In vitro differentiation of retinal cells from human pluripotent stem cells by small-molecule induction

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Accepted 19 June 2009
Journal of Cell Science 122, 3169-3179 Published by The Company of Biologists 2009
doi:10.1242/jcs.050393

Summary

The use of stem-cell therapy to treat retinal degeneration holds great promise. However, definitive methods of retinal differentiation that do not depend on recombinant proteins produced in animal or *Escherichia coli* cells have not been devised. Here, we report a defined culture method using low-molecular-mass compounds that induce differentiation of human embryonic stem (ES) cells and induced pluripotent stem (iPS) cells into retinal progenitors, retinal pigment epithelium cells and photoreceptors. The casein kinase I inhibitor CKI-7, the ALK4 inhibitor SB-431542 and the Rho-associated kinase inhibitor Y-27632 in serum-free and feeder-free floating aggregate culture induce retinal progenitors positive for RX, MITF, PAX6 and CHX10. The treatment induces hexagonal pigmented cells that express RPE65 and CRALBP, form ZO1-positive tight junctions and exhibit phagocytic functions. Subsequent treatment with retinoic acid and taurine induces photoreceptors that express recoverin, rhodopsin and genes involved in phototransduction. Both three-factor (OCT3/4, SOX2 and KLF4) and four-factor (OCT3/4, SOX2, KLF4 and MYC) human iPS cells could be successfully differentiated into retinal cells by small-molecule induction. This method provides a solution to the problem of cross-species antigenic contamination in cell-replacement therapy, and is also useful for in vitro modeling of development, disease and drug screening.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/122/17/3169/DC1

Key words: ES cell, Differentiation, iPS cell, Regeneration, Retina, Transplantation

Introduction

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of blastocyst stage embryos that can maintain an undifferentiated state indefinitely and differentiate into derivatives of all three germ layers: the ectoderm, mesoderm and endoderm (Evans and Kaufman, 1981). The pluripotency of ES cells has raised the possibility that they might be used to treat various degenerative diseases. However, the clinical application of human ES cell therapy faces ethical difficulties concerning the use of human embryos, as well as tissue rejection following implantation. One way to circumvent these issues is to generate pluripotent cells directly from somatic cells. Development normally proceeds irreversibly from embryo to adult as cells progressively differentiate into their final, specialized cell types. Remarkably, adult somatic cells can be reprogrammed and returned to the naive state of pluripotency found in the early embryo simply by forcing expression of a defined set of transcription factors. Forced expression of just three transcription factors, Oct4, Sox2 and Klf4, can reprogram somatic cells into induced pluripotent stem (iPS) cells (Nakagawa et al., 2008; Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Yu et al., 2007). iPS cells have been shown to be functionally equivalent to ES cells, as they express ES cell markers, have similar gene expression profiles, form teratomas, and contribute to all cell types in chimeric animals, including the germ line (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). These properties makes ES cells and iPS cells an attractive potential donor source for cell replacement therapies in tissues damaged by disease or injury (Hanna et al., 2007; Lindvall and Kokaia, 2006; Osakada and Takahashi, 2009; Wernig et al., 2008).

The adult mammalian central nervous system (CNS) contains endogenous neural stem cells that are capable of proliferation and differentiation, with newly generated neurons in the hippocampus and olfactory bulbs integrating into pre-existing neural circuits (Lledo et al., 2006; Zhao et al., 2008). In the adult retina, Müller glia act as endogenous progenitors in response to injury, but are too few in number to restore function after damage has occurred (Ooto et al., 2004; Osakada et al., 2007). Thus, transplantation of donor cells to replace damaged or lost cells is a promising approach for regeneration therapy. Retinas with photoreceptor degeneration can be repaired by transplantation of photoreceptor precursors or ES cell-derived progenitors, which are able to form synaptic connections to the host retina and improve visual function (Lamba et al., 2009; MacLaren et al., 2006). In addition, transplantation of ES cell-derived retinal pigment epithelia (RPE) has been reported to improve visual function in RPE degeneration diseases, such as age-related macular degeneration (Haruta et al., 2004; Lund et al., 2006). Unlike somatic stem cells, ES cells and iPS cells are able to propagate indefinitely. If photoreceptor and/or RPE cells could be differentiated from human ES cells or iPS cells under defined conditions, and the safety of their transplantation could be ensured, this approach would represent enormous potential for therapeutic treatment of retinal degeneration.

However, the use of human pluripotent stem cells as a donor cell source for transplantation therapy requires defined and controlled differentiation conditions. Although much progress has been made in the differentiation and propagation of human ES cells, definitive methods of retinal differentiation have not been devised (Hirano et al., 2003; Ikeda et al., 2005; Lamba et al., 2006; Osakada et al., 2008; Osakada et al., 2009; Zhao et al., 2002). Previously, we showed that retinal progenitors, photoreceptors and RPE cells can be generated from ES cells by mimicking developmental processes in a stepwise fashion in vitro (Ikeda et al., 2005; Osakada et al., 2008; Osakada et al., 2009). However, this culture method requires the addition of recombinant Dkk1 and Lefty-A (also known as Lefty2) proteins, which are produced in animal cells or *E. coli*, raising the possibility of infection or immune rejection due to cross-species contamination.

By contrast, using chemical compounds to induce differentiation offers several advantages compared with using recombinant proteins. Not only are they non-biological products, but they show stable activity, have small differences between production lots, and are low-cost. Thus, establishment of chemical compound-based culture systems will be necessary for human pluripotent cell-based transplantation therapies (Ding and Schultz, 2004). Here we show that chemical compounds can induce retinal progenitors, photoreceptors, and RPE cells from human ES and iPS cells.

Results

CKI-7 and SB-431542 induce retinal specification

We previously reported that ES cells differentiate into retinal progenitors when recombinant Dkk1 and Lefty-A proteins are added during suspension culture, known as serum-free culture of embryoid body-like aggregates, or SFEB (Ikeda et al., 2005; Osakada et al., 2008; Osakada et al., 2009; Watanabe et al., 2005). Since Dkk1 and Lefty-A inhibit Wnt and Nodal signaling, respectively, we focused on chemical inhibitors that block Wnt and Nodal signaling. The small molecule CKI-7 blocks Wnt signaling by inhibiting casein kinase I, a positive regulator of Wnt signaling (Chijiwa et al., 1989; Peters et al., 1999; Sakanaka et al., 1999). Similarly, SB-431542 blocks Nodal signaling by inhibiting activin receptor-like kinase (ALK)4, 5 and 7, which heterodimerize with activin type I receptors and are activated by phosphorylation upon binding of Nodal (Eiraku et al., 2008; Inman et al., 2002; Laping et al., 2002). Therefore, we postulated that the chemical inhibitors CKI-7 and SB-431542 could mimic the effects of Dkk1 and Lefty-A.

First, we examined the effects of CKI-7 and SB-431542 on Wnt and Nodal signaling in SEFB culture of mouse ES cells. Several lines of evidence indicate that Wnts, Nodal and Cripto inhibit neural commitment in ES cells (Aubert et al., 2002; Parisi et al., 2003). Real-time PCR demonstrated that the levels of *Wnt1*, *Wnt3*, *Nodal* and *Cripto* gradually increased during SFEB culture (Fig. 1A-D), suggesting the activation of Wnt and Nodal signaling during SFEB culture. We next determined the optimal concentrations of CKI-7 and SB-431542 for treatment of cells. Application of CKI-7 (0.1-10 μM) or SB-431542 (0.1-10 μM) from days 0-5 significantly increased the expression of the early neuroectodermal marker *Sox1* and the number of cells positive for the neural markers nestin and βIII-tubulin, in a concentration-dependent manner (data not shown). It should be noted that nestin is expressed in mitotically active areas of the developing and adult CNS, but is not a specific marker for neural stem cells, as it is expressed by differentiated astrocytes and neuronal progenitors and is also upregulated in glial cells after CNS injury (Chojnacki et al., 2009). Immunoblotting revealed that application of CKI-7 (5 μM) suppressed SFEB-induced β-catenin stabilization on day 5 (Fig. 1E), indicating that it inhibited Wnt signaling. In addition, application of SB-431542 (5 μM) abolished SFEB-induced Smad2 phosphorylation on day 5 (Fig. 1F), indicating that it inhibited Nodal signaling. We next examined the effects of CKI-7 and SB-431542 on neural differentiation. Combined application of CKI-7 (5 μM) and SB-431542 (5 μM) significantly increased *Sox1* expression on day 5 (Fig. 1G) and the number of nestin-positive and βIII-tubulin-positive cells on day 8 (Fig. 1H,I). Thus, we conclude that CKI-7 and SB-431542 block Wnt signaling and Nodal signaling, respectively, and thereby promote neural differentiation of ES cells.

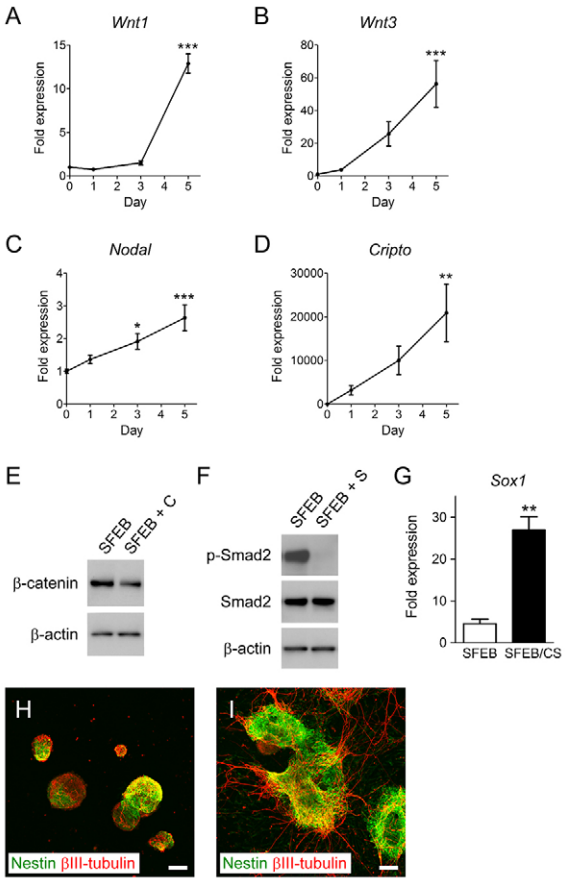

Fig. 1. CKI-7 and SB-431542 promote neural differentiation by blocking Wnt and Nodal signaling. (A-D) Wnt and Nodal signaling during SFEB culture. Levels of *Wnt1*, *Wnt3*, *Nodal* and *Cripto* were normalized to those on day 0. **P*<0.05, ***P*<0.01, ****P*<0.001, compared to day 0 (Dunnett's test). (E) CKI-7 inhibits Wnt signaling during SFEB culture. (F) SB-431542 inhibits Nodal signaling during SFEB culture. (G-I) Effects of CKI-7 and SB-431542 on neural differentiation. SFEB/CS treatment increases the expression of the early neuroectodermal marker *Sox1* (G) and the number of cells positive for neural markers Nestin (green) and βIII-tubulin (red; H, SFEB treatment; I, SFEB/CS treatment). ***P*<0.01, compared with SFEB (unpaired *t*-test, G). Scale bar, 100 μm (H,I).

Next, we examined whether CKI-7 and SB-431542 could induce neural differentiation in human ES cells. Undifferentiated human ES cells (khES-1) were dissociated into small clumps of five to ten cells and seeded as suspension cultures (Fig. 2A). CKI-7 (5 μM) and SB-431542 (5 μM) were added to the differentiation medium

of the suspension culture for 21 days (SEFB/CS culture). In addition, to improve cell survival during differentiation, the Rho-associated kinase inhibitor Y-27632 (10 μM), which prevents dissociation-induced cell death in human ES cells (Watanabe et al., 2007), was added 1 hour before dissociation and was maintained in the differentiation medium during the first 15 days of floating culture. Under these conditions, ES cells formed embryoid body-like aggregates. Expression of the undifferentiated ES cell markers *NANOG* and *OCT3/4* decreased during the suspension culture. SEFB/CS treatment significantly reduced the levels of *NANOG* and *OCT3/4* on day 21 (Fig. 2B,C). Subsequently, these aggregates were plated onto poly-D-lysine-laminin-fibronectin-coated slides on day 21, and cultured until day 40 (Fig. 2D). Immunostaining revealed that the neural progenitor marker nestin (NES) was strongly expressed by day 30, and 94.8±3.3% of colonies were positive for NES on day 40 (Fig. 2E). The neuronal marker βIII-tubulin was rarely detected on or before day 30, and substantially increased during days 35-40. By day 40, 92.7±3.3% of colonies were positive for βIII-tubulin (Fig. 2F,G). By contrast, expression of *NANOG* and *OCT3/4* had disappeared by day 40.

To characterize SFEB/CS-induced neural tissues, we next performed quantitative RT-PCR for regional gene markers along the rostral-caudal axis. SFEB/CS treatment increased the expression levels of the rostral CNS markers *BF1* (telencephalon), *RX* (retina and diencephalon), and *SIX3* (rostral diencephalon and brain tissue rostral to it), and decreased the caudal markers *IRX3* (caudal diencephalon and brain tissue caudal to it), *GBX2* (rostral hindbrain), and *HOXB4* (hindbrain and spinal cord), compared with SFEB treatment (Fig. 2H-M). These results indicate that SFEB/CS treatment preferentially induces the rostral-most CNS in human ES cells.

Following neural tube formation in vertebrates, progenitors in the optic vesicle and the optic cup express Mitf in the outer layer that will give rise to the RPE, Rx in the inner layer that will give rise to the neural retina, and Pax6 in both layers (Baumer et al., 2003; Furukawa et al., 1997; Ikeda et al., 2005; Mathers et al., 1997) (Fig. 2N). To determine whether CKI-7 and SB-431542 promote retinal specification of ES cells, we examined the expression of these markers in SFEB/CS-treated ES cells by immunocytochemistry. After 30 days in SFEB culture, colonies were rarely positive for MITF and RX. However, SFEB/CS treatment significantly increased the number of MITF-positive (MITF+) colonies (Fig. 2O,P; 32.5±3.0% of total colonies, 22.8±3.1% of total cells). SEFB/CS treatment was as efficient as treatment with recombinant proteins Dkk1 (100 ng/ml) and Lefty-A (500 ng/ml; SFEB/DL; Fig. 2P). On day 35, 25.4±2.9% of colonies were RX+ in SFEB/CS culture (Fig. 2O). The induced RX+ cells were frequently found in close proximity to MITF+ cells.

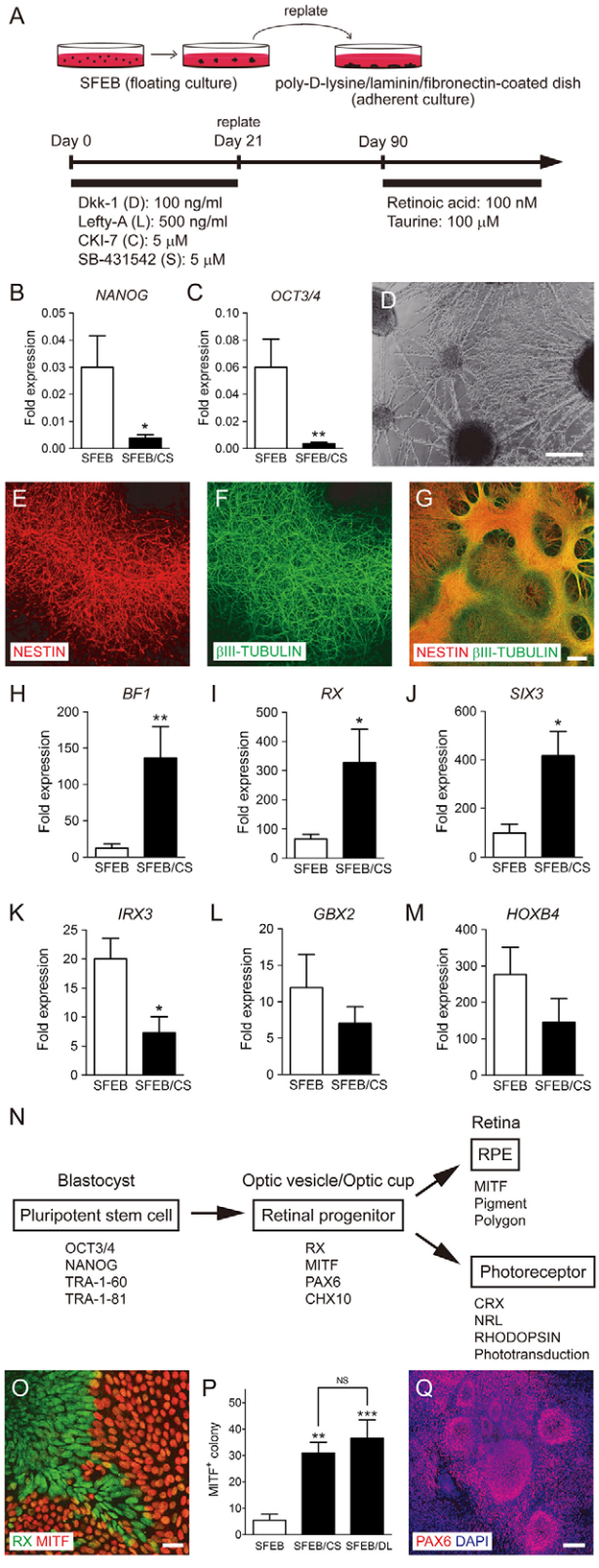

Fig. 2. Retinal specification of human ES cells by CKI-7 and SB-431542. (A) Schematic diagram of the culture procedure for retinal differentiation. (B,C) CKI-7 and SB-431542 decrease the expression of *NANOG* and *OCT3/4*, markers of the undifferentiated state. **P*<0.05, ***P*<0.01, compared with SFEB (unpaired *t*-test). (D) Phase-contrast image of human ES cells treated with CKI-7 and SB-431542. (E-G) Human ES cells treated with CKI-7 and SB-431542 express the neural markers NES (red) and βIII-tubulin (green) on day 40. (H-M) Regional characterization of SFEB/CS-treated neural tissues derived from human ES cells. Fold expression is the ratio of expression in differentiated versus undifferentiated ES cells. (N) Multi-step commitment in the development of retinal cells. Pluripotent stem cells derived from the inner cell mass (blastocyst) differentiate into retinal progenitors corresponding to those in the eye primordium (optic vesicle/optic cup) that give rise to RPE and photoreceptors (adult retina). (O) RX+ and MITF+ retinal progenitor cells develop from human ES cells under SFEB/CS culture conditions. (P) Effect of CKI-7 and SB-431542 on the percentage of MITF+ colonies. ***P*<0.01, ****P*<0.001, compared with SFEB. NS, not significant (Tukey's test). (Q) Formation of rosette-like clusters positive for PAX6. Scale bars: 300 μm (D), 100 μm (G,Q), 30 μm (O).

In addition, 79.2±5.1% of colonies were positive for PAX6, with some forming rosette-like clusters in SFEB/CS cultures (Fig. 2Q). Thus, SFEB/CS treatment is able to induce retinal progenitors from human ES cells.

Small molecule induced, ES-cell-derived retinal progenitors are competent to differentiate into RPE and photoreceptors

Next, we examined whether SFEB/CS-induced retinal progenitors could differentiate into RPE cells. On day 35, most MITF+ cells coexpressed PAX6, consistent with the in vivo marker profile of the embryonic RPE (Fig. 3A). On day 40, pigmented cells appeared. On day 60, pigmented cells with the squamous and hexagonal morphology characteristic of RPE cells were observed in SFEB/CS cultures (26.0±4.0% of colonies, 18.1±1.9% of total cells; Fig. 3B). No significant difference in the frequency of pigment cell induction was found between SFEB/CS treatment and SFEB/DL treatment (Fig. 3C). Immunostaining with an anti-ZO1 antibody showed that pigment cells derived from human ES cells formed tight junctions with a polygonal morphology by day 100 (Fig. 3D). We also examined the expression of genes related to the cellular functions of RPE cells. SFEB/CS-treated human ES cells expressed both *RPE65* and *CRALBP* on day 120 (Fig. 3E,F). Retinal pigment epithelium-specific protein 65 kDa (RPE65) is strongly expressed in RPE cells and is involved in the conversion of all-*trans* retinol to 11-*cis* retinal and in visual pigment regeneration, whereas cellular retinaldehyde-binding protein (CRALBP; also known as RLBP1) is involved in vitamin A metabolism. Furthermore, in the adult retina, RPE cells phagocytose the outer segment of photoreceptors to maintain photoreceptor function. We conducted a latex bead phagocytosis assay with our SFEB/CS-induced pigmented cells, and showed that Phalloidin-stained polygonal cells incorporated the latex beads (Fig. 3G). Thus, we conclude that SFEB/CS-treated cells are competent to differentiate into pigment cells with typical RPE characteristics.

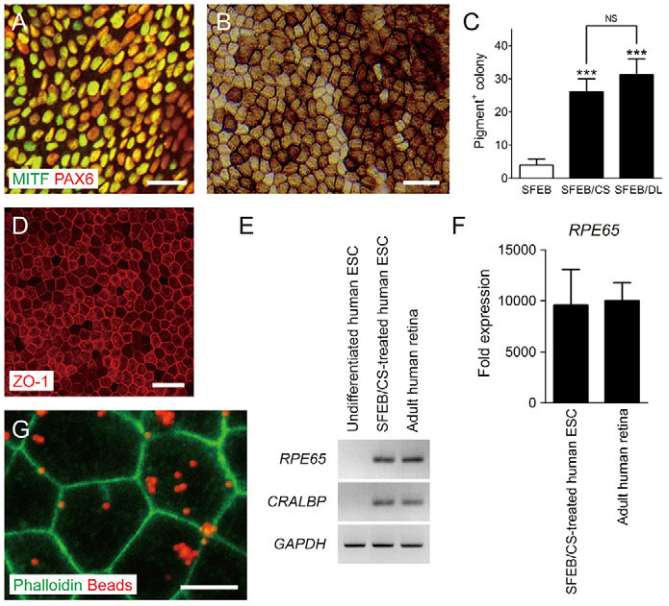

Fig. 3. Differentiation of RPE from SFEB/CS-treated human ES cells. (A) SB-431542 and CKI-7 treatment induced MITF+ (green)/PAX6+ (red) RPE progenitors from human ES cells. (B) Generation of polygonal pigment cells. (C) Effect of SB-431542 and CKI-7 on the percentage of pigment cells. ***P<0.001, compared with SFEB. NS, not significant (Tukey's test). (D) Tight junction formation of SFEB/CS-treated cells, as shown by anti-ZO1 antibody staining (red). (E) Maturity of human ES-cell-derived pigment cells. RPE65, retinal pigment epithelium-specific protein 65 kDa; CRALBP, cellular retinaldehyde-binding protein. (F) Quantitative RT-PCR analysis of *RPE65* in SFEB-CS-treated human ES cells. (G) Induced pigment cells have phagocytic function. Phalloidin-stained polygonal cells (green) incorporated latex beads (red). Scale bars: 30 μm (A,B,D), and 10 μm (G).

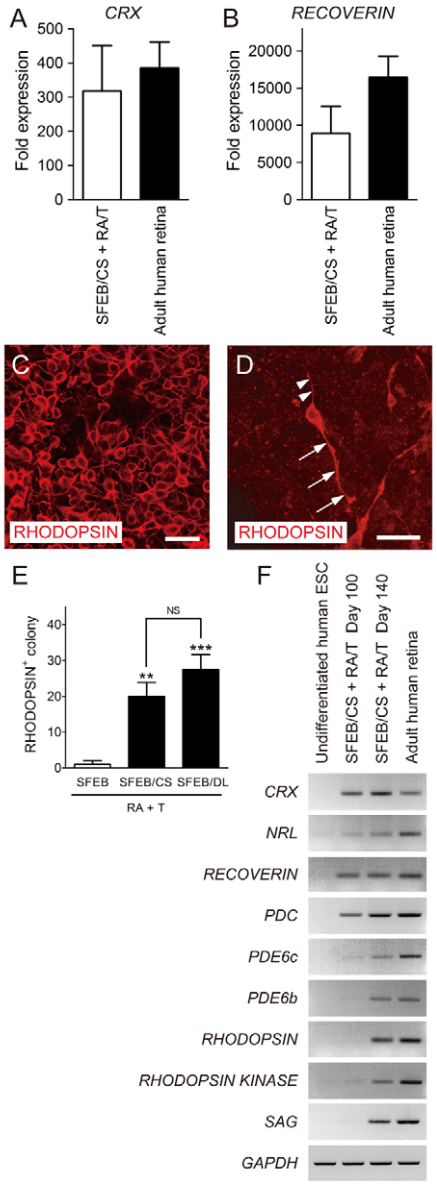

Fig. 4. Differentiation of photoreceptors from SFEB/CS-treated human ES cells. (A,B) Quantitative PCR analysis for the photoreceptor precursor marker *CRX* (A) and the mature photoreceptor marker *RCVRN* (B). Fold expression is the ratio of expression in differentiated versus undifferentiated human ES cells. SFEB/CS-cultured human ES cells were treated with retinoic acid and taurine (RA/T). (C,D) Immunostaining for the photoreceptor marker RHO. An outer process (arrows) and inner process (arrowheads) are present in SFEB/CS+RA/T-treated cells (D). Scale bars: 30 μm (C,D). (E) Effect of CKI-7 and SB-431542 on the percentage of RHO+ cells. Treatment with retinoic acid and taurine (RA+T). **P<0.01, ***P<0.001, compared with SFEB+RA/T. NS, not significant (Tukey's test). (F) RT-PCR analysis of human ES cells treated with SFEB/CS+RA/T. Expression of photoreceptor markers and phototransduction genes on days 100 and 140.

We then asked whether SFEB/CS-induced retinal progenitors could differentiate into retinal photoreceptors. We previously reported that the chemical compounds retinoic acid and taurine, both of which are critical for photoreceptor development, promote photoreceptor differentiation in an ES cell culture system (Osakada et al., 2008). When we treated SFEB/CS-induced ES cells with retinoic acid (100 nM) and taurine (100 μM) beginning at day 90 (SFEB/CS + RA/T treatment), the photoreceptor precursor marker *CRX* and the early photoreceptor markers *NRL* and recoverin (*RCVRN*) were detected on day 100 (Fig. 4A,B). By day 140, the differentiated cells expressed the mature photoreceptor marker rhodopsin (RHO; Fig. 4C). Cultured photoreceptors do not form outer segments, but putative outer and inner processes were observed in human ES cell-derived RHO+ cells (Fig. 4D), and SFEB/CS treatment significantly increased the number of RHO+ cells (20.1±3.9% of total colonies, 6.5±1.2% of total cells). The differentiation efficiencies of SFEB/CS and SFEB/DL treatment did not significantly differ (Fig. 4E). To determine the maturity of induced photoreceptors, we examined the expression of genes responsible for phototransduction. Human ES cells treated with SFEB/CS + RA/T expressed *RCVRN* (rods and cones), phosducin (*PDC*, rods and cones), phosphodiesterases (*PDE6b*, rods; *PDE6c*, cones), *RHO* (rods), rhodopsin kinase (*GRK1*, rods), and arrestin S-antigen (*SAG*, rods) by day 140 (Fig. 4F). These results indicate that the SFEB/CS + RA/T-treated ES cells are competent to respond to light. In addition, other types of retinal neurons were observed under these culture conditions at low efficiency, including HPC1+/PAX6+ amacrine cells, PKCα+ bipolar cells, and PAX6+/Islet1/2+ ganglion cells (<1%), as observed with SFEB/DL culture (Osakada et al., 2008). Thus, we conclude that SFEB/CS-induced retinal progenitors are competent to differentiate into photoreceptors in response to retinoic acid and taurine.

Retinal differentiation of human induced pluripotent stem (iPS) cells

Finally, we determined whether CKI-7 and SB-431542 could induce retinal differentiation in human iPS cells. iPS cells (clone 253G1) generated from human dermal fibroblasts by retroviral gene transfer of OCT3, SOX2, and KIF4 expressed pluripotent stem cell markers NANOG, OCT3/4, TRA-1-60, and TRA-1-81, but not pan-neural markers NES and βIII-tubulin (Fig. 5A,B; and data not shown). iPS cells were seeded as suspension cultures in the presence of Y-27632 (10 μM, days 0-14), CKI-7 (5 μM, days 0-20), and SB-431542 (5 μM, days 0-20). Under these conditions, human iPS cells grew as floating aggregates, in a manner similar to human ES cells treated with Y-27632 (10 μM, days 0-14), CKI-7 (5 μM, days 0-20), and SB-431542 (5 μM, days 0-20). On day 21, these aggregates were plated onto poly-D-lysine-laminin-fibronectin-coated slides. Immunostaining revealed that most (>80%) of the colonies are positive for the neural progenitor markers NES, βIII-tubulin, and NCAM on day 40 (Fig. 5C; and data not shown).

To characterize SFEB/CS-induced cells, we next performed quantitative RT-PCR for rostral-caudal CNS markers. SFEB/CS

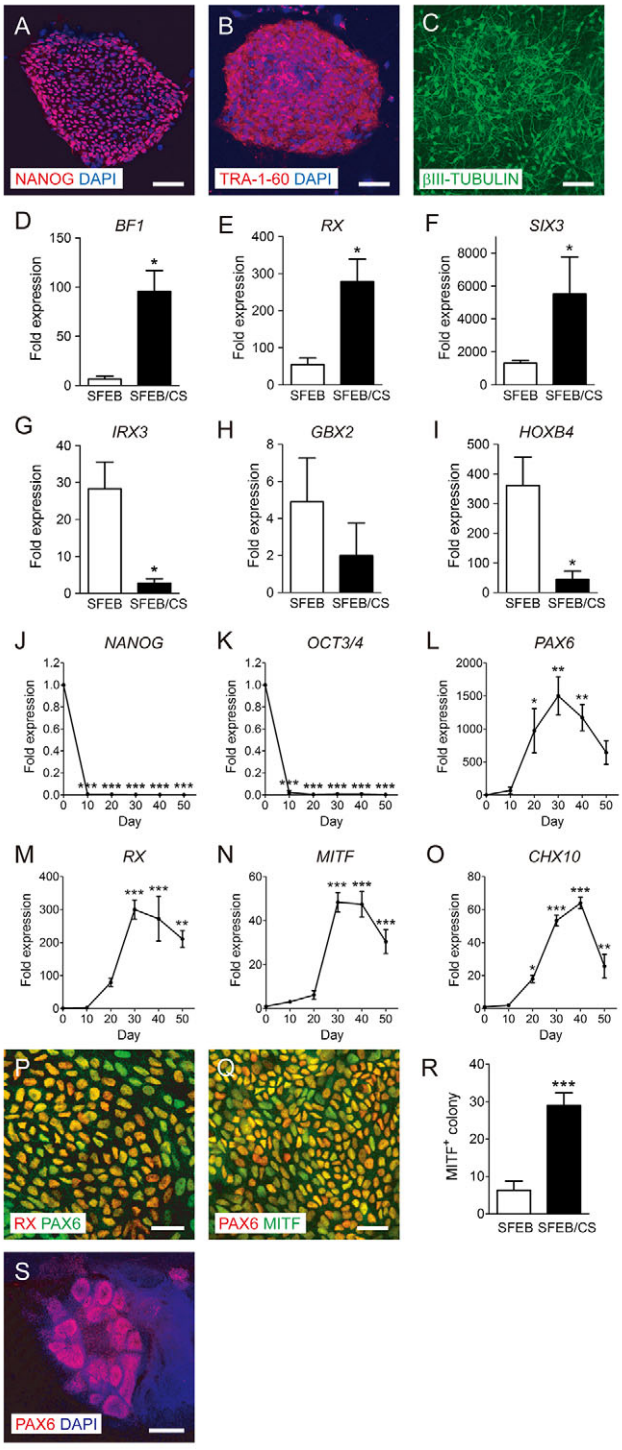

Fig. 5. Retinal specification of human iPS cells by CKI-7 and SB-431542. (A,B) Expression of pluripotent cell markers NANOG and TRA-1-60 in human iPS cells. (C) Neural induction of human iPS cells by SFEB/CS treatment. (D-I) Regional characterization of SFEB/CS-treated iPS cells. Fold expression is ratio of expression in differentiated versus undifferentiated iPS cells. **P*<0.05, compared with SFEB (unpaired *t*-test). (J-O) Time-course analysis of the expression of markers of the undifferentiated state, NANOG (J) and OCT3/4 (K) and retinal progenitor markers PAX6 (L), RX (M), MITF (N) and CHX10 (O) during SFEB/CS culture. Fold expression is ratio of expression on each day compared to day 0. **P*<0.05, ***P*<0.01, ****P*<0.001, compared to day 0 (Dunnett's test). (P) Differentiation of RX+/PAX6+ neural retina progenitors from SFEB/CS-treated human iPS cells. (Q) Differentiation of MITF+/PAX6+ RPE progenitors from SFEB/CS-treated iPS cells. (R) Effect of CKI-7 and SB-431542 on the percentage of MITF+ colonies. ****P*<0.001, compared with SFEB alone (unpaired *t*-test). (S) Formation of rosette-like clusters positive for PAX6. Scale bar, 100 μm (A-C), 30 μm (P,Q), and 300 μm (S).

treatment promoted expression of the rostral CNS markers *BF1* (telencephalon), *RX* (retina and diencephalon) and *SIX3* (rostral diencephalon and more rostral brain tissue) and suppressed expression of the caudal markers *IRX3* (caudal diencephalon and more caudal brain tissue), *GBX2* (rostral hindbrain) and *HOXB4* (hindbrain and spinal cord; Fig. 5D-I). Time-course analysis demonstrated that expression levels of the undifferentiated-state markers *NANOG* and *OCT3/4* decreased by day 10 (Fig. 4J,K). The levels of the retinal progenitor markers *PAX6*, *RX*, *MITF* and *CHX10* peaked on days 30-40 and gradually declined thereafter (Fig. 5L-O). Immunostaining demonstrated that SFEB/CS treatment induced retinal progenitors positive for both RX/PAX6 and MITF/PAX6 on day 35 (29.0±3.3% of colonies; Fig. 5P,Q). Cells positive for either RX (16.9±2.5% of total cells) or MITF (22.1±4.1% of total cells) were also generated. SFEB/CS treatment significantly increased the number of MITF+ colonies compared with SFEB treatment (Fig. 5R; supplementary material Fig. S1B). PAX6+ rosette-like clusters were also observed in SFEB/CS cultures (76.8±3.9% of colonies; Fig. 5S).

We then determined whether SFEB/CS-treated iPS cells could differentiate into retinal cells. On day 40, pigment cells appeared in SFEB/CS cultures (29.1±4.0% of colonies). These cells accumulated more pigment and had adopted a polygonal morphology with a squamous appearance by day 60 (27.2±4.4% of total cells; Fig. 6A; supplementary material Fig. S1C). These pigment cells formed polygonal actin bundles (Fig. 6B) and ZO1+ tight junctions by day 90, and expressed *RPE65* and *CRALBP* (Fig. 6C), consistent with characteristics of the RPE. We also examined photoreceptor differentiation from SFEB/CS-treated iPS cells. Human iPS cells were treated with Y-27632 (10 μM, days 0-14), CKI-7 (5 μM; days 0-20), and SB-431542 (5 μM; days 0-20), and subsequently with retinoic acid (100 nM; days 90-140) and taurine (100 μM; days 90-140). On day 120, 26.5±8.3% of total colonies were immunopositive for the photoreceptor marker RCVRN in SFEB/CS + RA/T culture (Fig. 6D). On day 140, 5.4±1.9% of total cells expressed RHO, a rod photoreceptor marker (Fig. 6E). We performed RT-PCR to test for expression of genes responsible for phototransduction. SFEB/DL- and RA/T-treated iPS cells expressed *PDC*, *PDE6b*, *PDE6c*, *RHO*, *GRK1* and *SAG*, indicating that human iPS cell-derived photoreceptor cells possess the functional components required for light response. Taken together, these results indicate that small molecule induction of human iPS cells can cause differentiation into retinal cells.

In addition, we compared the differentiation potential of four lines of human iPS cells (253G1, 253G4, 201B6, and 201B7) and one line of human ES cells (khES-1). 253G1 and 253G4 were generated by retroviral transduction of three factors, OCT3, SOX2, and KIF4 (3F hiPSC), and 201B6 and 201B7 were generated by transduction of four factors, OCT3, SOX2, KIF4 and MYC (4F hiPSC). These pluripotent stem cells were treated with Y-27632 (10 μM, days 0-14), CKI-7 (5 μM; days 0-20) and SB-431542 (5 μM; days 0-20), and plated onto poly-D-lysine-laminin-fibronectin-coated slides on day 21. All lines of human iPS cells tested differentiated into neural cells positive for NES and βIII-tubulin on day 40 (supplementary material Fig. S1A). The khES-1 (hESC), 253G1, 253G4 (3F hiPSC) and 201B7 (4F hiPSC) lines generated pigment cells that expressed *RPE65* and *CRALBP* (Fig. 6C; supplementary material Fig. 1B,C). However, 201B6 (4F hiPSC) did not differentiate into pigment cells. The efficiencies of MITF+ cell, ZO1+ cell, and pigment cell differentiation did not differ significantly between khES-1, 253G1(3F hiPSC) and 201B7 (4F hiPSC) cells in SFEB/CS culture (Fig. 6E). These results suggest that 201B6 is a pseudo iPS cell colony or does not maintain pluripotency under our conditions, despite the expression of markers of the undifferentiated state. Thus, we conclude that the selection of iPS cell colonies rather than the presence or absence of MYC affects the differentiation capacity of iPS cells.

Taken together, our data show that the SFEB/CS method can induce retinal specification in human iPS and ES cells.

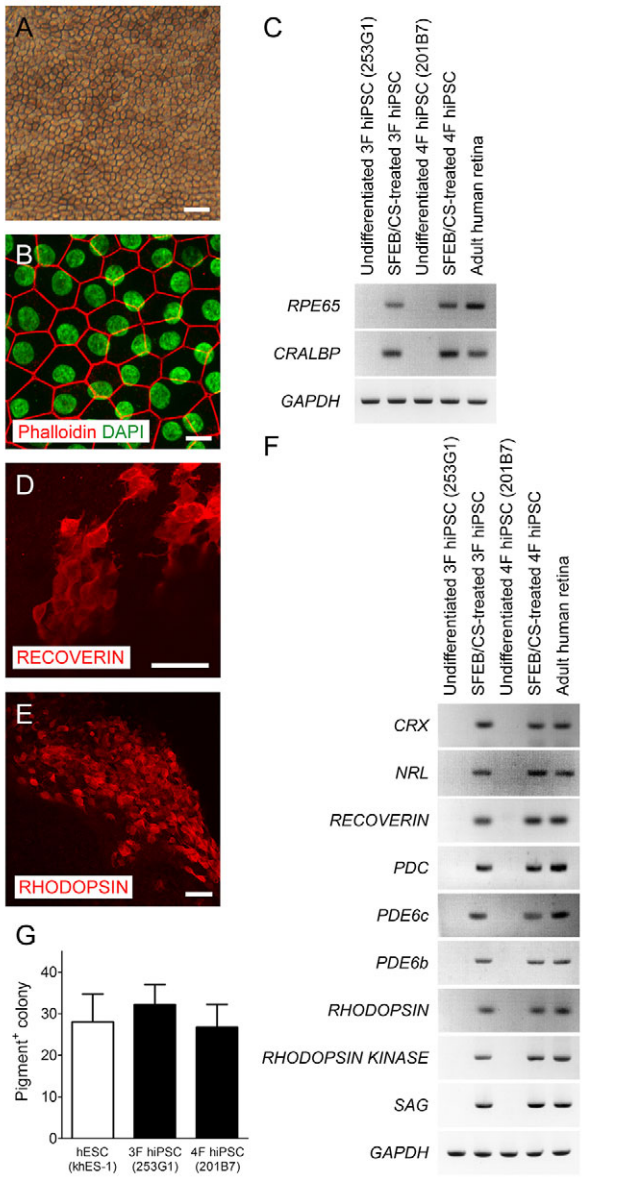

Fig. 6. Generation of RPE and photoreceptors from both three- and four-factor human iPS cells. (A) Generation of pigment cells from human iPS cells in SFEB/CS cultures. (B) Phalloidin staining shows the polygonal shape of the pigment cells. (C) RT-PCR analysis for markers of the mature RPE, *RPE65* and *CRALBP* in two lines of human iPS cells. (D,E) Generation of RCVRN+ (D) and RHO+ (E) photoreceptors from human iPS cells in SFEB/CS + RA/T culture. (F) RT-PCR analysis of phototransduction genes in two lines of human iPS cells. (G) Comparison of the differentiation of one human ES cell line (khES-1) and two human iPS cell lines (253G1 and 201B7). The 253G1 line was generated by three-factor induction (OCT3/4, SOX2, and KLF4) and the 201B7 line by four-factor induction (OCT3/4, SOX2, KLF4 and MYC). Scale bars: 30 μm (A,D,E) and 10 μm (B).

Discussion

Photoreceptor loss in retinal degeneration is the major cause of blindness (Hartong et al., 2006; Rattner and Nathans, 2006). Cell transplantation of photoreceptors and/or RPE cells is one of the most promising therapeutic strategies for incurable retinal degenerative diseases (MacLaren and Pearson, 2007; Osakada and Takahashi, 2009). However, the clinical application of cell transplantation is hampered by the fact that cells are often cultured with materials from other animals, such as feeder cells, serum and recombinant proteins, and this poses a risk in terms of adverse immune responses and potential exposure to xenopathogens (Martin et al., 2005). In the present study, we have established a method of inducing retinal differentiation of human ES cells and iPS cells using the chemical compounds CKI-7, SB-431542 and Y-27632. These chemical compounds are non-biological, do not trigger immune responses, have stable activity, show little difference between production lots, and are inexpensive. Therefore, retinal differentiation methods using chemical compounds are ideal for clinical applications. Our small molecule-based differentiation method provides a solution to the problem of cross-species antigenic contamination in cell replacement therapy, and also contributes to in vitro modeling of development, disease and drug screening (Ding and Schultz, 2004; Pouton and Haynes, 2007).

In the present study, we conclude that inhibition of β-catenin (Wnt signaling) and pSmad (Nodal signaling) is important for retinal cell differentiation. To determine whether other signaling pathways might be involved in retinal specification, we also tested the effects of Shh, Wnt, BMP4, Nodal (without Lefty-A), IGF, FGF1, FGF2, and FGF antagonists (data not shown). However, addition of these proteins showed only marginal effects, if any, on retinal cell differentiation in our ES cell system. The patterning signals that induce the retinal primordia in the embryo have not yet been elucidated. How Wnt and Nodal signaling pathways control the expression of eye field transcription factors such as Six3, Pax6, Rx, Chx10 and Mitf deserve further investigation. In vitro methods of studying mouse and human pluripotent stem cells will pave the way to understanding the molecular and cellular mechanisms of retinal specification during development, and also bridge the gap between mouse embryology and human development.

We have used potent and specific inhibitors to block Wnt and Nodal signaling. CKI-7 is a specific inhibitor of casein kinase I, and does not inhibit other kinases such as protein kinase A, protein kinase C, Ca^{2+}/CaM kinase II, and myosin light chain kinase at concentrations as high as 100 μM (Chijiwa et al., 1989). SB-431542 is a specific inhibitor of ALK4, 5 and 7, and has no effect on more divergent ALK family members that recognize bone morphogenetic proteins (BMPs) (Inman et al., 2002). SB-431542 also exhibits no effect on components of the ERK, JNK or p38 MAP kinase pathways (Laping et al., 2002). Accumulating evidence indicates that CKI-7 and SB-431542 suppress Wnt and Nodal signaling, respectively. However, we found that the differentiation efficiency of CKI-7 and SB-431542 was lower than that of Dkk1 and Lefty-A, although the difference was not statistically significant. We also observed that the aggregates formed by cells treated with CKI-7 and SB-431542 were smaller than those made by cells treated with Dkk1 and Lefty-A (data not shown). As far as we have examined, SB-431542 increased the expression levels of Hes5 and Hesr2, downstream components of Notch signaling (Louvi and Artavanis-Tsakonas, 2006), but not Hes1, Hesr1 or Hesr3 (supplementary material Fig. S2). These observations raise the possibility that non-specific effects of SB-431542 might affect ES cell differentiation. Identification of specific inhibitors should help our understanding of signaling pathways in retinal specification, and also contribute to establishment of efficient and selective differentiation methods (Ding and Schultz, 2004).

From the therapeutic point of view, direct reprogramming of somatic cells to generate iPS cells provides an invaluable resource for regenerative medicine, enabling the generation of patient-specific cells of any lineage without the use of embryonic material. We found that the efficiency of retinal differentiation of human iPS cells was comparable to that of human ES cells. In addition, retinal differentiation of three-factor human iPS cells (OCT3/4, SOX2 and KLF4) was similar to that of four-factor human iPS cells (OCT3/4, SOX2, KLF4 and MYC), indicating that the differentiation capacity of human iPS cells does not depend on the specific combination of reprogramming factors. One line of human iPS cells never generated pigmented cells, suggesting that partial or aberrant reprogramming results in impaired ability to differentiate into the required cell type. Indeed, abnormal expression of a single gene, such as *Nat1*, *Grb2*, *Apc* or *Nanog*, renders ES cells refractory to differentiation (Yamanaka et al., 2000). Thus, we conclude that the selection and validation of iPS cells rather than the sets of reprogramming factors used are critical for generation of iPS cells.

Another concern for transplantation therapy is the possibility of tumor formation as a result of contamination with undifferentiated ES cells or iPS cells (Choo et al., 2008; Fukuda et al., 2006). Thus, purification to remove undifferentiated cells is required for donor cell preparation. Moreover, although transgenes are largely silenced in iPS cells, reactivation of transgenes, in particular Myc, can lead to tumorigenesis (Okita et al., 2007). We have been able to generate mouse and human iPS cells using recombinant Oct4, Sox2, Klf4 and Myc proteins without transfection of viral vectors or plasmids (Kim et al., 2009; Zhou et al., 2009). However, if human iPS cells can be generated with only small molecules, feeder-cell-free, animal-product-free, and gene-insertion-free retinal cells could be obtained from patients' cells using our small molecule induction system. Thus, identification of small molecules that induce reprogramming is important for clinical grade preparation of iPS cells.

In addition, choosing the proper cell type and stage for donors is critical for successful transplantation. MacLaren et al. have demonstrated that integration of donor rod photoreceptors in the host retina requires rod photoreceptors of a corresponding stage to postnatal days 3-6 (MacLaren et al., 2006). These studies suggest that the ontogenic stage of transplanted photoreceptors determines the ability of these cells to integrate into the diseased retina, further underscoring the importance of cell type- and stage-specific purification of differentiated ES and/or iPS cells. Selection of specific types of ES-cell-derived progenitors for transplantation into host mice can be easily achieved using mouse ES cells with knocked-in fluorescence or antibiotic-resistance genes at specific marker loci. However, knock-in technology is not suitable for human ES cells or iPS cells. Thus, identification of surface antigens marking postnatal days 3-6 rod photoreceptors and purification of ES and iPS cell-derived rod photoreceptors corresponding postnatal days 3-6 stage are crucial (Osakada and Takahashi, 2009).

Finally, the host environment is also crucial for photoreceptor transplantation (Fisher et al., 2005). Retinal degeneration is characterized by microglial activation and glial scar formation, which may impede integration and survival of transplanted cells. Robust integration of transplanted retinal cells into the retinas of host mice deficient in both vimentin and glial fibrillary acidic protein

has been reported (Kinouchi et al., 2003). Moreover, matrix metalloproteases and chondroitinases that degrade the extracellular matrix in the diseased retina aid in the integration of transplanted photoreceptors (Suzuki et al., 2007; Suzuki et al., 2006). Disruption of the outer limiting membrane also increases photoreceptor integration following transplantation (West et al., 2008). These studies indicate that the glial barrier in the host retina prevents integration of donor photoreceptors. Therefore, in addition to immunosuppression, the host retinal environment must be modulated for successful transplantation.

In conclusion, this small molecule-based method provides a solution to the problem of cross-species antigenic contamination in cell replacement therapy, which represents a significant step toward clinical application of human ES cell or iPS cell-based transplantation therapy for retinal diseases (Ding and Schultz, 2004). Additionally, patient-specific iPS cell-derived retinal cells will facilitate the development of transplantation therapies without immune rejection, and promote an improved understanding of disease pathogenesis (Dimos et al., 2008; Ebert et al., 2009; Park et al., 2008). For successful retinal regeneration, methods of purifying donor retinal cells and optimizing host conditions, as well as use of animal models of human diseases to determine the efficacy (functional recovery) and safety (immune response and tumor formation) of treatments will be crucial.

Materials and Methods

Mouse ES cell culture

Mouse ES cells were maintained as described previously (Ikeda et al., 2005; Osakada et al., 2008; Osakada et al., 2009; Ueno et al., 2006; Watanabe et al., 2005). For the SFEB (serum-free culture of embryoid body-like aggregates) method, ES cells were incubated at 5×10^4 cells/ml in a bacterial-grade dish with differentiation medium [Glasgow minimal essential medium (GMEM), 5% KnockOut Serum Replacement (KSR), 0.1 mM non-essential amino acids, 1 mM pyruvate, and 0.1 mM 2-mercaptoethanol]. CKI-7 (0.1-10 μM; Sigma) or SB-431542 (0.1-10 μM; Sigma) was applied to the medium for 5 days while cells were in suspension culture.

Human ES cell culture

Human ES cells were used in accordance with the human ES cell research guidelines of the Japanese government. Human ES cells (khES-1) were maintained as previously described (Osakada et al., 2008; Osakada et al., 2009; Ueno et al., 2006). Briefly, undifferentiated human ES cells were maintained on a feeder layer of mitomycin-C-treated mouse embryonic fibroblasts in a humidified atmosphere of 2% CO_2 and 98% air at 37°C. ES cells were passaged every 3-4 days.

For differentiation into retinal cells, ES colonies were treated with Y-27632 (10 μM) for 1 hour, and dissociated into clumps (5-10 cells per clump) with 0.25% trypsin and 0.1 mg/ml collagenase IV in PBS containing 1 mM $CaCl_2$ and 20% KSR. Feeders were removed by incubation of the ES cell suspension on a gelatin-coated dish. ES cell clumps, at a density of 8.8×10^2 clumps/ml, were incubated in a non-adhesive 2-methacryloyloxyethyl phosphorylcholine (MPC)-treated dish (Nunc) in DMEM/F-12 supplemented with 0.1 mM 2-mercaptoethanol, 0.1 mM non-essential amino acids, 2 mM L-glutamine, and 20% KSR for 3 days, in 20% KSR-containing ES differentiation medium (GMEM, 0.1 mM non-essential amino acids, 1 mM pyruvate, and 0.1 mM 2-mercaptoethanol) for 3 days, then in 15% KSR-containing ES differentiation medium for 9 days, and finally in 10% KSR-containing ES differentiation medium for 6 days. Y-27632 (10 μM) was added for the first 15 days of suspension culture. CKI-7 (5 μM) and SB-431542 (5 μM) were applied to the medium for 21 days during suspension culture. The medium was changed every 3 days. ES cell aggregates were then re-plated en bloc on poly-D-lysine-laminin-fibronectin-coated eight-well culture slides (BD Biocoat) at a density of 15-20 aggregates/cm^2. In adherent cultures, cells were incubated in 10% KSR-containing ES differentiation medium. For photoreceptor differentiation, SFEB/DL- or SFEB/CS-treated differentiated cells were further incubated in photoreceptor differentiation medium [GMEM, 5% KSR, 0.1 mM non-essential amino acids, 1 mM pyruvate, 0.1 mM 2-mercaptoethanol, N2 supplement, 100 nM retinoic acid (Sigma), 100 μM taurine (Sigma), and 50 units/ml penicillin, 50 μg/ml streptomycin] for 50 days. The medium was changed daily.

Human iPS cell culture

Retroviral transduction of Oct3/4, Sox2, Klf4, and Myc into human cells and the culture conditions for these cells were previously described (Hirami et al., 2009; Nakagawa et al., 2008; Takahashi et al., 2007). Briefly, the human iPS cell lines 253G1and 253G4 were established by retroviral transduction of OCT3/4, SOX2 and KLF4. The human iPS cell lines 201B6 and 201B7 were established by retroviral transduction of OCT3/4, SOX2, KLF4 and MYC. Undifferentiated human iPS cells were maintained on a feeder layer of mitomycin-C-treated SNL cells (a mouse fibroblast STO cell line expressing the neomycin-resistance gene cassette and LIF) in DMEM-F-12 supplemented with 0.1 mM 2-mercaptoethanol, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 20% KSR, and 4 ng/ml basic fibroblast growth factor (Upstate Biotechnology) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. These iPS cells were passaged with 0.25% trypsin and 0.1 mg/ml collagenase IV (Gibco) in PBS containing 1 mM $CaCl_2$ and 20% KSR every 3-4 days.

For retinal differentiation, iPS colonies were treated with Y-27632 (10 μM) for 1 hour, and dissociated into clumps (5-10 cells per clump) with 0.25% trypsin and 0.1 mg/ml collagenase IV in PBS containing 1 mM $CaCl_2$ and 20% KSR. Feeders were removed by incubation of the iPS cell suspension on a gelatin-coated dish for 1 hour. iPS cell clumps, at a density of 8.8×10^2 clumps/ml, were incubated in a non-adhesive MPC-treated dish (Nunc) in DMEM-F-12 supplemented with 0.1 mM 2-mercaptoethanol, 0.1 mM non-essential amino acids, 2 mM L-glutamine, and 20% KSR for 3 days, in 20% KSR-containing ES differentiation medium (GMEM, 0.1 mM non-essential amino acids, 1 mM pyruvate, and 0.1 mM 2-mercaptoethanol) for 3 days, then in 15% KSR-containing ES differentiation medium for 9 days, and finally in 10% KSR-containing ES differentiation medium for 6 days. Y-27632 (10 μM) was added for the first 15 days of suspension culture. CKI-7 (5 μM) and SB-431542 (5 μM) were added to the medium for 21 days during suspension culture. The medium was changed every 3 days. Formed cell aggregates were then re-plated en bloc on poly-D-lysine-laminin-fibronectin-coated eight-well culture slides (BD Biocoat) at a density of 15-20 aggregates/cm^2. In adherent cultures, cells were incubated in 10% KSR-containing ES differentiation medium. For photoreceptor differentiation, SFEB/CS-treated differentiated cells were further incubated in the photoreceptor differentiation medium [GMEM, 5% KSR, 0.1 mM non-essential amino acids, 1 mM pyruvate, 0.1 mM 2-mercaptoethanol, N2 supplement, 100 nM retinoic acid (Sigma), 100 μM taurine (Sigma), and 50 units/ml penicillin, 50 μg/ml streptomycin] for 50 days. The medium was changed daily.

Immunocytochemistry

Cells were immunolabeled as described previously (Ikeda et al., 2005; Mizuseki et al., 2003; Osakada et al., 2008; Osakada et al., 2009; Osakada et al., 2007; Ueno et al., 2006). The primary antibodies used were as follows: mouse anti-βIII-tubulin (1:500, Sigma), mouse anti-CD133 (1:100, Miltenyi Biotec), rat anti-Crx (1:200), rat anti-E-cadherin (1:50, Takara), mouse anti-microtubule-associated protein-2(a+b) (1:500, Sigma), mouse anti-Mitf (1:30, Abcam), goat anti-Nanog (1:20, R&D), mouse anti-N-cadherin (1:500, BD pharmingen), rabbit anti-NCAM (1:200, Chemicon), rabbit anti-nestin (1:1000, Covance), mouse anti-Oct3/4 (1:200, BD pharmingen), mouse anti-Pax6 (1:500, R&D), rabbit anti-Pax6 (1:600, Covance), mouse anti-rhodopsin (RET-P1, 1:2000, Sigma), rabbit anti-Rx (1: 200), mouse anti-TRA-1-60 (1:200, Chemicon), anti-TRA-1-81 (1:200, Chemicon), and rabbit anti-ZO1 (1:100, Zymed). Antibodies against Crx and Rx were obtained as previously described (Ikeda et al., 2005). The secondary antibodies used were as follows: anti-mouse IgG, anti-rabbit IgG, anti-rat IgG, anti-goat IgG, and anti-mouse IgM antibodies conjugated with Cy3 or Cy2 (1:300, Jackson). For enhancement of immunoreactive signal, specimens were incubated with biotinylated secondary antibodies (1: 200, Vector), and then with Texas Red-Avidin or FITC-Avidin (1:1000, Vector). Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 1 μg/ml, Molecular Probes). Labeled cells were imaged with a laser-scanning confocal microscope (Zeiss).

Real-time PCR

Total RNA was extracted with the RNeasy kit (Qiagen), treated with RNase-free DNase I, and reverse-transcribed with a first-strand cDNA synthesis kit (Amersham) as previously described (Osakada et al., 2008; Osakada et al., 2007). Quantitative PCR was performed with the StepOnePlus Real-Time PCR system (Applied Biosystems). Specific primers and their corresponding probes were designed with the Universal ProbeLibrary system (Roche). The expression levels were normalized to those of β-actin. The primers used for quantitative PCR are listed in Table 1.

RT-PCR analysis

Total RNA was extracted with the RNeasy kit (Qiagen), treated with RNase-free DNase I, and reverse-transcribed with a first-strand cDNA synthesis kit (Amersham) as previously described (Osakada et al., 2008; Osakada et al., 2007). The cDNA was used as a template for PCR with ExTaq (Takara). Human adult retinal cDNA (Clontech) was used as a positive control. The PCR products were separated by electrophoresis on an agarose gel and detected under UV illumination. The primers used for RT-PCR are listed in Table 2.

Western blot analysis

Cells were harvested and homogenized in ice-cold lysis buffer. After normalization of protein concentrations and denaturation, samples were subjected to 4-12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Wako), followed by transfer to polyvinylidene difluoride membranes (GE). The membranes were probed with rabbit anti-Smad2/3 (1:1000; Cell Signaling), rabbit anti-phospho

Table 1. Primers used for real-time PCR

Gene	Primer sequence (5′-3′) Forward	Reverse
Mouse		
Cripto	GCCTATGGGATTCCCTTCC	ACAGCGGGATACAGGGACT
Hes1	ACACCGGACAAACCAAAGAC	CGCCTCTTCTCCATGATAGG
Hes5	GATGCTCAGTCCCAAGGAGA	AGCTTCAGCTGCTCTATGCTG
Hesr1	CATGAAGAGAGCTCACCCAGA	CGCCGAACTCAAGTTTCC
Hesr2	GTGGGGAGCGAGAACAATTA	GTTGTCGGTGAATTGGACCT
Hesr3	CTGAATTGCGACGATTGGT	GCAAGACCTCAGCTTTCTCC
Nodal	CCAACCATGCCTACATCCA	CACAGCACGTGGAAGGAAC
Sox1	GTGACATCTGCCCCCATC	GAGGCCAGTCTGGTGTCAG
Wnt1	TACTGGCACTGACCGCTCT	CTTGGAATCCGTCAACAGGT
Wnt3	CTCGCTGGCTACCCAATTT	GAGGCCAGAGATGTGTACTGC
β-actin	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA
Human		
BF1	TACTACCGCGAGAACAAGCA	TCACGAAGCACTTGTTGAGG
CHX10	GCTGGACACCAGCCAGAC	GCAGATTTGGACATTTTTCGAT
CRALBP	AGATCTCAGGAAGATGGTGGAC	GAAGTGGATGGCTTTGAACC
CRX	CACCAGGCTGTGCCCTAC	CTTCCAGCTCCTCCAGTTG
GBX2	AAAGAGGGCTCGCTGCTC	ATCGCTCTCCAGCGAGAA
HOXB4	TGGATGCGCAAAGTTCAC	GCTGGACACCAGCCAGAC
IRX3	AAAAGTTACTCAAGACAGCTTTCCA	GAAATTCCTTCTCCAGCTCCA
MITF	AGAGTCTGAAGCAAGAGCACTG	TGCGGTCATTTATGTTAAATCTTC
NANOG	ATGCCTCACACGGAGACTGT	AGGGCTGTCCTGAATAAGCA
OCT3/4	GCAAAACCCGGAGGAGGAGTC	CCACATCGGCCTGTGTATATC
PAX6	TCACCATGGCAAATAACCTG	CAGCATGCAGGAGTATGAGG
RCVRN	TAACGGGACCATCAGCAAG	CCTCGGGAGTGATCATTTTG
RPE65	CAATGGGTTTCTGATTGTGGA	CCAGTTCTCACGTAAATTGGCTA
RX	GGCAAGGTCAACCTACCAGA	CTTCATGGAGGACACTTCCAG
SIX3	CCGGAAGAGTTGTCCATGTT	CTCCTCCAGCGTCTCACAG
β-actin	ATTGGCAATGAGCGGTTC	GGATGCCACAGGACTCCA

Table 2. Primers used for RT-PCR

Gene	Primer sequence (5′-3′) Forward	Reverse
CRALBP	AGATCTCAGGAAGATGGTGGAC	GAAGTGGATGGCTTTGAACC
CRX	GCCCCACTATTCTGTCAACG	CTTCCAGCTCCTCCAGTTG
NRL	GAGCCCAGAGGAGACAGGA	TTTAGCTCCCGCACAGACAT
PDC	TCAAAGGAACGAGTCAGCAG	CTGCTGCAAGGCATGTTAAA
PDE6b	CAGTGATGAACACCGACACC	ATTTGACCAGGTCCAGTTCG
PDE6c	CTGAGGTGGCCTCTAGGTTG	GCTGGTGTGATGAAGCCTTAG
RCVRN	TAACGGGACCATCAGCAAG	CCTCGGGAGTGATCATTTTG
RHO	CACCAGGCTGTGCCCTAC	GCCTCATCGTCACCCAGT
GRK1	GGACTGGTTCCTGGACTTCA	AAGCCAGGGTTCTCCTCATT
RPE65	CAATGGGTTTCTGATTGTGGA	CCAGTTCTCACGTAAATTGGCTA
SAG	CTGATCCGCAAAGTACAGCA	TCAGCGTCTTGGTCAAAGTG
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

Smad2/3 (1:1000; Cell Signaling), or rabbit anti-β-catenin (1:1000; Upstate) antibodies, and then with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000; Dako). The bound antibodies were detected with an enhanced chemiluminescence detection system (Amersham).

Phagocytosis assay

Cells were incubated in medium containing Cy3-conjugated 1 μm polystyrene microspheres at a concentration of 1.0×10^8 beads/ml for 6 hours at 37°C as described previously (Osakada et al., 2008). For visualization of F-actin, the cells were stained with Alexa-Fluor-488-conjugated Phalloidin (Molecular Probes). The fluorescence signal was observed with a laser-scanning confocal microscope.

Statistical analysis

Values are expressed as means ± s.e.m. 100-200 colonies were examined in each experiment. All statistical analyses were performed using GraphPad PRISM version 5.0 (GraphPad Software Inc.). The statistical significance of differences was determined by one-way analysis of variance followed by Dunnett's test or Tukey's test, or with an unpaired *t*-test. Probability values less than 5% were considered significant.

We thank H. Suemori and N. Nakatsuji (Kyoto University, Kyoto, Japan) for the human ES cell line, K. Takahashi and S. Yamanaka (Kyoto University) for the human iPS cell line, M. Kikkawa, A. Nomori and K. Iseki for technical assistance, and members of the Takahashi laboratory and the Sasai laboratory for helpful discussions. This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, and the Leading Project (M.T). This study was also supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, the Mochida Memorial Foundation for Medical and Pharmaceutical Research, the Kanae Foundation for the Promotion of Medical Science, the Uehara Memorial Foundation, and the Naito Foundation (F.O.).

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