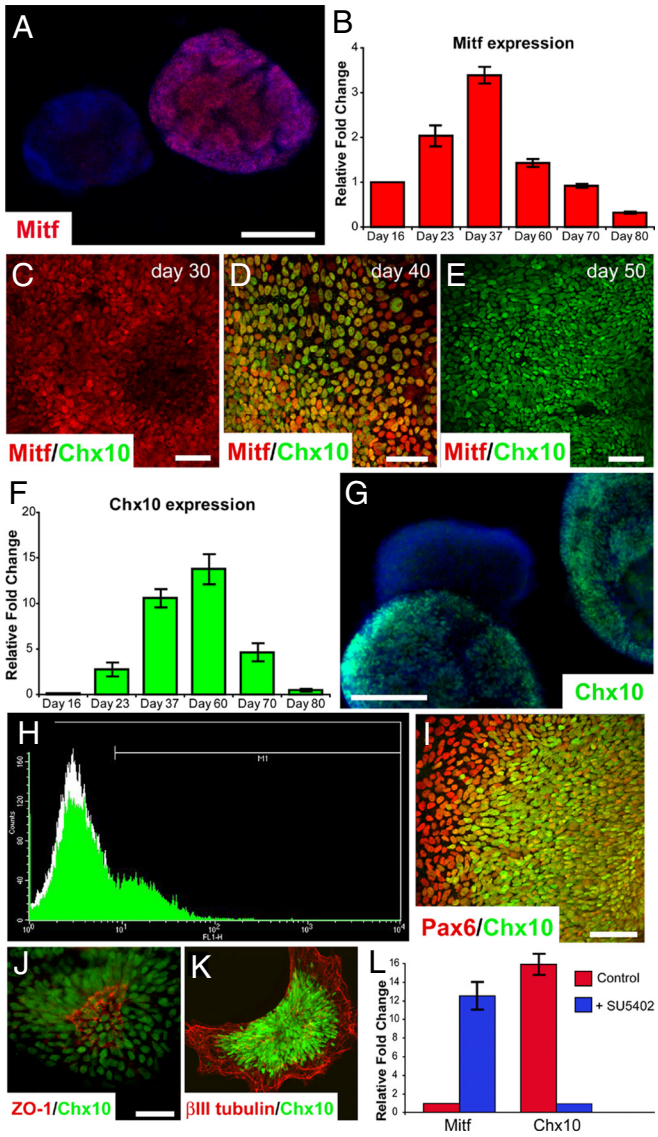

Fig. 3. Acquisition of optic vesicle and optic cup cell phenotypes. (*A*) Mitf protein expression in neurospheres after 30 days of differentiation. (*B*) qPCR analysis of Mitf gene expression over the first 80 days of differentiation. (*C–E*) Immunocytochemical analyses of the time course of Mitf and Chx10 protein expression in neurospheres at 30 (*C*), 40 (*D*), or 50 (*E*) days of differentiation. (F) qPCR analysis of Chx10 gene expression over the first 80 days of differentiation. (*G*) Uniform Chx10 expression throughout a subset of neurospheres by day 40. (*H*) FACS analysis demonstrating the percentage of all cells expressing Chx10 at day 40. (*I*) Immunocytochemical analysis showed all Chx10+ cells coexpressed Pax6 at day 40. (*J*) Rosettes of Chx10+ cells expressed the tight junction protein ZO-1 within their core. (*K*) Rare Chx10+ cells coexpressed βIII tubulin at day 40. (*L*) qPCR demonstrating increased Mitf expression and corresponding decreased Chx10 expression in adherent cultures treated with the FGF inhibitor SU5402. qPCR values were expressed as fold change relative to cultures at day 16 (*B* and *F*) or day 10 (*L*) of differentiation. (Scale bars, 500 μm in panels *A* and *G*; 50 μm in panels *C–E*, *J*, and *K*; and 75 μm in panel *I*; blue stain in *A* and *G* is Hoechst nuclear dye.)

Prolonged maintenance of the hESC-derived retinal progenitors as neurospheres allowed for further maturation of these cells toward a photoreceptor phenotype. Among the first differentiated neural retinal phenotypes observed during development are cone photoreceptors (22, 23), whose precursors express the primitive cone and rod photoreceptor-specific transcription factor Crx (24). By day 80 of differentiation, 19.4 ± 3.1% of all

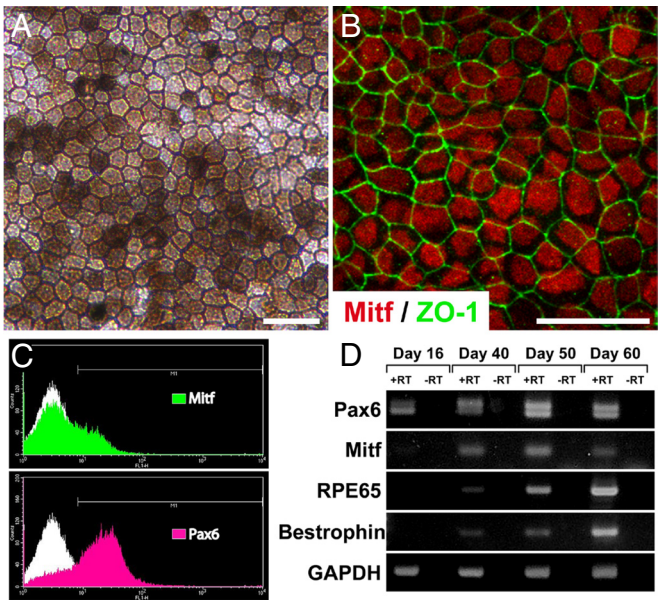

Fig. 4. Generation of retinal pigment epithelium. (*A*) Photomicrograph of adherent cultures showing pigmented, hexagonal RPE-like cells. (*B*) Immunostaining revealing expression of Mitf within RPE-like cells, as well as the tight junction protein ZO-1. (*C*) FACS analysis demonstrating the percentage of all adherent cells expressing Mitf and Pax6 at day 40 of differentiation. (*D*) RT-PCR studies showing expression of genes associated with an RPE fate. (Scale bars, 100 μm.)

neurospheres contained Crx+ photoreceptor precursors (Fig. 5*A*). Within these neuropheres, 63.0 ± 7.6% of all cells expressed Crx. Furthermore, 46.4 ± 7.9% of Crx+ cells expressed more mature photoreceptor markers, such as recoverin (Fig. 5*B*) and/or the cone photoreceptor-specific protein opsin (Fig. 5*C*). Recoverin and opsin expression was not observed in Crx-negative cells.

To analyze the time course and sequential acquisition of neuroretinal- and photoreceptor-associated gene expression, RT-PCR analysis was performed (Fig. 5*D*). Throughout the differentiation process from day 16 through day 80, Pax6 gene expression was detected. Rx gene expression was also present early in differentiation, followed by the consecutive expression of Chx10, Crx, and opsin. Overall, the timing of expression of the gene and protein markers used in this study coincided with that of normal human retinal development (22, 23) (Fig. S5).

During normal retinogenesis, the Pax6(+5a) isoform is expressed in increasing abundance relative to total Pax6 (25). RT-PCR results from the present study similarly suggested that the Pax6(+5a) isoform became more prevalent during hESC differentiation (Figs. 2*A* and 5*D*). To verify this observation, qPCR of the Pax6(+5a) isoform relative to total Pax6 expression was performed (Fig. S6). This analysis confirmed the onset of Pax6(+5a) expression between days 4 and 16 of differentiation and demonstrated a relative increase in the expression of this isoform between days 60 and 70, which corresponded to the appearance of photoreceptor-like cells in culture.

Differentiation of Retinal Cell Types from Human iPS Cells. To determine the potential for stepwise derivation of retinal cell types from human iPS cells, we applied the hESC differentiation protocol to four different human iPS cell lines. Consistent with a previous report (11), considerable variation was found in the ability of these lines to produce Pax6+ neuroectodermal cells at day 10 of differentiation, with efficiencies ranging from 5% to 56% of the total cell population. Based on these results, we chose to study the IMR90–4 cell line in greater detail. Upon differentiation, the appearances of the iPS cell colonies, iPS cell aggregates, neural rosettes and neurospheres were indistinguishable from those of hESCs (Fig. S7). During differentiation, immunocytochemistry revealed early eye field cells coexpressing Pax6 and Rx by day 10 (Fig. 6*A*). These cells also expressed a full complement of eye field and neuroepithelial transcription factors (Fig. S8). Discrete populations of Mitf+ cells were observed upon further differentiation of eye field colonies as neurospheres (Fig. 6*B*). Like their hESC counterparts, many of these iPS cell neurospheres appeared to lose Mitf expression in favor of Chx10 expression (Fig. 6*C*), yielding neurospheres that were highly enriched for Chx10+ cells (Fig. 6*D*). Among the total population, 12.9 ± 4.3% of all neurospheres expressed Chx10 at 40 days of differentiation, within which 90.1 ± 1.2% of all cells expressed Chx10. Over time, photoreceptor markers appeared, such as the rod- and cone-specific transcription factor Crx, which was present in 14.4 ± 5.1% of all neurospheres by day 80 (Fig. 6*E*). Similar to the expression of earlier markers of retinal differentiation, Crx+ cells were common within individual positive neurospheres, constituting 65.5 ± 9.3% of cells. At day 80, 44.6 ± 8.1% of cells within Crx+ clusters expressed recoverin (Fig. 6*F*) and/or opsin (Fig. 6 *G* and *H*). As with hESCs, recoverin and opsin expression was not found in Crx-negative cells. PCR analysis confirmed the sequence and timing of gene expression of these markers, along with the early loss of Oct4 expression (Fig. 6*I*). RPE cells were present within iPS cell cultures as well, with pigmentation first apparent at approximately day 35 of differentiation and typical monolayers arising by day 50 (Fig. 6*J*). Like hESC-derived RPE, these cells possessed morphological characteristics of mature RPE and expressed Mitf and ZO-1 (Fig. 6*K*).

Discussion

The results presented here demonstrate that human pluripotent stem cells can adopt signature features associated with all major stages of early eye and retinal development, while following an expected timeline for human retinal development (22, 23). Although previous reports have shown that hESCs can acquire retinal characteristics at various times during differentiation (3–7), many markers used to identify primitive retinal cell types are also expressed in other developing neural cells. This makes it difficult to unequivocally assign immature cell types to the retinal lineage without knowledge of their dynamic behavior in culture. Thus, it is important to monitor each stage of cellular maturation to ensure that critical developmental checkpoints are met in order and within a predictable time frame.

In the first few weeks of human development, a portion of the primitive anterior neuroepithelium gives rise to the eye field (13, 16, 26, 27), a cell population characterized by the expression of numerous transcription factors including Pax6, Rx, Six3, Six6, Tll, and Lhx2. We have demonstrated that the production of Pax6+/Rx+ cells is highly efficient, with 95% of all cells coexpressing these essential transcription factors. This efficiency is likely due in part to a relative lack of influence from endogenous BMP and Wnt signaling, since both pathways are known to antagonize neural specification (28, 29). In support of this theory, increasing expression of BMP and Wnt antagonists (Noggin and Dkk-1, respectively) was observed in hESC cultures shortly after the onset of differentiation. Early exposure of differentiating hESCs to recombinant BMP4 and Wnt3a eliminated the expression of Pax6 and Rx, as well as the subsequent formation of neural rosettes.

Although Pax6 and Rx have been used to identify retinal progenitor cells in differentiating ESC cultures (7, 16), during development these factors are intially coexpressed in a broad region of the anterior neural plate that includes the eye field and future forebrain (16). Thereafter, Pax6+/Rx+ cells become restricted to more specific areas of the developing CNS (16), predominantly the retina (26, 30). In the present study, the majority of the early Pax6+/Rx+ population did not subsequently adopt cellular phe-

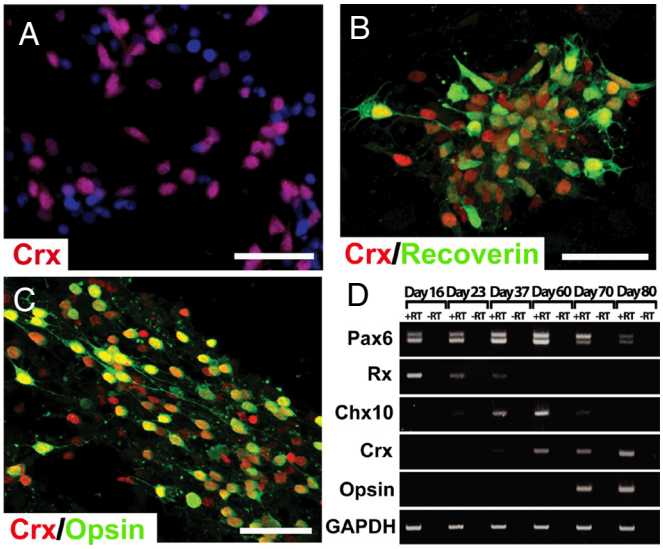

Fig. 5. Generation of early photoreceptor phenotypes. (*A*) Immunocytochemical detection of cells expressing the photoreceptor-specific transcription factor Crx at 80 days of differentiation. (*B* and *C*) Expression of the photoreceptor protein recoverin (*B*) and the cone photoreceptor-specific protein opsin (*C*) among Crx-expressing cells at day 80. (*D*) RT-PCR demonstrated the stepwise acquisition of a cone photoreceptor fate from an eye field population. (Scale bars, 50 μm.) Blue stain in A is Hoechst nuclear dye.

notypes of the optic vesicle or optic cup despite retaining an anterior neural identity. Therefore, the enriched Pax6+/Rx+ cell population derived in this study most closely resembled a primitive stage of human eye field development, which preceded the appearance of committed retinal progenitors.

After the optic vesicles evaginate from the paired eye fields, expression of Mitf occurs throughout cells fated to become retina (12, 17). However, the decision to differentiate toward either a neural retina or RPE fate is revealed during the late optic vesicle and optic cup stages, in part via differential expression of the transcription factor Chx10 (18, 19). Neural retinal progenitors destined for the inner layer of the optic cup express Chx10 and down-regulate Mitf in response to FGFs secreted by the overlying surface ectoderm. Thus, Chx10 is the earliest specific marker of neural retinal progenitor cells (19). Conversely, cells destined for the outer layer of the optic cup remain Mitf+ and Chx10-negative and subsequently differentiate into RPE. Our results provide evidence that hESCs proceed through analogous stages of early retinal differentiation, as indicated by the spatiotemporal expression of Mitf and Chx10 in neurospheres. Furthermore, inhibition of endogenous FGF signaling during the optic vesicle and optic cup stages of hESC differentiation resulted in a profound increase in Mitf gene expression and a corresponding decrease in Chx10 gene expression. This suggests that mechanisms governing cell fate choice in the developing retina may also function in differentiating hESC cultures.

After adopting a retinal fate, individual neurospheres yielded a high percentage of photoreceptor precursors in a time frame predicted by normal human retinogenesis. As with earlier stages of retinal differentiation, this was achieved without the addition of specific exogenous agents. Previously, retinoic acid and taurine had been used to induce differentiation of photoreceptor-like cells (7). By eliminating such agents, the present system is suited for the investigation of endogenous factors and mechanisms that affect differentiation and maturation of specific retinal cell types.

Taking into account the entire hESC population present at the start of the differentiation process, we observed a decrease in targeted cell production with each subsequent stage of retinal

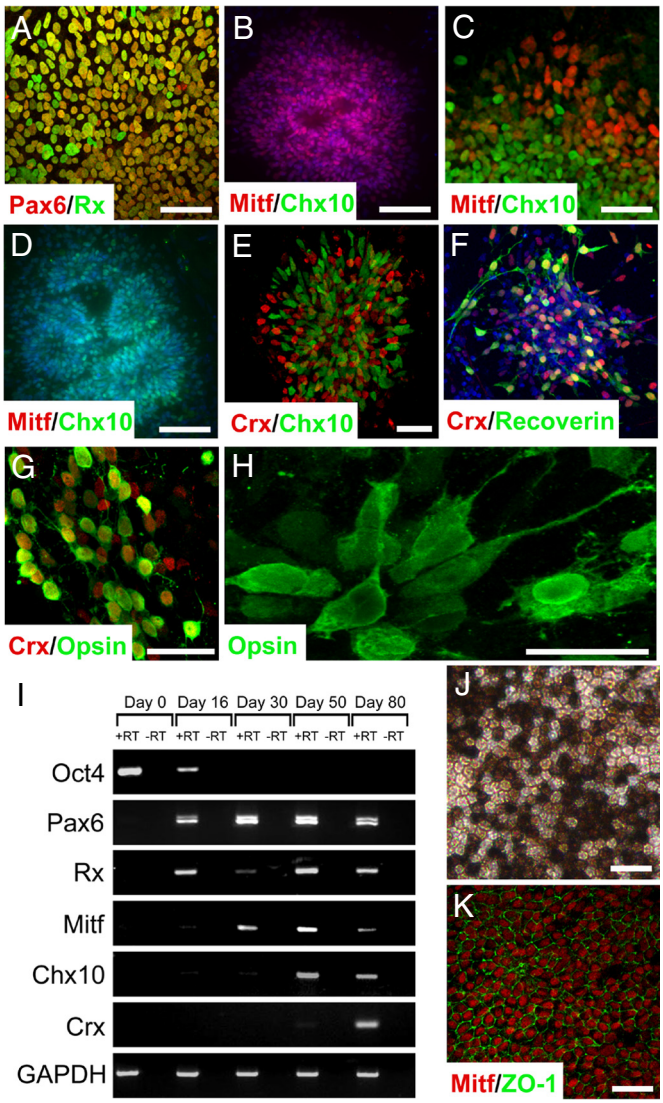

Fig. 6. Stepwise retinal specification from human iPS cells. (*A*) Various stages of retinal differentiation were observed, beginning with Pax6+/Rx+ eye field cells by day 10. (*B–D*) Mitf+ and Chx10+ cells, indicative of the optic vesicle/optic cup stages, are evident by day 40. (*E*) By day 80, clusters were present containing Chx10+ retinal progenitors and Crx+ photoreceptor precursor cells. (*F–H*) Many Crx-expressing cells expressed the photoreceptor protein recoverin (*F*) and the cone-specific protein opsin (*G* and *H*). (*I*) RT-PCR analysis demonstrating the stepwise expression of retina- and photoreceptor-associated genes in differentiating iPS cells over time. (*J* and *K*) RPE cells derived from iPS cells acquired a typical hexagonal morphology and pigmentation (*J*) and expressed Mitf and ZO-1 (*K*). (Scale bars, 50 μm.) Blue stain in *B* and *D* is Hoechst nuclear dye; blue stain in *F* is To-Pro-3 nuclear dye.

differentiation. This observation is consistent with normal retinal development, where early cell types often give rise to multiple distinct progeny of the same lineage. However, there now exist opportunities to introduce exogenous factors for defined time periods to augment production of retinal cell types at specific developmental stages. Such precision is likely to be important, since a single factor can have diverse effects on cellular fate choice depending on the stage of development (31). For example, we observed that early inhibition of endogenous FGF signaling in differentiating hESCs resulted in a loss of eye field specification, whereas later inhibition differentially regulated genes important for the induction of RPE and neural retina progenitors. Manipulation of the culture environment with

signaling factors may also alter the time course of retinal cell differentiation from hESCs. This is suggested by the striking differences in the timing of photoreceptor marker expression observed in previous studies, in which the onset of Crx expression ranged from one to thirteen weeks (6, 7).

Given the ability of hESCs to mimic normal human retinogenesis, we investigated whether another source of human pluripotent stem cells, iPS cells, displayed a similar potential. A previous report by Yu et al. (11) showed that human iPS cell lines differed in their early expression of Pax6, a finding confirmed here. Since Pax6 expression is necessary for retinal development, it is not surprising that one of the highest Pax6-expressing lines from that study, IMR90–4, was efficient at producing retinal cell populations. Other iPS cell lines displayed reduced competency to produce neural and retinal cell types, a phenomenon also observed by Hirami et al. (8). Therefore, present techniques for deriving iPS cells from somatic cells do not always yield uniform lineage competencies between lines.

A detailed knowledge of the stages and time course of retinal differentiation from hESCs and iPS cells not only provides an opportunity to study fundamental questions of human retinal development, but may also aid efforts to use pluripotent stem cell derivatives for pharmaceutical testing and retinal repopulation studies. A previous report by MacLaren et al. (32) demonstrated that cells from a specific stage of mouse retinal development were capable of functionally integrating into degenerate adult mouse retinas. More recently, Lamba et al. (33) noted similar results using a mixture of retinal cell types derived from hESCs. The hESC differentiation protocol described in the present study provides access to human retinal cells at all major stages of retinal development. The near absence of contamination from non-neural cell types and the potential to enrich for discrete retinal cell types further add to the possible clinical utility of these differentiating cultures. The potential for iPS cells to generate multiple retinal cell types will aid in the development of in vitro models of human retinal degenerative diseases and stimulate investigation into customized stem cell therapies for patients afflicted by these disorders (34, 35).

In summary, we have shown that hESCs meet the criteria (1, 2) to serve as a comprehensive in vitro model system for human retinogenesis. Using an identical culture method, human iPS cells show a similar potential, although variation can occur between lines. On a broader level, this study supports a role for pluripotent stem cells to test concepts in human developmental biology that were previously extrapolated from animal models. In turn, this ability could narrow the gap between our understanding of human development and that of other mammalian species.

Methods

Maintenance of hESCs and Human iPS Cells. Pluripotent stem cells were maintained as previously described for hESCs (15). Detailed protocols are available in the *SI Text*.

Differentiation of hESCs and Human iPS Cells. The initial differentiation of hESCs and human iPS cells toward an eye field fate was performed with modifications to previously described protocols (15, 36). Thereafter, a chemically-defined retinal differentiation medium was used to promote the stepwise production of retina-specific cell types from free-floating neurospheres (36). Detailed protocols are available in the *SI Text*.

RT-PCR. RT-PCR and qPCR experiments were performed as previously described (36). More detailed methods, including primer sequences, can be found in the *SI Text* as well as Table S1.

Immunocytochemistry. Cell aggregates were plated onto coverslips, fixed with 4% paraformaldehyde, and then immunostained as described (15). Detailed procedures are provided in the *SI Text* as well as Table S2.

FACS. Staining and sorting of cells were performed as previously described (15). Detailed procedures are provided in the *SI Text*.

Western Analysis. Western blots were performed as previously described (36). Detailed procedures are provided in *SI Text* as well as Table S2.

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