Supplementary Data

Retinal Differentiation

Summary

We initiated neural induction of pluripotent human embryonic stem cells (hESCs) with noggin (as we published earlier [1–3], also Supplementary Fig. S1) when hESC colonies reached 75%–80% density. To do this, we replaced (at day 0) hESC medium [containing basic fibroblast growth factor (bFGF)] with hESC medium/Neurobasal complete*(NB) medium (1:1 ratio) with no bFGF and 100 ng/mL noggin morphogen (Sigma-Aldrich), then (on day 3) replaced the medium with 100% NB with 1xN2, 1xB27, and 100 ng/mL noggin, and cultured for another 3 days. The recipe is described [2], except for the replacement of 1× Pen-Strep with 1× amphotericin-B, 1× gentamicin. We continued replacing ½ of the conditioned medium every third day with fresh NB/N2/B27/noggin. At +2 weeks after initiating the protocol, we applied bFGF (10 ng/mL; Sigma-Aldrich). At +4 weeks, we applied the retinal induction protocol (DKK-1 and IGF-1, 10 ng/mL each; Sigma-Aldrich) [1,4] for 1 week, and then maintained retinally induced cells for up to 12 weeks in Neurobasal complete medium (recipe below) with noggin (100 ng/mL), bFGF, and FGF9 (both at 10 ng/mL) [1,5,6] to promote neural retinal differentiation.

3D hESC-derived retinal tissue aggregates started to appear by about week 4 after initiation of the differentiation protocol (Supplementary Fig. S1) and rapidly increased in size by 6 weeks. We did not observe further growth between weeks 7 and 12. Maintaining hESC-derived retinal tissue on the plates at later time points (around weeks 10–12) was challenging due to the visible degradation of Matrigel and dislodging from the plates. Therefore, we characterized hESC-derived retinal tissue by quantitative reverse transcription–coupled polymerase chain reaction, immunoblot, immunohistochemistry (IHC), and electrophysiology at 6 weeks and kept only some remaining large 3D hESC-derived retinal tissue aggregates for electrophysiology testing only.

The 3D growth of retina-like tissue aggregates in cultures was not synchronous, producing various shapes and sizes, and the number of such aggregates varied between 2–3 and 15 or more per 35-mm plate.

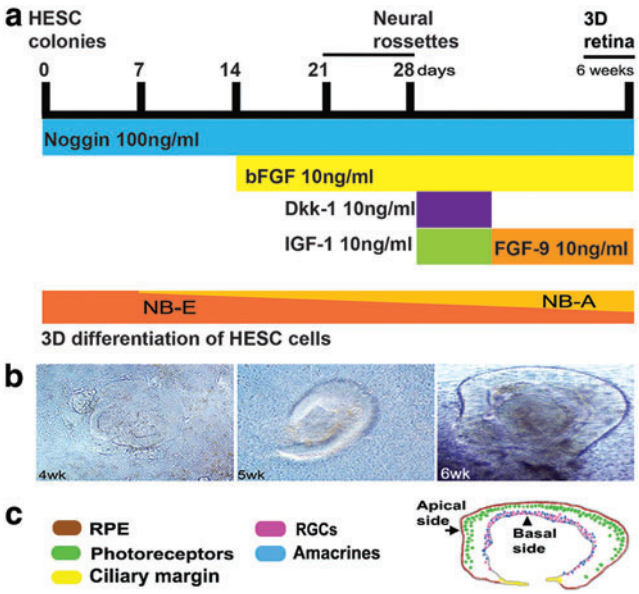
Composition of Neurobasal complete medium. 1xN2, 1xB27 without retinoic acid, 1× L-glutamine (1%), 1% Minimal Essential Medium nonessential amino acid solution (MEM), 1× amphotericin-B/gentamicin (Life Technologies), BSA fraction V (0.1%) (Sigma-Aldrich), β-mercaptoethanol (0.1 mM; Sigma-Aldrich), and 94.8% (volume/volume) of Neurobasal medium*.

Two types of Neurobasal media (both from Life Technologies) were used: standard Neurobasal (more suitable for culture of embryonic neural tissue labeled as NB-E) and Neurobasal-A (NB-A), formulated for long-term culture of postnatal and adult neurons.

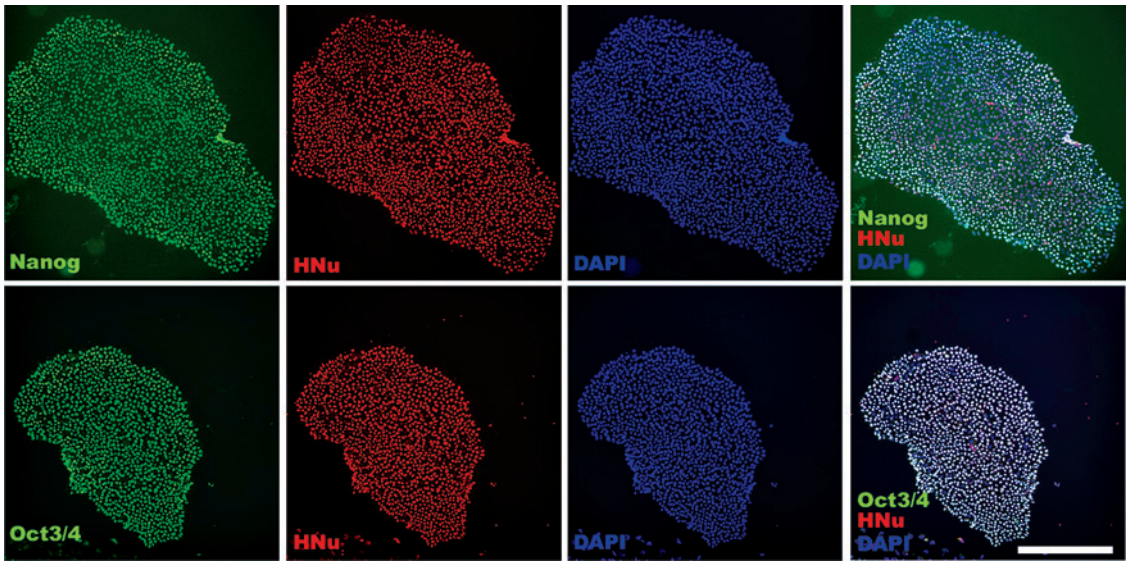
The composition of Neurobasal medium in Neurobasal complete was very gradually changed weekly to increase the percentage (volume/volume) of NB-E from 2% at day 7 to 60% at 6–12 weeks to promote the survival of already differentiated postmitotic neurons while maintaining the differentiating progenitors.

**Composition of Neurobasal medium, Neurobasal complete recipe.* Days 0–7: NB-E, no NB-A; days 8–14: 97% NB-E/3% NB-A; days 15–21: 93% NB-E/7% NB-A; days 21–28: 85% NB-E/15% NB-A; days 29–35: 70% NB-E/30% NB-A; and

Days 36+: 40% NB-E/60% NB-A. The variation of these ratios still produced 3D retina-like tissue aggregates. NB-A is expected to promote the survival of mature retinal



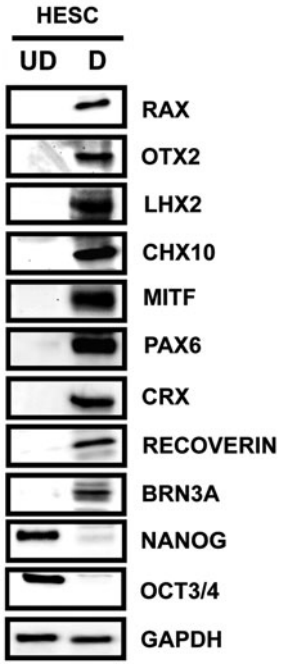
SUPPLEMENTARY FIG. S1. **(a)** Schematic diagram illustrates protocol of human embryonic stem cell (hESC) differentiation to 3D retinal tissue in adherent conditions (*top*). We initiated neural induction of pluripotent hESCs with noggin when hESC colonies reached 75%–80% density. Day 0: hESC medium [containing basic fibroblast growth factor (bFGF)] was replaced with hESC medium/Neurobasal (NB) medium containing N2 and B27/no retinoic acid (1:1 ratio) with no bFGF and 100 ng/mL noggin morphogen. Day 3: the medium was replaced with 100% NB and 100 ng/mL noggin. Half of the medium was replaced every 3 days. bFGF was added at day 14. Retinal induction was done at day 28. Two types of NB media were used: standard Neurobasal (more suitable for culture of embryonic neural tissue, labeled as NB-E) and Neurobasal-A (NB-A), formulated for long-term culture of postnatal and adult neurons. The composition of Neurobasal medium in Neurobasal complete was very gradually changed weekly (see Supplementary data). **(b)** Dynamics of 3D retinal tissue growth in adherent conditions. **(c)** Cartoon depicts localization of retinal cell types [retinal pigment epithelium (RPE), photoreceptors (PRs), Amacrine, retinal ganglion cells (RGCs)] within the 6-week-old hESC-derived retinal tissue growing in the adherent conditions.



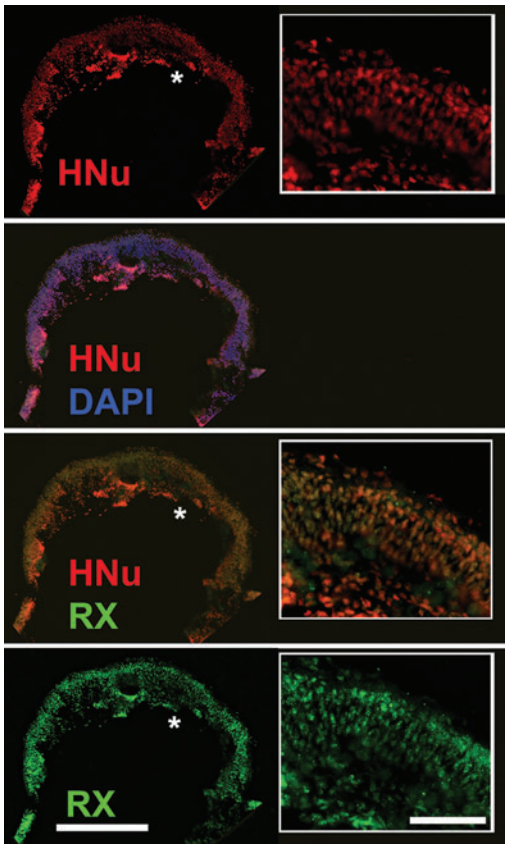
SUPPLEMENTARY FIG. S2. Immunostaining of hESC colonies (HESC line WA01) with pluripotency markers. hESC colonies were stained with antibodies to OCT3/4 and NANOG confirming the initial pluripotent state of hESC colonies cultured on growth factor-reduced Matrigel according to TeSR1 protocol (WiCell). HNu–human nuclei antibody, confirming human origin of cells. DAPI (4′, 6-diamidino-2-phenylindole)-stained nucleus. Scale bar: 50 μm.

neurons. About 50% of the medium was renewed every 3 days with fresh Neurobasal complete supplemented with noggin, bFGF, and FGF9.

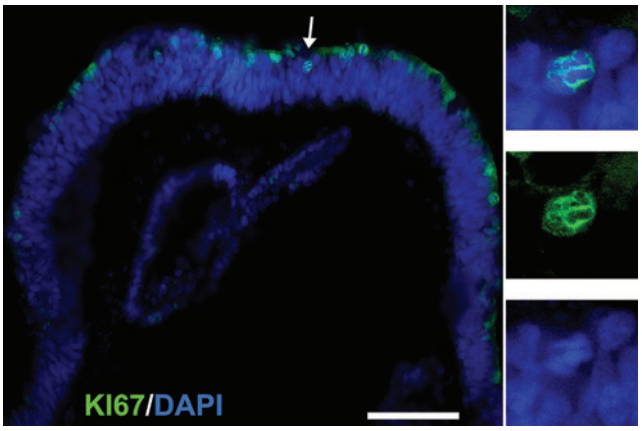
We compared the dynamics of retinal differentiation in hESC-derived retinal tissue growing as attached aggregates (the focus of this article) and in hESC-retinal cells derived in the 2D attached monolayer (reported earlier [1]).



SUPPLEMENTARY FIG. S3. Immunoblot from undifferentiated (UD) hESCs and differentiated (D) hESC-derived retinal tissue collected at 6 weeks.

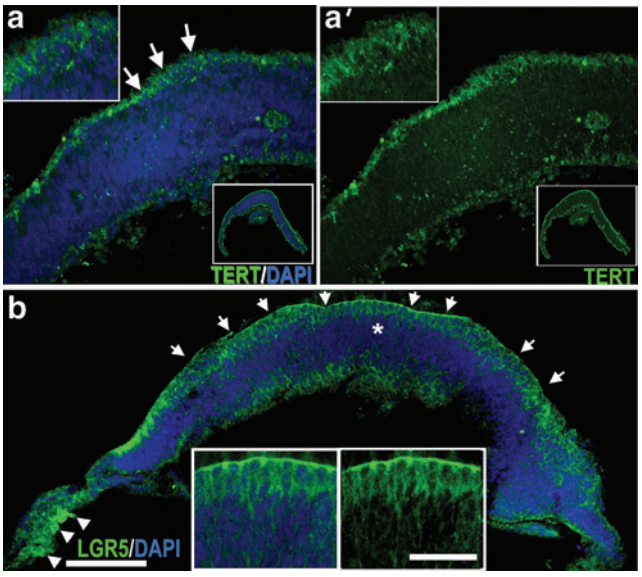


SUPPLEMENTARY FIG. S4. Presence of RAX [+] cells in hESC-derived retinal tissue. Immunostaining of hESC-derived retinal tissue with antibody to retinal cell fate marker, RAX, and human cell marker, HNu (human nuclei), counterstained with nuclear stain DAPI. The *insets* represent the magnification of the areas marked with an *asterisk*. Scale bars: 50 and 20 μm, respectively.

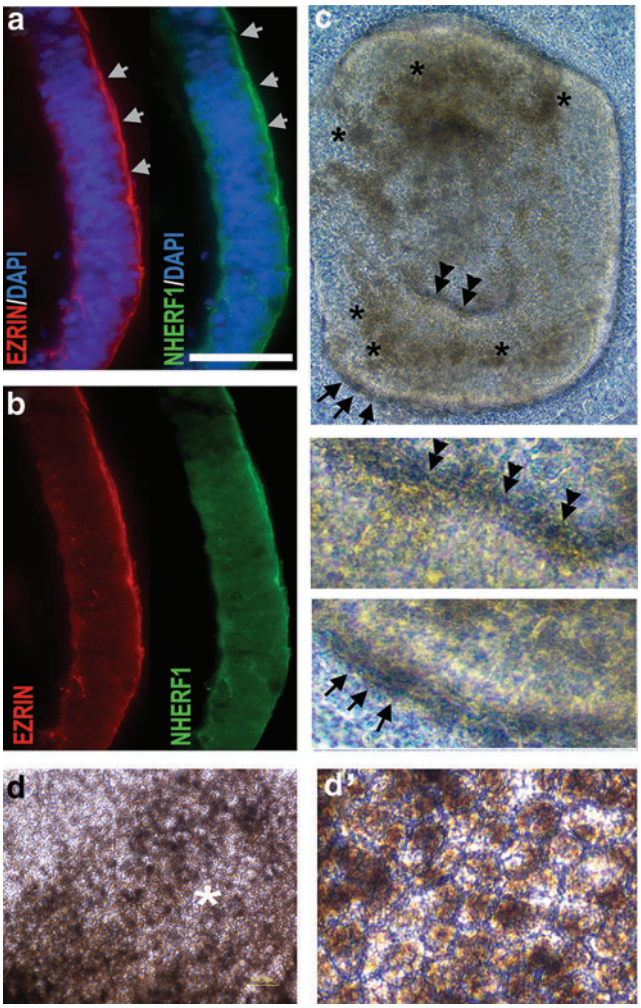


SUPPLEMENTARY FIG. S5. Representative images of Ki67-stained cells in hESC-derived retinal tissue. Dividing cells [4.5% ± 0.8% ($n = 4$)] were localized to the apical side. The *inset* represents the magnification of the areas marked with an *arrow* and shows the mitotic cell. Scale bars: 50 and 20 μm, respectively. DAPI-stained nucleus.

For 3D retinal tissue derivation: We maintained differentiating cells in adherent conditions from day 0 (hESCs) to 6 weeks and longer and collected largest retinal organoids for IHC and quantitative reverse transcription-coupled polymerase chain reaction (RT-PCR) at 6 weeks. Some additional



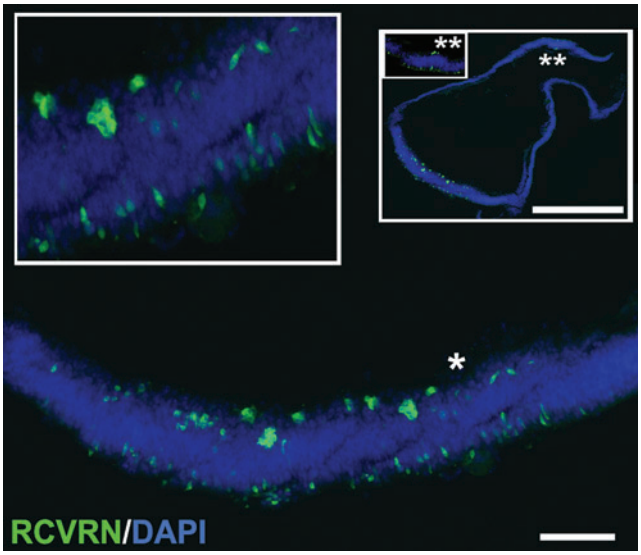
SUPPLEMENTARY FIG. S6. Strong presence of stem/progenitor cell markers, TERT and LGR5, in the apical side of hESC-derived retinal tissue. **(a–a′)** Immunostaining of hESC-derived retinal tissue with antibody to Telomerase catalytic (protein) subunit (TERT). TERT [+] cells (*arrows*) demarcate the apical side where progenitors/dividing cells are localized. DAPI-stained nucleus. The *top insets* represent the magnification of the area marked with *arrows*. The *bottom insets* are the overview of the hESC-derived retinal section stained with anti-TERT antibody. **(b)** Immunolabeling of hESC-derived retinal tissue with anti-LGR5 antibody shows strong signal in the apical side (*arrows*) and demarcates the area where retinal progenitors localize. *Arrowheads* point to ciliary margin-like zone. The *insets* represent the magnification of the area marked with an *asterisk*. Scale bars: 50 and 20 μm, respectively.



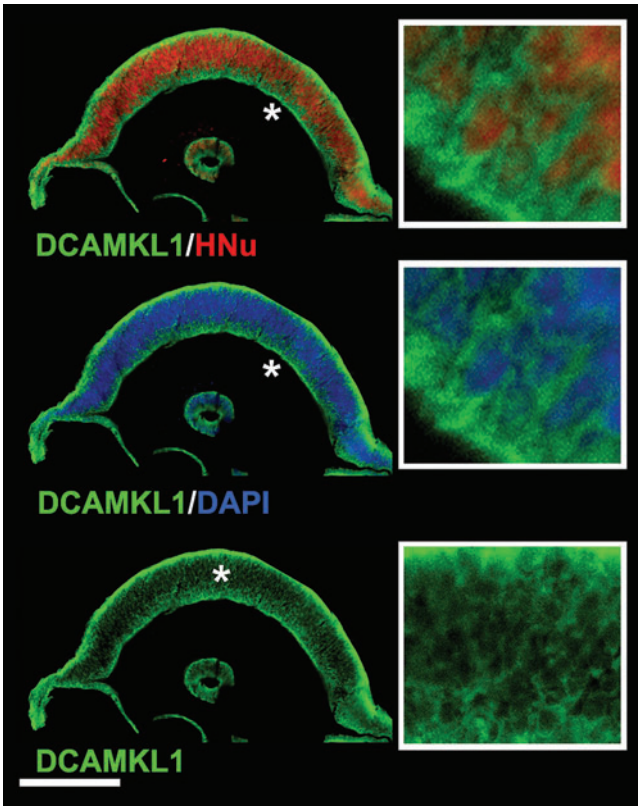
SUPPLEMENTARY FIG. S7. Presence of RPE layer in hESC-derived retinal tissue. **(a)** Immunostaining of hESC-derived retinal tissue with antibodies to two mature RPE markers, EZRIN, an actin-binding protein, and ERM-binding phosphoprotein50/sodium-hydrogen exchanger regulatory factor1 (EBP50/NHERF1). *Arrow* indicates strong staining of EZRIN and NHERF1 in the apical side. DAPI-stained nucleus. Scale bars: 50 and 20 μm, respectively. **(b)** Staining with antibodies to EZRIN and NHERF1, no DAPI counterstaining. **(c)** RPE cells are present in hESC-derived retinal tissue growing in adherent conditions. *Arrows* point to RPE cells in the apical side. *Double arrowheads* point to some RPE cells present on the basal side. The *insets* represent the magnification of the area in **c** shown with *arrows* and *arrowhead*, respectively. *Asterisk* shows RPE pigmentation in hESC-derived retinal tissue. Scale bars: 50 and 20 μm, respectively. **(d–d′)** Strong pigmentation of RPE cells growing in 2D adherent conditions. The cells exhibit hexagonal shape typical for RPE cells.

electrophysiology experiments were done with 12-week-old aggregates.

For derivation of hESC retinal cells in 2D monolayer: We followed our earlier published protocols [42,45]. At 4 weeks, neural rosettes were excised mechanically and replated as clusters of neuroepithelial cells [30,42,43] on gelatin/laminin-coated plates (0.1% gelatin/10 μg/mL laminin



SUPPLEMENTARY FIG. S8. Recoverin [+] immature PRs in hESC-derived retinal tissue. Immunostaining with anti-RCVRN antibody shows the presence of RCVRN [+] cells in the apical side and clusters of RCVRN [+] cells in the basal side. The *left inset* represents the magnification of the area marked with an *asterisk*. The *right inset* is an overview of hESC-derived retinal tissue section stained with anti-RCVRN antibody. *Double asterisk* depicts RCVRN [+] cells present in the periphery. DAPI-stained nucleus. Scale bar: 50 and 20 μm, respectively.



SUPPLEMENTARY FIG. S9. Strong neuronal staining in hESC-derived retinal tissue. Immunostaining of section with anti- DCAMKL1 antibody and human tissue-specific antibody HNu (human nuclei). DCAMKL1 is a marker of young neurons. Note prominent staining in the apical and basal sides. The *insets* represent the magnification of the area marked with an *asterisk*. DAPI-stained nucleus. Scale bar: 50 μm.

in phosphate-buffered saline, coated overnight at 4°C) and cultured at high density (70%–80%) between weeks 4 and 12, using the same media and retinal induction protocol (Supplementary Fig. S1).

Morphogens and mitogens

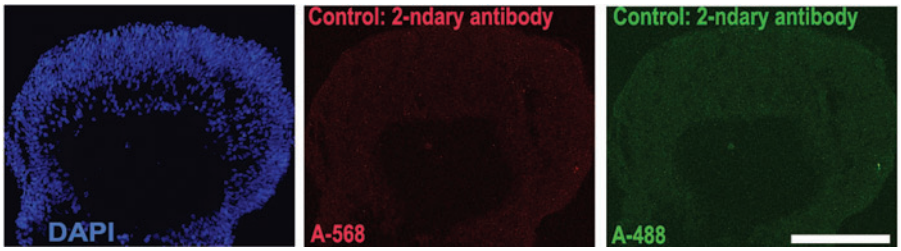
Neurobasal medium and Neurobasal-A with B27/N2 supplementation were from Life Technologies. Plastic Petri dishes were from Corning, Inc. Gelatin, laminin, Noggin, DKK-1, IGF-1, FGF9, and bFGF were from Sigma-Aldrich.

Cryosectioning and slides

The Microm HM550 cryostat (Thermo Scientific) was used to generate 12 μm serial sections of hESC-derived retinal tissue. Histological sections were generated by cutting through the central plane of each selected aggregate (parallel to the surface of the Petri dish, the midsagittal sections). Microscope SuperFrost™ Plus slides were purchased from Fisher Scientific. Glass coverslips were purchased from Brain Research Laboratories.

Immunoblot

Total lysates of undifferentiated and differentiated hESCs were homogenized by motorized handheld homogenizer (VWR) in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Roche complete). At least 40 individual large hESC-derived retinal tissue aggregates were collected for immunoblotting. The protein concentration was measured by using Bradford assay. Equal concentrations (40 μg) of protein samples were resolved in 4%–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to the Immobilon-FL membrane (Bio-Rad). Membranes were blocked with blocking buffer (BLOTTO) for 30 min at room temperature. After blocking, membranes were incubated with primary antibodies to OTX2 (anterior neuroectoderm marker [47] induction, maintenance [48] and anterior brain development [49,50]), RAX [51,52], LHX2 [53,54], PAX6 [55] (the eye field markers [53,56], the presence of which is important for optic identity), CHX10 (multipotential retinal progenitors [57]), CRX (cone–rod homeobox, key rod and cone photoreceptor (PR) progenitor and young PR marker [58]), RECOVERIN (young PRs [27,59]), MITF (key retinal pigment epithelial determining cell fate marker [60,61]), BRN3A (one of several key retinal ganglion cell (RGC) fate determining markers, postmitotic RGCs [62]), NANOG, OCT3/4 (the two key pluripotency markers), and GAPDH (housekeeping marker, loading control) at 1:1,000 dilution for 4 h at room temperature. The secondary antibodies, horse radish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse (all from Jackson Immunoresearch), were used at 1:50,000 dilution for 30 min at room temperature. Membranes were scanned using the Thermofisher MyECL chemiluminescence imager. All antibodies are listed in Supplementary Table S2.



SUPPLEMENTARY FIG. S10. Immunostaining of hESC-derived retinal tissue with secondary antibodies Alexa-488 and Alexa-568. DAPI was used for counterstaining the nuclei. Scale bar: 50 μm.

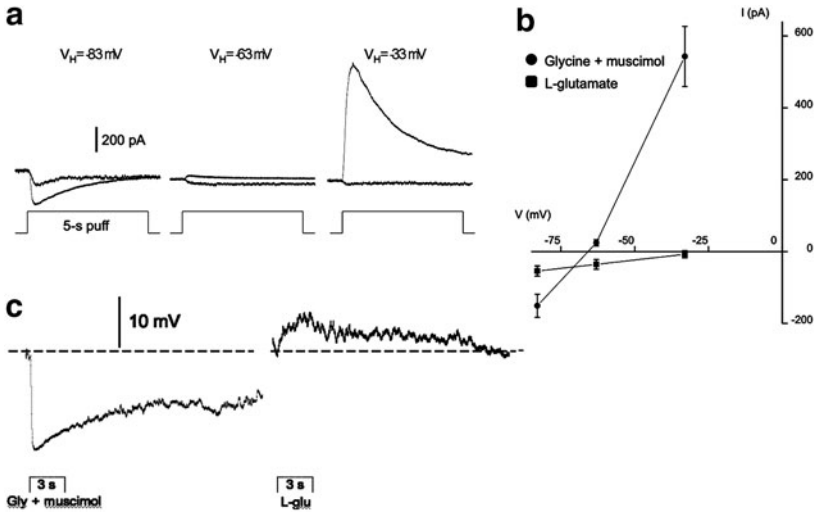
Electrophysiological recording—whole-cell recording

hESC-derived retinal tissue aggregates grown in 35-mm cell culture dishes were positioned on a fixed-stage upright microscope (Eclipse FN1; Nikon Instruments) and superfused by Ames' medium gassed with 95% O_2 5% CO_2. The superfusate was delivered to the dish at 2–3 mL/min and maintained at 32°C using a temperature controller (Warner Instruments). hESC-derived retinal tissue aggregates were visualized using infrared transillumination and NIS Elements D imaging software (Nikon Instruments). Whole-cell recordings were obtained from medium to large round somas located on the interior of the organoids using either a MultiClamp 700A or 700B amplifier (Molecular Devices). Glass micropipettes with tip resistances, 6–8 MΩ, were pulled from thick-walled borosilicate tubings on a Narishige PC-10 puller and were filled with either a K^+- or Cs^+-based intracellular solution (described below). PCLAMP 9 or PCLAMP 10 software (Molecular Devices) was used for data acquisition.

Whole-cell recordings were performed on cells from hESC-derived retinal tissue of two ages: 6 and 12 weeks. In some experiments, intrinsic electrophysiological behavior was assessed using either a series of voltage steps (500 ms duration, −103 to +37 mV in 10 mV increments) or a series of current steps (400 ms duration, −300 to +250 pA in 50 pA increments). In other experiments, we probed for the presence of functional neurotransmitter receptors by measuring responses to exogenous agonists. Cells were either voltage clamped at several holding potentials or current clamped at I = 0, and a double-barrel micropipette was used to puff (PicoPump PV820; World Precision Instruments) Ames' medium containing either L-glutamate (2 mM) or muscimol plus glycine (both at 1 mM) onto the recorded neuron. In the voltage-clamp experiments, series resistances were 15–40 MΩ and were compensated by up to 50%.

Electrophysiological recording—chemicals and solutions

Two different intracellular solutions were used. The K^+-based solution contained (in mM) 120 K-gluconate; 5 NaCl; 4 KCl; 10 HEPES; 2 EGTA; 4 Mg-ATP; 0.3 Na-GTP; 7 Tris-phosphocreatine; 0.1% Lucifer Yellow; and KOH to adjust pH to 7.3. The Cs^+-based solution contained (in mM) 120 Cs-methanesulfonate; 3 NaCl; 2 QX-314 chloride; 5 tetraammonium chloride; 10 HEPES; 10 BAPTA tetrapotassium; 2 Mg-ATP; 0.3 Na-GTP; 0.1% Lucifer Yellow;



SUPPLEMENTARY FIG. S11. Inner retinal neurons in 12-week-old hESC-derived retinal tissue have functional neurotransmitter receptors. **(a)** Responses of one cell to puffed agonists, recorded under voltage clamp at three different holding potentials. *Black recording traces* represent responses to 2 mM L-glutamate, and *gray traces* are responses to 1 mM muscimol + 1 mM glycine. **(b)** I-V plots summarizing data from all cells tested ($n = 6$). Error bars show S.E.M. **(c)** Current-clamp responses of another cell to muscimol + glycine (*left*) and L-glutamate (*right*).

and NaOH to adjust pH to 7.3. Using CLAMPEX software (Molecular Devices), the liquid junction potential was calculated to be ~ 13 mV (K^+ internal solution) or ~ 10 mV (Cs^+ internal solution), which was taken into account in all recordings. Unless stated otherwise, the K^+ internal solution was used. QX-314 chloride was purchased from Alomone Labs and muscimol from Tocris. All other chemicals were purchased from Sigma-Aldrich.

Supplementary References

1. Hambright D, KY Park, M Brooks, R McKay, A Swaroop and IO Nasonkin. (2012). Long-term survival and differentiation of retinal neurons derived from human embryonic stem cell lines in un-immunosuppressed mouse retina. Mol Vis 18:920–936.
2. Nasonkin I, V Mahairaki, L Xu, G Hatfield, BJ Cummings, C Eberhart, DK Ryugo, D Maric, E Bar and VE Koliatsos. (2009). Long-term, stable differentiation of human embryonic stem cell-derived neural precursors grafted into the adult mammalian neostriatum. Stem Cells 27:2414–2426.
3. Nasonkin IO and VE Koliatsos. (2006). Nonhuman sialic acid Neu5Gc is very low in human embryonic stem cell-derived neural precursors differentiated with B27/N2 and noggin: implications for transplantation. Exp Neurol 201:525–529.
4. Lamba DA, MO Karl, CB Ware and TA Reh. (2006). Efficient generation of retinal progenitor cells from human embryonic stem cells. Proc Natl Acad Sci U S A 103: 12769–12774.
5. Pittack C, GB Grunwald and TA Reh. (1997). Fibroblast growth factors are necessary for neural retina but not pigmented epithelium differentiation in chick embryos. Development 124:805–816.
6. Zhao S, FC Hung, JS Colvin, A White, W Dai, FJ Lovicu, DM Ornitz and PA Overbeek. (2001). Patterning the optic neuroepithelium by FGF signaling and Ras activation. Development 128:5051–5060.
7. Meyer JS, SE Howden, KA Wallace, AD Verhoeven, LS Wright, EE Capowski, I Pinilla, JM Martin, S Tian, et al. (2011). Optic vesicle-like structures derived from human pluripotent stem cells facilitate a customized approach to retinal disease treatment. Stem Cells 29:1206–1218.
8. Nakano T, S Ando, N Takata, M Kawada, K Muguruma, K Sekiguchi, K Saito, S Yonemura, M Eiraku and Y Sasai. (2012). Self-formation of optic cups and storable stratified neural retina from human ESCs. Cell Stem Cell 10: 771–785.
9. Zhong X, C Gutierrez, T Xue, C Hampton, MN Vergara, LH Cao, A Peters, TS Park, ET Zambidis, et al. (2014). Generation of three-dimensional retinal tissue with functional photoreceptors from human iPSCs. Nat Commun 5:4047.
10. Reichman S, A Terray, A Slembrouck, C Nanteau, G Orieux, W Habeler, EF Nandrot, JA Sahel, C Monville and O Goureau. (2014). From confluent human iPS cells to self-forming neural retina and retinal pigmented epithelium. Proc Natl Acad Sci U S A 111:8518–8523.
11. Nag TC and S Wadhwa. (2006). Morphological and neurochemical development of the human neural retina. Neuroembryol Aging 4:19–30.
12. Glubrecht DD, JH Kim, L Russell, JS Bamforth and R Godbout. (2009). Differential CRX and OTX2 expression in human retina and retinoblastoma. J Neurochem 111:250–263.
13. O'Brien KM, D Schulte and AE Hendrickson. (2003). Expression of photoreceptor-associated molecules during human fetal eye development. Mol Vis 9:401–409.
14. Larsen KB, M Lutterodt, MF Rath and M Moller. (2009). Expression of the homeobox genes PAX6, OTX2, and OTX1 in the early human fetal retina. Int J Dev Neurosci 27:485–492.
15. Hendrickson A, H Djajadi, A Erickson and D Possin. (2006). Development of the human retina in the absence of ganglion cells. Exp Eye Res 83:920–931.
16. Hendrickson A, K Bumsted-O'Brien, R Natoli, V Ramamurthy, D Possin and J Provis. (2008). Rod photoreceptor differentiation in fetal and infant human retina. Exp Eye Res 87:415–426.
17. Engelsberg K and F Ghosh. (2011). Human retinal development in an in situ whole eye culture system. Dev Neurosci 33:110–117.
18. Yan XX and AF Wiechmann. (1997). Early expression of recoverin in a unique population of neurons in the human retina. Anat Embryol (Berl) 195:51–63.
19. McLeod DS, T Hasegawa, T Prow, C Merges and G Lutty. (2006). The initial fetal human retinal vasculature develops by vasculogenesis. Dev Dyn 235:3336–3347.

Supplementary Table S1. List of Q-RT PCR Primers and Target Genes

No.	*Gene*	*Forward*	*Reverse*
1	*SOX1*	ACCAGGCCATGGATGAAG	CTTAATTGCTGGGGAATTGG
2	*NES*	TACACCCCGATCCTGGAAG	TAGCCTCCTGACTCCCTTCA
3	*NCAM1*	CTTGATGTTCGGCACTATTTGT	GGGGGTGAGAACCTCACTG
4	*DCX*	GGATCCAGGAAGATCGGAAG	TTGTCTGAGGAACAGACATAGCTT
5	*MSI1*	CCAATGGGTACCACTGAAGC	CACTCGTGGTCCTCAGTCAG
6	*PROM1*	AAGGCATATGAATCCAAAATTGA	CCACCAGAGGCATCAGAATAA
7	*TUBB3*	ACGTGTGAGCTGCTCCTGT	AAAAACAAAACCGTAAAACGTCA
8	*MAP2*	CGAACTTTATATTTTACCACTTCCTTG	CCGTTCATCTGCCATTCTTC
9	*OLIG2*	AGCTCCTCAAATCGCATCC	ATAGTCGTCGCAGCTTTCG
10	*GFAP*	GGTTGAGAGGGACAATCTGG	AGGTTGTTCTCGGCTTCCA
11	*FOXG1*	AGAGCAGCACGTCCATGAG	AAAACTTGGCAAAGAGGGTCT
12	*LHX2*	CCAAGGACTTGAAGCAGCTC	AAGAGGTTGCGCCTGAACT
13	*MITF*	CAGGTGCCGATGGAAGTC	GCTAAAGTGGTAGAAAGGTACTGCTT
14	*PAX6*	TCACCATGGCAAATAACCTG	CAGCATGCAGGAGTATGAGG
15	*RX*	TTCGAGAAGTCCCACTACCC	ACTTAGCCCGTCGGTTCTG
16	*SIX3*	TGATGTGGAGCCTGTGTCTTT	CCTGCTCCCTGAAGTACACC
17	*SIX6*	GGACACTGCAAGCCCAGTAT	ATGATTCGCGCCCTTTCT
18	*ATOH7*	CCCTAAATTTGGGCAAGTGA	TCGGCCTTCTGTTCTACTGG
19	*POU4F1*	CTCCCTGAGCACAAGTACCC	GGCGAAGAGGTTGCTCTG
20	*POU4F2*	CCTTTCCTCGTCCGCTCTTT	GTTCGCTCCCTCTTCAGTCC
21	*POU4F3*	CCAAATTCTCCAGTCTGCACT	CATCAAAGCTTCCAAATATATTACCC
22	*ISL1*	AAGGACAAGAAGCGAAGCAT	TTCCTGTCATCCCCTGGATA
23	*DLX2*	CTCCCTCAGCTCTCTCCTCA	CCGCAAAGGCACCTAAACT
24	*THY1*	AGGAGCCGGACACTTCTCA	AGTCACAGAACAGGAAGAACCAC
25	*CHX10*	CCGGAAGACAGGATACAGGT	ACTCCGCCATGACACTGC
26	*OTX2*	GGGTATGGACTTGCTGCAC	CCGAGTGAACGTCGTCCT
27	*CRX*	CGAGTTGGTACACACCGTCA	TCTCTTCACATCTCGCCTTTC
28	*ASCL1*	GCTCTTACGACCCGCTCA	ATGCAGGTTGTGCGATCA
29	*NEUROD1*	ACCTCGAAGCCATGAACG	CTTCCAGGTCCTCATCTTCG
30	*NRL*	TCCTCTCGGCCATTTCTG	CTCAAACTTCATCAAGTCAAAGTCA
31	*NR2E3*	CAGAGGCTGCCCTGTAACC	CAGCCACTGTGGAGCTCAT
32	*RCVRN*	TAACGGGACCATCAGCAAG	CCTCGGGAGTGATCATTTTG
33	*RHO*	TATGGGCAGCTCGTCTTCA	TTCTGTGTGGTGGCTGACTC
34	*PDE6B*	GAAGAAACTGAGCCCTGAGAATG	GCTCCATGTTGATGCTCTCCTG
35	*PROX1*	GAGCCTCCGTGGAACTCA	TGGGCACAGCTCAAGAATC
36	*CALB1*	CACAGCCTCACAGTTTTTCG	CCTTTCCTTCCAGGTAACCA
37	*CALB2*	AAGCACTTTGACGCAGACG	CATGCCAGAGCCTTTCCTT
38	*LGR5*	GGGTGGCAGCAAGTATGG	ATGAGAGCGACCATGTAGCC
39	*OPN1SW*	CCCTCATCTGCTTCTCCTACA	GTTCAGCCTTCTGGGTCGTA
40	*OPN1MW*	GGCTACACCGTCTCCCTGT	GCAGACCACCATCCATCTCT
41	*OPN1LW*	CATGGTGGTGGTGATGATCT	CATGCGAAGAAGGTGTAGGG
42	*ARR3*	CCAACCTGGCCTCTAGCAC	CTGACTTTGTAGGACACCAGGA
43	*THRB*	GACAAAGTCACGCGAAATCA	GCCATGCCAACATAGATGC
44	*PDE6C*	ACCAAGTTGCCGTGGAGAAA	ACCCGCAACTTCCTGTCAAA
45	*CCND3*	GGGATCACTGGCACTGAAG	CCTGAGGCTCTCCCTGAGT
46	*GLUL*	CAGTATACTCTTACCAGTGCGAGGT	CACAGTTGCCTGAAGAACTTATCT
47	*MSX1*	CTCGTCAAAGCCGAGAGC	CGGTTCGTCTTGTGTTTGC
48	*GATA4*	GCAGCCAGAGTCCCTCAG	CTGGCTTTTTGCCTCCTG
49	*SOX17*	ACGCCGAGTTGAGCAAGA	TCTGCCTCCTCCACGAAG
50	*FOXA2*	GGGTGATTGCTGGTCGTTT	ATACTGGAAGCCGAGTGCAT
51	*BDNF*	GTAACGGCGGCAGACAAA	GACCTTTTCAAGGACTGTGACC
52	*GDNF*	CCGCTAAAAGGTGTGGATGT	TTGCACTGTAGCAGGAATGC
53	*NGF*	TCCGGACCCAATAACAGTTT	GGACATTACGCTATGCACCTC
54	*VEGFA*	GGATTTTGGAAACCAGCAGA	CCGTCTCTCTCTTCCTCGAC
55	*FGF2*	GGCAGGATTTTTATTGCCATT	GGGACCATCTGGATGTGTAGA
56	*SERPINF1*	GTGTGGAGCTGCAGCGTAT	TCCAATGCAGAGGAGTAGCA
57	*CNTF*	AGGGATGGCTTTCACAGAGC	GCCAGATAGAGCGGCTACAG
58	*DROSHA*	TCATGCCACGTTTTGTAAGATT	GCTTTGCTGCACCTTAACAAG
59	*DICER1*	CAGTCGGCTTCTTCAGTCG	GGATTCCAGTGATCCTCTGC
60	*DGCR8*	AAAACTTGCGAAGAATAAAGCTG	TCTGTTTAACAAAGTCAGGGATGA

(*continued*)

Supplementary Table S1. (Continued)

No.	*Gene*	*Forward*	*Reverse*
61	*TYR*	GCTGCCAATTTCAGCTTTAGA	CCGCTATCCCAGTAAGTGGA
62	*RPE65*	CAATGGGTTTCTGATTGTGGA	CCAGTTCTCACGTAAATTGGCTA
63	*BEST1*	CTGGGACCAGAAACCAGGAC	CTGTGCCAGGAACTCTCCAG
64	*TYRP1*	GCTCCAGACAACCTGGGATAC	GCAACCAGTAACAAAGCGCC
65	*DCT*	CGACTCTGATTAGTCGGAACTCA	GGTGGTTGTAGTCATCCAAGC
66	*GAPDH*	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
67	*SCN1A*	ACTCAGGACTTCTGGGAAAATCT	GAGCCCAAGAAAATGACCAA
68	*SCN2A*	GACCCATATGGCACTAGAACTGT	ACTACCTGGTTTTGGGGGAGAG
69	*KCNA1*	GTTACCCAGCCCAGAGAATG	TGTGTTCCGTGGCTCTCTTT
70	*KCNA6*	AAGGGAGACCAGGGGACTAC	ACGATGTGGGAGGTGCTC

Supplementary Table S2. List of Primary Antibodies

Target cells	*Target proteins/epitope*	*Host*	*Dilutions*	*Vendor*
HESC marker	Oct3/4	Rabbit	1:500	Abcam
	Nanog	Rabbit	1:1,000	Abcam
RPE marker	Ezrin	Mouse	1:250	Abcam
	NHERF1-H100	Rabbit	1:250	Santacruz
Eye field marker	RAX	Rabbit	1:250	Abcam
	OTX2	Rabbit	1:250	Abcam
	MAP2	Mouse	1:500	Abcam
	PAX6	Rabbit	1:500	Covance
	CRX	Mouse	1:500	Abnova
	LHX2	Rabbit	1:250	Gift from Edwin Monuki
	CHX10	Rabbit	1:500	Gift from Connie Cepko
Cell proliferation	Ki67	Rabbit	1:500	Abcam
	Ki67	Mouse	1:500	BD Pharm
Photoreceptor	Recoverin	Rabbit	1:500	Millipore
	HNu	Mouse		Chemicon
Horizontal Axons	NF200	Rabbit	1:500	Chemicon
Amacrine	Calretinin	Rabbit	1:250	Millipore
	LGR5	Rabbit	1:250	Abgent
Ganglion	Brn3b	Rabbit	1:250	gift from Tudor
	Brn3a	Rabbit	1:250	Millipore
	Synaptophysin	Mouse	1:250	Chemicon
Stem cell	TERT	Rabbit	1:250	Abgent
	DCAMLK1	Rabbit	21:250	Abcam

Supplementary Table S3. Gene Expression in hESC-derived Retinal Cells Differentiated in 2D Adherent Monolayer Conditions (2, 3, 4, and 12 weeks) and in 3D Retinal Tissue (6 weeks)

Neuroectoderm, Eye Field

Fate	*NE*	*ANE*	*ANE*	*EYF*	*EYF*	*EYF*	*EYF*	*EYF*
Genes	*SOX1*	*FOXG1*	*OTX2*	*PAX6*	*SIX3*	*SIX6*	*LHX2*	*RX*
2wk	9.5±0.6	29.5±4.0	3.3±0.1	8.4±0.4	26.5±1.0	6.4±1.8	2.6±0.1	4.3±0.3
3wk	5.0±0.4	44.3±7.1	4.0±0.6	13.2±0.2	10.2±0.2	15.8±6.3	6.0±0.4	7.9±1.1
4wk	14.0±1.1	43.3±3.8	7.5±0.2	16.6±2.1	26.3±1.2	35.2±12.0	7.7±0.1	20.0±3.4
6wk 3D	11.2±0.3	218.2±10.7	1.2±0.1	43.7±1.1	9.9±1.4	13.1±1.1	88.4±4.8	206.9±31.3
12wk 2D	43.1±4.8	355±31.2	2.42±0.1	19.5±0.2	13.5±2.4	3.3±0.79	214.6±9.5	17.3±1.4

Early Neuronal, glial

Fate	*EN*	*EN*	*EN*	*EN*	*ASTR*	*OLIG*	*NCR*
Genes	*MAP2*	*DCX*	*ASCL1*	*NEUROD1*	*GFAP*	*OLIG2*	*MSX1*
2wk	4.8±0.3	12.5±0.3	58.5±0.6	244.7±9.6	0.4±0.0	0.6±0.0	0.6±0.0
3wk	6.2±0.2	15.5±1.0	47.1±3.0	240.9±20.1	0.7±0.1	1.1±0.0	4.6±0.1
4wk	10.8±0.1	26.0±0.1	134.2±12.5	377.3±5.5	1.0±0.0	3.4±0.2	1.7±0.0
6wk 3D	3.0±0.05	9.1±0.3	74.2±5.4	249±6.1	1.4±0.05	1.4±0.31	1.7±2.3
12wk 2D	21.4±0.4	33.4±1.9	144.2±6.4	160.4.0±6.6	1.9±0.08	1.1±0.06	0.1±3.2

RPE markers

Genes	*MITF*	*BEST1 (VMD2)*	*TYR*	*TYRP*	*RPE65*	*DCT*	*PMEL*
2wk	67.2±4.6	0.46±0.0	85.8±2.5	277.6±5.4	902±1.6	494.2±41	1.0±0.004
3wk	101.5±1.4	1.1±0.0	4562.7±89.4	2815.7±151.7	8520±33.4	2037.6±69.9	1.4±0.1
4wk	264.3±7.1	2.0±0.0	8173.8±480.5	5106.9±25.0	1063.2±10.4	4116.9±80.7	2.6±0.1
6wk 3D	359.3±22.9	0.63±0.1	13676.8±402	4446.5±8.4	3035.2±267.4	11462.7±280.9	6.9±0.2
12wk 2D	16.9±0.2	1.9±0.0	693.0±40.1	488.9±4.8	253.2±5.0	161.2±2.4	0.2±0.0

Retinal progenitors, retina ganglion cell markers

Fate	*Ret.progen.*	*Ret.progen.*	*RGC*	*RGC*	*RGC*	*RGC*	*RGC*	*RGC*
Genes	*CHX10*	*IKZF1*	*MATH5 (ATOH7)*	*ISL1*	*BRN3A*	*BRN3B*	*BRN3C*	*DLX2*
2wk	1.0±0.0	3.9±1.3	0.3±0.0	6.0±0.4	0.7±0.0	2.2±0.0	2.7±0.3	1.0±0.0
3wk	0.7±0.2	1.9±0.0	0.9±0.1	8.9±1.6	1.3±0.1	4.0±1.2	2.3±0.2	1.3±0.1
4wk	1.5±0.5	2.3±0.0	1.3±0.2	14.8±1.9	1.7±0.2	2.3±0.1	2.1±0.1	4.9±0.0
6wk 3D	98.7±10.1	0.5±0.0	48.1±4.2	17.2±2.3	1.4±0.2	119.3±5.8	5.3±2.6	3.6±0.1
12wk 2D	314.0±12.3	1.4±0.3	15.0±0.2	3.7±0.4	4.0±1.0	18.3±0.1	9.7±1.0	17.2±2.9

Photoreceptor markers

Fate	*Cone/Rod*	*Cone/Rod*	*Rod*	*Rod*	*Rod*	*Rod*	*Cone*	*Cone*	*Cone*	*Cone* *THRB*	*Cone* *ARR3*
Genes	*CRX*	*RCVRN*	*NRL*	*NR2E3*	*PDE6B*	*RHO*	*PDE6C*	*OPN1SW*	*OPN1MW*	(*Trβ2*)	(*CAR*)
2wk	1.7±0.3	1.5±0.0	1.4±0.1	0.7±0.1	2.1±0.0	0.4±0.1	0.3±0.0	8.7±0.2	2.2±0.5	1.5±0.05	0.7±0.05
3wk	3.2±0.2	7.2±0.1	1.1±0.4	1.8±0.1	2.1±0.1	0.9±0.1	0.8±0.0	4.1±1.5	5.2±1.3	1.6±0.06	0.8±0.07
4wk	6.6±0.5	5.5±0.5	2.3±0.8	5.6±0.5	4.9±0.4	2.0±0.1	0.9±0.1	31.4±2.2	8.9±0.4	2.8±0.04	0.9±0.03
6wk, 3D	11.2±0.1	49.2±1.7	2.0±0.2	2.1±0.1	6.9±0.1	1.7±0.4	0.1±0.0	48.4±4.3	1.2±0.1	2.3±0.09	1.5±0.0
12wk, 2D	13.4±0.5	24.0±0.9	4.9±0.0	2.0±0.1	5.6±0.3	1.1±0.0	2.2±0.1	3.5±0.0	0.5±0.2	3.2±0.09	0.8±0.05

Inner Nuclear Layer markers

Fate	*Mu.Glia*	*Mu.Glia*	*Horiztl*	*Horiztl*	*Amacrn*	*Amacrn*	*Rod bipolar*
Genes	*GLUL*	*CCND3*	*PROX1*	*CALB1*	*CALB2*	*LGR5*	*PRKCA*
2wk	1.4±0.2	0.9±0.1	11.7±0.3	14.5±5.6	0.7±0.1	306.8±6.0	1.1±0.3
3wk	1.3±0.1	1.7±0.3	18.7±0.5	28.4±0.4	0.6±0.0	280.3±2.8	0.7±0.1
4wk	4.6±0.4	3.4±0.3	38.4±4.5	30.6±0.3	1.5±0.1	275.6±9.5	1.2±0.0
6wk 3D	1.0±0.0	0.8±0.1	9.2±0.7	4.3±0.1	0.3±0.1	493.7±21.8	0.6±0.0
12wk 2D	1.4±0.0	3.4±0.1	41.4±2.3	35.9±2.8	4.8±0.3	68.3±1.0	2.3±0.1

(continued)

Supplementary Table S3. (Continued)

Trophic factors

Genes	*BDNF*	*GDNF*	*NGF*	*CNTF*	*PEDF = SERPIN-F1*	*VEGFA*	*FGF2*
2wk	0.2 ± 0.2	1.3 ± 0.1	2.4 ± 0.1	3.3 ± 0.7	2.6 ± 0.3	0.8 ± 0.1	0.3 ± 0.1
3wk	0.4 ± 0.0	1.9 ± 0.0	4.5 ± 0.2	5.5 ± 0.5	0.0 ± 0.0	2.1 ± 0.1	0.4 ± 0.1
4wk	0.9 ± 0.1	1.7 ± 0.1	4.7 ± 0.3	7.3 ± 0.3	8.5 ± 0.6	2.1 ± 0.1	0.5 ± 0.1
6wk 3D	0.09 ± 0.0	0.9 ± 1.2	2.49 ± 0.07	1.37 ± 0.0	7.43 ± 0.3	2.07 ± 0.1	0.54 ± 0.1
12wk	0.8 ± 0.0	1.7 ± 0.1	6.1 ± 0.4	4.5 ± 0.3	41.4 ± 8.9	3.7 ± 0.2	0.5 ± 0.1

MicroRNA-processing genes and Telomerase (Neural Retina plates)

Genes	*DICER*	*DROSHA*	*LIN28*	*DGCR8 (PASHA)*	*AGO2*	*TERT*
2wk	1.5 ± 0.1	1.4 ± 0.1	0.1 ± 0.0	0.66 ± 0.0	0.69 ± 0.0	0.05 ± 0.0
3wk	2.1 ± 1.0	1.2 ± 0.0	0.3 ± 0.0	1.3 ± 0.0	0.9 ± 0.0	0.12 ± 0.0
4wk	3.4 ± 0.1	2.4 ± 0.1	0.2 ± 0.0	1.6 ± 0.0	1.2 ± 0.0	0.19 ± 0.1
6wk 3D	2.0 ± 0.1	1.4 ± 0.1	0.19 ± 0.0	1.2 ± 0.0	1.1 ± 0.0	0.18 ± 0.1
12wk 2D	2.0 ± 0.0	1.5 ± 0.0	0.001 ± 0.0	2.0 ± 0.1	0.7 ± 0.1	0.12 ± 0.0

wk, week.

Supplementary Table S4. Expression Pattern of Genes in Different In Vitro Models and Human Fetal Development

Source	*Lamba et al. [4] hESC (H1)*	*Meyer et al. [7] hiPSC (fibroblast)*	*Nakano et al. [8] hESC (KhES-1)*	*Zhong et al. [9] hiPSC (CB-iPSC6.2, KA.1, IMR90-4)*	*Reichman et al. [10] Human dermal fibroblast-derived iPSC*
Genes					
CRX	D21	D20	D28-34	—	D14
PAX6	D21	D20	D34	D12	D14
OTX2	—	D20	—	D49	D21
BRN3A	—	D80	D30	D35	D21
CALRETININ	—	D80	—	D35	D21
RECOVERIN	—	D80	D43	D63	D42
RHODOPSIN	—	—	D126	D119	D112
R/G OPSIN	—	—	D126	D119	D112
SYNAPTOPHYSIN	D28-35	—	—	—	—
Ki67	—	—	—	D14	D14

Gene	*Fetal week retina*	*Reference*
CRX	10/11	[11–13]
PAX6	6–10	[14]
OTX2	6–11	[12,14]
BRN3A	19–21	[15]
CALRETININ	11–25	[11,15,16]
RECOVERIN	10.5–13	[13,16–18]
RHODOPSIN	14.5	[16]
R/G OPSIN	11.5	[16]
SYNAPTOPHYSIN	14–25	[11,15]
NF200	19–21	[15]
KI67	6	[19]