



Review Article

Nuclear reprogramming to treat retinal degenerative diseases

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Cells can be reprogrammed to assume a different fate *in vitro*. Oct3/4, Sox2, and Klf4 have been used to direct cells to a pluripotent state. These induced pluripotent stem (iPS) cells, which are able to differentiate into any cell type, may provide a novel therapeutic avenue for a wide range of disorders. Photoreceptors or retinal pigment epithelia derived from patient iPS cells, for example, may be useful in transplantation therapy for retinal degenerative diseases, and *in vitro* disease models and drug screening. Recent evidence, however, has revealed significant differences between embryonic stem (ES) cells and iPS cells. iPS cells have helped elucidate the underpinnings of pluripotency, in particular similarities and differences among mouse ES cells, human ES cells, and epiblast stem cells. Intriguingly, cellular plasticity suggests that a specific set of factors could potentially convert somatic cells directly to retinal progenitors or retinal cells. Cellular reprogramming methodologies should markedly contribute to both our understanding of retinal degenerative diseases and the development of novel therapies, including cell transplantation and new drugs.

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Nuclear reprogramming

The human body is generated from a single cell, the fertilized egg. This totipotent stem cell produces all of the required cell types, including such extraembryonic tissue as the placenta. As embryonic cells divide and differentiate, they lose the ability to form some cell types and progressively become more specialized. For example, although the inner cell mass (ICM) of the mammalian blastocyst is pluripotent, giving rise to cells from all three embryonic germ layers—endoderm, ectoderm, and mesoderm—it does not form the extraembryonic trophoblast lineage. Then, within each germ layer, stem cells or progenitor cells develop into several different terminal cell types. It is thought that, during normal development, cellular differentiation and lineage commitment are unidirectional and irreversible.

Many lines of evidence, however, indicate that cells can be reprogrammed into a different fate *in vitro*. Gurdon demonstrated that frog somatic cells can be reprogrammed after they are fused with enucleated oocytes, resulting in not only tadpoles but also normal adult frogs^{1,2}. Campbell et al. and Wakayama et al. demonstrated vertebrate reprogramming by creating cloned animals from sheep and mouse somatic cells^{3,4}. In addition to oocytes, mouse and human ES cells (or their extracts) also can be used to reprogram somatic cells into an ES cell-like state^{5,6}. These findings indicate that somatic cells become pluripotent in response to certain factors from oocytes and ES cells.

Takahashi and Yamanaka identified reprogramming factors from a number of candidate genes⁷. The authors introduced Oct3/4, Sox2, Klf4, and c-Myc into mouse fibroblasts using retroviral vectors. The fibroblasts carried a fusion of β -galactosidase and the neomycin-resistant gene under the control of Fbx15 and ES cell-like cells that were resistant to G418 were selected. The identified cells were named iPS (induced pluripotent stem) cells. iPS cells selected based on Fbx15 expression (Fbx15-iPS cells) showed ES-like morphology, expressed several ES cell marker genes (*e.g.*, Oct3/4, E-Ras, Dax1, Zfp296, FGF4, and Esg1), and formed teratomas. Yet these cells failed to generate viable adult chimeras when they were injected into blastocysts, and clear differences

from ES cells in gene expression profiles and differential potentials were observed. In contrast to ES cells, the Oct3/4 and Nanog promoter regions were not fully demethylated in the Fbx15iPS cells. Instead, endogenous expression of Nanog or Oct4 was used to select induced cells that were fully reprogrammed (Nanog iPS cells or Oct4 iPS cells)^{8,9}. These high-quality iPS cells were morphologically similar to ES cells, and showed comparable proliferation, gene expression profiles, and epigenetic modifications. Moreover, the endogenous Nanog and Oct4 promoters were unmethylated in these cells. Importantly, the reprogrammed cells were able to contribute to the germline of adult chimeric mice and generate live late-term embryos when they were injected into tetraploid blastocysts^{8,9}. The molecular mechanisms that govern iPS cell reprogramming remain poorly understood, however¹⁰⁻¹².

Three distinct approaches to cellular reprogramming are used: cell fusion, nuclear transfer, and transcription factor transduction¹³. The technical difficulty, reprogramming speed, reprogramming efficiency, and therapeutic potential of each of these techniques differ. Cell fusion rapidly and easily induces reactivation of pluripotency factors and tissue-specific genes, and the efficiency of this method is the highest among the three reprogramming options. Thus, cell fusion provides a powerful system for understanding the molecular mechanisms underlying nuclear reprogramming, although it is not suitable for clinical use. Nuclear transfer involves transplantation of a nucleus from a differentiated somatic cell into an enucleated oocyte. Nuclear transfer results in pluripotent stem cells, which are used for both basic research and therapeutic applications; however, the method is associated with some ethical concerns and the technique is difficult. Finally, transcription factor transduction is relatively easy and lacks significant ethical dilemmas, yielding pluripotent cells that can be used clinically or experimentally to model human diseases or screen potential treatments. But it takes longer to generate iPS cells with this method. It should be noted that some of the reprogramming mechanisms of the three approaches may differ. The three should share epigenetic changes, but cell division and DNA replication are required for



nuclear transfer and transcription factor transduction but not cell fusion¹⁴). Further studies are required to understand nuclear reprogramming and cellular plasticity in detail.

Importantly, iPS cell technology has opened up the possibility that a somatic cell can be converted directly to another cell type. Several studies have demonstrated that fibroblasts can be directed to a new fate, including neurons, cardiomyocytes, hematopoietic progenitors, and macrophage-like cells¹⁵⁻¹⁸). For instance, a combination of neural lineage-specific transcription factors—Ascl1, Brn2, and Myt1l—was used to convert fibroblasts directly into functional

neurons¹⁶). Introducing the transcription factors Gata4, Mef2c, and Tbx5 reprogrammed postnatal cardiac or dermal fibroblasts directly into differentiated cardiomyocytes¹⁷). Ectopic expression of Oct4 and treatment with the cytokines Fms-related tyrosine kinase 3 ligand and Stem cell factor reprogrammed dermal fibroblasts into CD45⁺ hematopoietic progenitors¹⁸). A number of groups are now trying to identify a set of transcription factors that will convert one type of somatic cell to another as a means for regeneration therapy. Indeed, it appears that methods for directly reprogramming cells into therapeutically significant cell types will be devised in the near future.

Table. 1 Pluripotent states in ES cells, iPS cells and epiblast stem cells.

	Mouse ES cell	Mouse iPS cell	Epiblast stem cell	Human ES cell	Human iPS cell	Naive Human ES cell	Naive Human iPS cell
Origin	ICM	Somatic cell	Epiblast	ICM	Somatic cell	ICM	Somatic cell
Morphology	Dome-shaped	Dome-shaped	Flat	Flat	Flat	Dome-shaped	Dome-shaped
Growth	LIF/STAT3	LIF/STAT3	FGF/Activin	FGF/Activin	FGF/Activin	LIF GSK3 inhibitor ERK inhibitor	LIF GSK3 inhibitor ERK inhibitor
Enzymatic dissociation	Yes	Yes	No	No	No	Yes	Yes
X chromosome inactivation	XaXa	XaXa	XaXi	XaXi	XaXi	XaXa	XaXa
Germline transmission	Yes	Yes	No	?	?	?	?

Table 1. Pluripotent states in ES cells, iPS cells, and epiblast stem cells.

Mouse ES cells are derived from E3.5 mouse blastocysts in the presence of BMP and LIF. Epiblast stem cells are derived from E5.5–E5.75 postimplantation epiblasts in the presence of activin and bFGF. Mouse ES cells and iPS cells are germline competent, whereas epiblast stem cells cannot contribute to chimera formation. Mouse and rat epiblast stem cells resemble conventional human ES cells in terms of morphology, growth factor requirement, and gene expression patterns. Mouse ES cells, mouse iPS cells, “naïve” human ES cells, and “naïve” iPS cells formed dome-shaped colonies, whereas epiblast stem cells, conventional human ES cells, and conventional human iPS cells formed flat colonies. When dissociated into single cells, mouse ES cells and mouse iPS cells survive, whereas conventional “primed” human ES cells and human iPS cells undergo cell death. Mouse ES cells and iPS cells have two active X chromosomes (XaXa), whereas conventional “primed” human ES cells and iPS cells carry one inactive X chromosome (XiXa). “Naïve” human ES cells and iPS cells resemble mouse ES cells in signaling responses, colony morphology, single-cell survival after dissociation, and the state of X chromosome activation (XaXa).



Various states of pluripotency

Mouse and human ES cells, which were first established *in vitro* in 1981 and 1998, respectively^{19,20}, are both derived from blastocyst stage embryos, but have a number of different biologic properties (Table 1). For instance, mouse ES cells depend on leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP), whereas human ES cells rely on activin/nodal and fibroblast growth factor (FGF). Mouse ES cells form dome-shaped colonies, whereas colonies of human ES cells are flat. Mouse ES cells can be dissociated into single cells, a process that kills human ES cells. In mice, the parental X chromosome is imprinted during spermatogenesis, becoming silenced during cleavage and reactivated in ICM cells of the postimplantation blastocyst. ICM-derived ES cells have two active X chromosomes (XaXa). Once ES cells begin to differentiate, one X chromosome is inactivated through Xist (XaXi). Although mouse ES cells have two active X chromosomes (XaXa), conventional human ES cells exhibit partial or complete X chromosome inactivation (XaXi). Intriguingly, the pluripotent states of epiblast stem cells derived from mice at E5.5–5.75 or rat at E7.5 were similar to that of human ES cells^{21,22}. Mouse ES cells contribute functionally differentiated progeny to chimeras. By contrast, epiblast stem cells are unable to colonize an embryo even though they express the core pluripotency genes *Oct4*, *Sox2*, and *Nanog*. The pluripotent states of mouse epiblast stem cells and conventional human ES cells are less primitive or more primed than that of mouse ES cells. Similar to “primed” human ES cells, conventional human iPS cells generated using Oct3/4, Sox2, and Klf4 combined with bFGF also seem to mimic the pluripotent state of epiblast stem cells.

Mouse epiblast stem cells can be reprogrammed to become mouse iPS cells that can contribute to germline-competent chimeras via forced expression of Klf4 or Nanog^{23,24}. Remarkably, “primed” human ES cells can be directed to a pluripotent state resembling mouse ES cells via forced expression of both Oct3/4 and Klf4 or both Oct3/4 and Klf2 in the presence of LIF, the ERK1/2 inhibitor PD0325901, the GSK3inhibitor CHIR99021, and the adenylate cyclase activator forskolin²⁵. Mouse ES cell-like “naïve” human ES

cells are generated after dissociation of “primed” human ES cells into single cells and subsequent treatment with two small molecules: thiazovivin and tyrintegin²⁶. These converted “naïve” human ES cells have growth, X chromosome activation, and gene expression profiles that are similar to those of mouse ES cells. “Naïve” human ES cells are characterized by XaXa, whereas “primed” human ES cells are XaXi. In the presence of LIF, human fibroblasts can be reprogrammed to “naïve” human iPS cells that display some of the morphologic, molecular, and functional properties of mouse ES cells²⁷. Recently, human ES cells with two active X chromosomes (XaXa) were isolated under physiologic 5% oxygen²⁸. “Naïve” human iPS cells established in the presence of LIF and the signaling pathway inhibitors PD0325901 and CHIR99021 show characteristics of growth, X chromosome activation (XaXa), and gene expression that markedly resemble those of mouse ES cells²⁵.

Accumulating evidence has demonstrated significant differences between ES cells and iPS cells, including differentiation potential, tumorigenic potential, gene expression profiles, epigenetic modification, and expression of imprinted genes. Nextgeneration sequencing technology has revealed DNA methylation patterns in iPS cells on a genome-wide scale^{29–33}. iPS cell generation require the proper reprogramming of histone modification. In both ES cells and iPS cells, histone H3 and H4 are hyperacetylated in the promoter regions of pluripotency-associated genes. In contrast, differentiated cells have hypoacetylated H4. Histone methylation is another epigenetic modification that influences gene expression and chromatin structure. In both ES cells and iPS cells, H3 is methylated at lysine 4 (H3K4) and demethylated at lysine 9 (H3K9) in the promoter regions of pluripotency-associated genes, whereas fibroblasts have the opposite patterns. The trimethylation of H3K4 and H3K27 has been associated with the promoter regions of active and inactive genes, respectively, so-called bivalent chromatin structure. The degree and distribution of H3K4 and H3K27 methylation across the genome provides a distinctive signature for different cell types³⁴. Interestingly, Deng et al. demonstrated that DNA methylation patterns in ES cells and iPS cells are quite different from that in fi-



broblasts, and that iPS cells display more methylation than ES cells³¹). Several lines of evidence indicate the presence of epigenetic memory of the origin cells owing to incomplete reprogramming at some genomic loci in iPS cells^{29,33}). High-throughput array-based relative methylation analysis revealed that the differentiation and methylation of nuclear transfer-derived pluripotent stem cells were more similar to ES cells than iPS cells²⁹). Somatic cell nuclear transfer and transcription factor-based reprogramming have been used to revert adult cells to pluripotent stem cells. These two reprogramming methods use different mechanisms and kinetics to reset genomic methylation, an epigenetic modification of DNA. DNA demethylation takes place faster in nuclear-transfer ES cells than in iPS cells. iPS cells obtained using factor-based reprogramming harbor residual DNA methylation signatures characteristic of their somatic tissues of origin, which favors differentiation along lineages related to the donor cell and restricts alternative cell fates. One report also showed that iPS cells obtained from different cell types exhibit distinct transcriptional and epigenetic profiles and different *in vitro* differentiation potentials³³). Several studies have demonstrated decreased efficiency and increased variability in differentiation potential of iPS cells compared to ES cells^{29,35-37}). Notably, continuously passing iPS cells attenuate these differences. In line with these studies, iPS cells derived from retinal pigment epithelia (RPE) tend to differentiate into RPE³⁷).

Stadtfield et al. showed that the overall mRNA and microRNA expression profiles of genetically identical mouse ES cells and iPS cells are markedly similar; the few exceptions include the non-coding RNA Gtl2 (Meg3) and the small nucleolar RNA Rain³⁸). These transcripts are encoded within the imprinted Dlk1–Dio3 gene cluster on chromosome 12qF1, members of which were aberrantly silenced in most of the iPS cell clones. Crucial for germ-line transmission and tetraploid aggregation, activation of the Dlk1–Dio3 region is positively correlated with the pluripotency of stem cells³⁹). Because the pluripotent states of iPS cell clones vary, selection and validation of reprogrammed cells are critical for iPS cell generation and subsequent use. The Dlk1–Dio3 region may serve as a marker to identify fully

pluripotent iPS cells from partial pluripotent counterparts.

iPS cells can be generated from various somatic cells, including fibroblasts, hepatic cells, gastric epithelium cells, keratinocytes, peripheral blood cells, cord blood cells, neural stem cells, and adipose stem cells⁴⁰⁻⁴⁶). Importantly, the origins of iPS cells determine not only their differentiation potential but also their tumorigenic potential³⁵). For example, teratomas are more likely to form from iPS cells obtained from adult mouse tail tip fibroblasts than those created from mouse embryonic fibroblasts. In addition to optimizing iPS cell generation, selection of origin cells and evaluation of iPS cell clones are critical steps to safe and effective therapies.

Studies have demonstrated that iPS cells are similar, but not identical, to ES cells. ES cells and iPS cells can assume various pluripotent states (Table 1). In mice and rats, the potential to form chimeras is important during the generation of knock-in and knock-out animals. With the exception of fertilization treatment, however, clinical use of human ES cells and human iPS cells should not require chimeras. Selection of safe nontumorigenic iPS cell clones is critical for transplantation therapies⁴⁷). It is essential to determine the best choice of origin cells, how cells can be reprogrammed into iPS cells, and how to best evaluate iPS clones.

In vitro retinal differentiation of ES cells and iPS cells

In this section, we describe our differentiation system to obtain retinal cells from pluripotent stem cells, including mouse, monkey, and human ES and iPS cells. Not surprisingly, *in vitro* differentiation of ES cells, in part, recapitulates *in vivo* embryogenesis. Acquisition of neural fates (neural induction) by ES cells can be controlled using BMP, FGF, and Wnt signaling, whereas the production of specific neural cell types (neural patterning) can be controlled using exogenous patterning signals, such as Wnt, BMP, Shh, FGF, and retinoic acid⁴⁸⁻⁵²). In response to the appropriate signals, ES cells will differentiate into a wide range of neural cell types that correlate with their positions along the anterior–posterior and dorsal–ventral axes^{53,54}). Thus, based on our knowledge of retinal development, we developed methods to create retinal cells from



ES cells *in vitro*.

During early embryogenesis, the retinal primordium forms in the rostral-most region of the Six3-expressing diencephalon. The transcription factor Rx, an early marker of the eye field, plays an essential role in specification of the retinal primordium within the rostral Six3⁺ central nervous system. At E10.5 in mice, neural retina progenitors in the inner layer of the optic cup express both Rx and Pax6, whereas RPE progenitors in the outer layer of the optic cup express Pax6 and Mitf, but not Rx. We first attempted to induce the development of the rostral Six3⁺ central nervous system progenitors and Rx⁺/Pax6⁺ retinal progenitors by applying exogenous patterning signals⁵⁵. Because the extracellular patterning signals that induce the retinal primordia have not yet been identified, we took a candidate approach to identify soluble factors that drive Rx and Pax6 expression. In serum-free and feeder-free aggregate culture (SFEB culture), strong expression of Six3 was detected on culture day 5, but not in cells cultured with the caudalizing factor retinoic acid. The strongest induction of Rx⁺/Pax⁺ cell development was observed when ES cells were treated with Dkk-1, Lefty-A, FCS, and Activin (referred to as SFEB/DLFA cells). We next tested whether RPE differentiation was observed in SFEB/DLFA culture by examining the expression of the early RPE marker Mitf. Mitf⁺ cell aggregates were observed in SFEB/DLFA cultures, whereas SFEB-treated cells rarely expressed Mitf. Consistent with the *in vivo* expression profile of RPE markers, most Mitf⁺ cells in the SFEB/DLFA culture were Pax6⁺ and Otx2⁺. Thus, we concluded that the SFEB/DLFA treatment preferentially induced the development of retinal progenitors from mouse ES cells.

The neural retina consists of seven types of cells: rod photoreceptors and cone photoreceptors in the outer nuclear layer (ONL); bipolar cells, horizontal cells, amacrine cells and Müller glia in the inner nuclear layer; and retinal ganglion cells in the ganglion nuclear layer. During retinal development, the competency of retinal progenitors changes in response to intrinsic regulators (such as transcription factors) and extrinsic regulators (such as neurotrophic factors), resulting in the seven retinal cell types in the

following temporal sequence: retinal ganglion cells, cone photoreceptors, amacrine cells, and horizontal cells, followed by rod photoreceptors, bipolar cells, and Müller glia. We next sought to generate mature retinal cells from ES cell-derived retinal progenitors *in vitro*^{56,57}. We introduced GFP to the Rx locus of mouse ES cells and purified Rx⁺ retinal progenitors that differentiated from ES cells. After SFEB/DLFA treatment, Rx-GFP⁺ cells were collected using fluorescence activated cell sorting. The purified Rx-GFP⁺ cells expressed Rx, Pax6, Notch (Notch1–4), and the downstream mediators of Notch signaling Hes1, Hes5, and Hey1. Consistent with critical roles for Notch signaling in photoreceptor specification, inhibition of Notch signaling using the γ secretase inhibitor DAPT promoted differentiation of ES cell-derived Rx-GFP⁺ cells into Crx⁺ photoreceptor precursors. These Crx⁺ precursors differentiated efficiently into cone photoreceptors, but less efficiently into Rhodopsin⁺ rod photoreceptors. Acidic FGF, basic FGF, taurine, Shh, and retinoic acid, which reportedly promote rod differentiation, significantly increased the number of Rhodopsin⁺ rod photoreceptors in mouse ES cell cultures. *In vitro* generation of rod photoreceptors is particularly valuable for retinal degenerative diseases, because approximately 95% of photoreceptors in humans are rod photoreceptors, and these cells are the major cell type lost in patients with retinitis pigmentosa.

The retinal differentiation methods used for mouse ES cells required fetal bovine serum (FBS), which hinders transplantation therapies. To overcome this problem, we sought to create a defined culture system that allowed us to generate retinal cells from monkey and human ES cells⁵⁶. Treatment of monkey and human ES cells with Dkk-1 or Lefty-A during suspension culture increased the percentage of colonies that were positive for retinal progenitor markers Mitf, Rx, Pax6, and Chx10. Subsequently, the progenitors developed into hexagonal pigment epithelium and were polarized with apical microvilli and basal membranes. The pigment cells expressed the mature RPE markers RPE-65 and CRALBP, formed ZO-1⁺ tight junctions, and performed phagocytosis, a characteristic of the RPE. Photoreceptor differentiation, however, occurred only infrequently under these conditions. Additional treatment with



retinoic acid and taurine significantly promoted differentiation of ES cell-derived progenitors into photoreceptors that were positive for Crx, Nrl, Recoverin, Rhodopsin, Blue

opsin, or Red/Green opsin (Figure 1A). Moreover, the induced photoreceptors expressed genes involved in phototransduction.

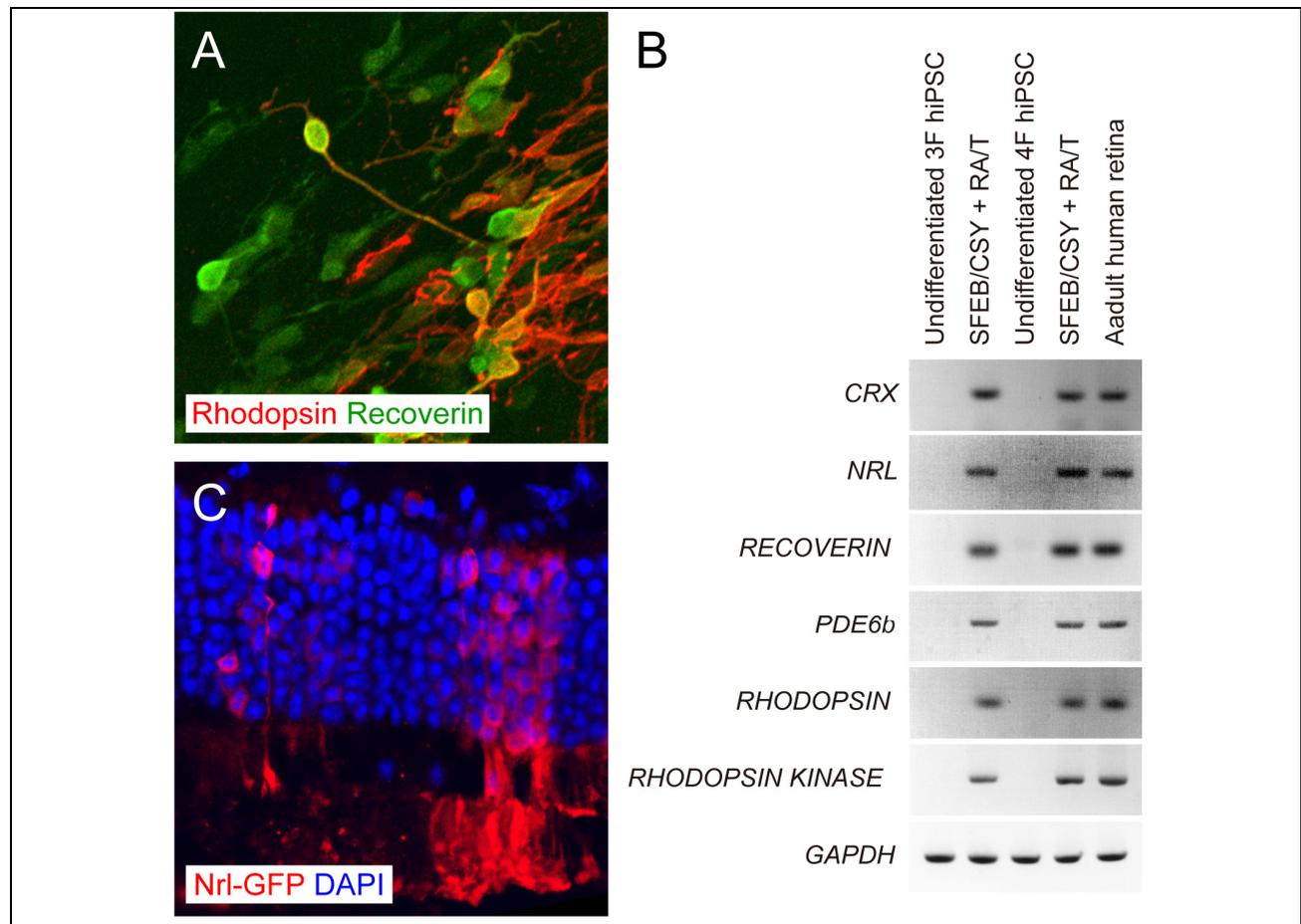


Figure 1. Transplantation and generation of photoreceptors

(A) Human ES cells differentiated into rod photoreceptors that were positive for both Rhodopsin and Recoverin.

(B) Human iPS cells generated using three factors (Oct3/4, Sox2, and Klf4: 3F hiPSC) or four factors (Oct3/4, Sox2, Klf4, and c-Myc: 4F hiPSC) can differentiate into rod and cone photoreceptors. Human iPS cells were treated with CKI-7, SB-431542, and Y-27632 during SFEB culture and subsequently with retinoic acid and taurine (SFEB/CSY + RA/T).

(C) Transplantation of rod photoreceptors into the adult retina. Nrl-GFP+ rod photoreceptors isolated from P5 mice integrate into the normal mouse retina.

These differentiation methods for ES cells can also be used to create iPS cells⁵⁸). It should be noted that the selection and validation of iPS cells are critical for their generation and further differentiation. We found that mouse iPS cells, selected based on Nanog expression (Nanog-iPS cells), are able to differentiate into photoreceptors and RPE. Fbx15-iPS cells, however, differentiated into nestin⁺ and β III-tubulin⁺ neurons, but not retinal cells. As mentioned above, Nanog-iPS cells are fully reprogrammed, and contribute to the germline of adult chimeric mice and to live-late term embryos when injected into

tetraploid blastocysts. In contrast, Fbx15-iPS and ES cells show similar morphologies, proliferation, and teratoma formation, but clear differences in their global gene expression profiles and differentiation potentials. Similarly, we tested many human iPS cell lines that developed with four factors (Oct3/4, Sox2, Klf4, and c-Myc) or three factors (Oct3/4, Sox2, and Klf4). Although all of the human iPS cell lines expressed the pluripotent cell markers Nanog, SSEAs and TRA-1s and formed teratomas, one of the human iPS cell lines could not generate retinal cells. These results indicate that selection and va-



Validation of reprogrammed cells are critical steps when generating iPS cells.

It is also important to note that, because xenogenic factors may induce immune responses following transplantation, differentiated cells should be generated from human ES cells or iPS cells without contamination if the cells are to be used clinically for transplantation strategies. For retinal differentiation, the above-mentioned method requires the addition of recombinant Dkk-1 and Lefty-A proteins, which are produced in animal cells or *E. coli*, raising the possibility of infection or immune responses owing to contamination. By contrast, using chemical compounds to induce differentiation offers several advantages compared with using recombinant proteins. Chemicals are usually stable, can be produced relatively uniformly, and generally cost less to create. Thus, establishment of chemical compound-based culture systems would likely be beneficial for human pluripotent cell-based transplantation therapies. We have succeeded in establishing chemical compound-based culture systems for generating retinal cells from human ES cells and iPS cells⁵⁹. This represents another significant step towards clinical application of retinal transplantation therapy. Application of the casein kinase I inhibitor CKI-7, the ALK-4 inhibitor SB-431542, and the Rho-associated kinase inhibitor Y-27632 to SFEB cultures produced retinal progenitors that were positive for Rx, Mitf, Pax6, and Chx10. This treatment resulted in hexagonal pigmented cells that expressed RPE-65 and CRALBP, formed ZO-1⁺ tight junctions, and exhibited phagocytosis (Figure 2A, B). Subsequent treatment with retinoic acid and taurine induced photoreceptors that expressed Crx, Nrl, Recoverin, Rhodopsin, and genes involved in phototransduction (Figure 1B).

In vitro differentiation systems for pluripotent stem cells should provide material for transplantation therapy to treat retinal degenerative diseases, insights into embryogenesis and diseases, and a means to evaluate the efficacy and toxicity of novel drugs.

Directly reprogramming cells to become retinal cells

iPS cell generation is one example of cellular plasticity. As stated earlier, fibroblasts can be induced to acquire new cell fates, in-

cluding neurons, cardiomyocytes, macrophage-like cells, and hematopoietic progenitors¹⁵⁻¹⁸. Cellular reprogramming technology may allow direct conversion of specific human cell types into retinal cells.

Lens regeneration in newts is a clear example of nuclear reprogramming occurring naturally in adult vertebrates⁶⁰. After lens removal, pigment epithelial cells in the dorsal iris re-enter the cell cycle, lose the pigment granule, and generate a structurally and functionally complete lens. On the other hand, the avian iris pigment epithelia (IPE) cannot regenerate a lens *in vivo* after lens removal or damage. Surprisingly, however, the avian IPE can generate lens cells *in vitro*⁶¹, an observation also reported for mammals⁶². The newt IPE can generate a lens *in vivo* in response to injury, whereas IPE in chicks and mammals lack an appropriate environment to differentiate into other cell types *in vivo*. The fate of the cells differentiating *in vitro* is, not surprisingly, dictated by the conditions.

IPE and the neural retina are developmentally derived from the inner layer of the optic cup. Long established as a safe and uncomplicated ophthalmic surgery to obtain autologous tissue, peripheral iridectomy allows autotransplantation while minimizing the risk of immune rejection. Therefore, we sought to generate photoreceptors from iris tissue. When rat iris-derived cells were maintained in serum-free media containing FGF, pigment cells proliferated and gradually lost pigmentation⁶³. After two weeks, some of these iris-derived cells differentiated into cells expressing neurofilament, a neural marker. Rat iris-derived cells do not differentiate into cells that express photoreceptor-specific markers. Crx is a homeobox gene that is essential for photoreceptor fate determination. Otx2, a homeobox gene with a high degree of amino-acid sequence homology with Crx, is a key regulator of photoreceptor genesis. Thus, both Crx and Otx2 are important intrinsic factors for photoreceptor generation. We introduced Crx or Otx2 into iris-derived cells to induce a photoreceptor phenotype. Adenoviral transfer of Crx into iris cells *in vitro* created cells expressing the photoreceptor markers Rhodopsin and Recoverin⁶³. Retroviral transfer of Otx2 also resulted in cells expressing these photoreceptor markers⁶⁴. Because NeuroD and Nrl are also



important for photoreceptor development, we tested whether NeuroD and Nrl induced a photoreceptor phenotype⁶⁵; these two genes, however, did not induce photoreceptor marker expression in iris-derived cells. Combination of both Crx and NeuroD or both Crx and Nrl induced a photoreceptor-like phenotype in iris cells, although the efficiency was similar to that observed with only Crx⁶⁵. Next, we used monkey iris tissue to create photoreceptors. In monkeys, Crx by itself did not induce Rhodopsin expression, and a combination of Crx and NeuroD was

required to obtain a photoreceptor phenotype⁶⁵. We also found that NeuroD expression was induced by Crx gene transfer in rat iris-derived cells. In primate cells, however, Crx did not induce NeuroD expression, and coexpression of Crx and NeuroD was needed to produce a photoreceptor-like phenotype. The Crx-transfected rat cells and Crx- and NeuroD-transfected monkey cells expressed several photoreceptor markers, including phototransduction genes, and electrophysiologically responded to light stimulation⁶⁵.

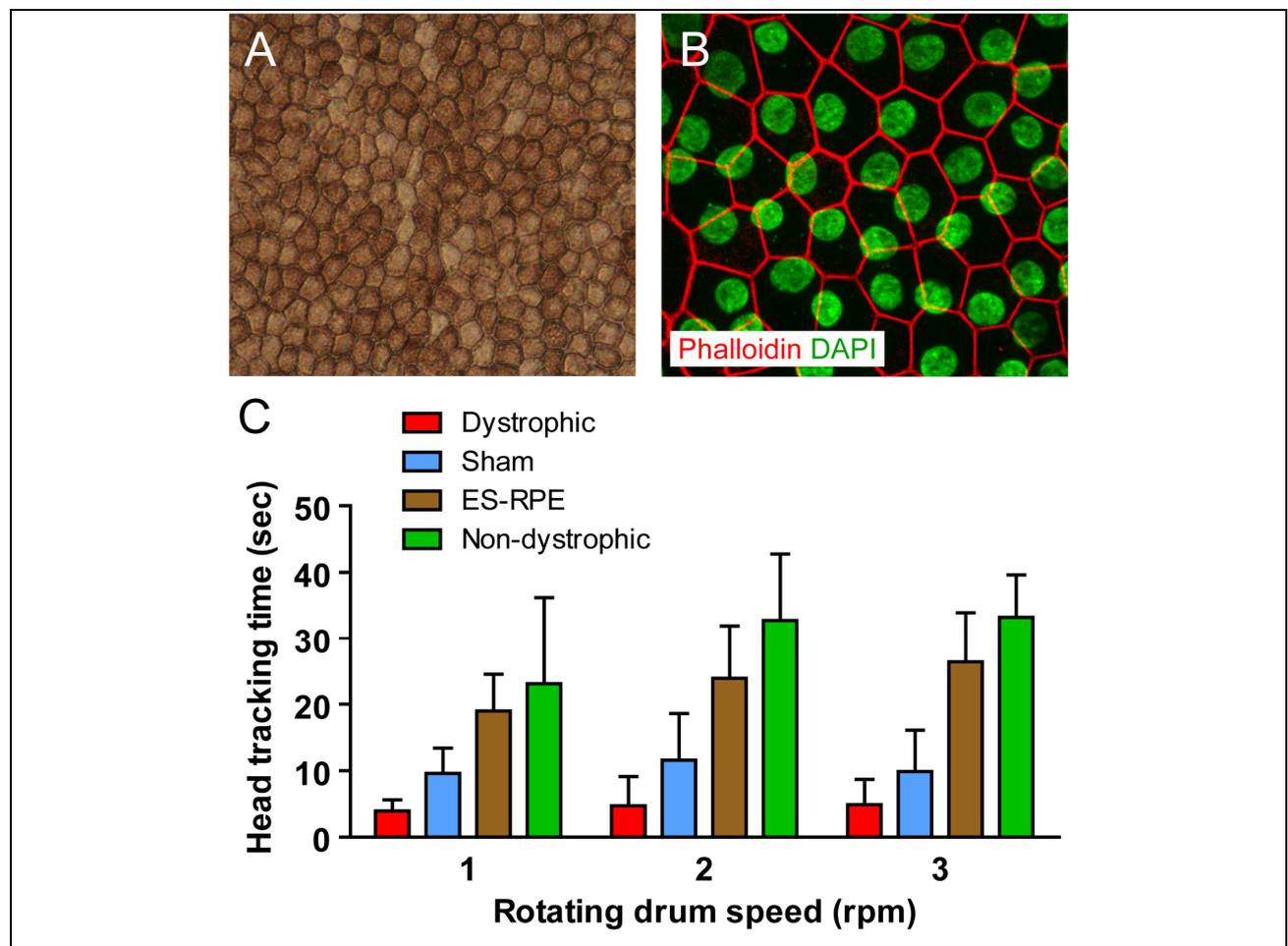


Figure 2. RPE transplantation rescues photoreceptor degeneration.

(A) Differentiation of pigment cells from human iPS cells.

(B) Polygonal human iPS cell-derived RPE. For retinal differentiation, human iPS cells were treated with CKI-7, SB-431542, and Y-27632 during SFEB culture.

(C) Functional recovery by subretinal transplantation of monkey ES cell-derived RPE (ES-RPE) into RCS dystrophic rats. The total time for head tracking in response to rotating black and white stripes during the 4-min test period was measured at speeds of 2, 4, and 8 rpm.

The iris-derived photoreceptor-like cells, however, did not express all of the genes required for a functional photoreceptor (our unpublished data). Using multiple factors

appears to reprogram cell fates more efficiently. Creation of a complete photoreceptor from iris cells will likely require additional intrinsic transcription factors and/or differ-



ent culture conditions. A detailed understanding of transcription networks that govern photoreceptor development and appropriate *in vitro* culture conditions will help allow direct reprogramming to photoreceptors.

Regenerative medicine for retinal degenerative diseases

In retinitis pigmentosa, photoreceptors are lost due to genetic mutations^{66,67}. In age-related macular degeneration, degeneration of the RPE is followed by loss of photoreceptors⁶⁸. Thus, some retinal degeneration disorders are caused by specific loss of cell populations within the retina. Once retinal neurons are lost, no radical treatments are available. Clearly, regeneration of photoreceptors or RPE is an important therapeutic avenue in these retinal degenerative diseases^{54,69-71}.

We have shown that human ES cells can differentiate into retinal progenitors, photoreceptors, and RPE *in vitro*^{56,57}. Lamba et al. showed that transplantation of photoreceptor cells derived from ES cells was therapeutically beneficial in mice with photoreceptor degeneration⁷². When transplanted into the vitreous of newborn wild-type mice, ES cell-derived retinal cells integrated into all retinal layers and expressed markers appropriate for the lamina in which they settled. When transplanted into the subretinal space of adult mice, the cells integrated into the ONL, expressed photoreceptor markers, and formed outer segments required for efficient photon capture. When human ES cell-derived retinal cells were transplanted into the subretinal space of 4- to 6-week-old *Crx*^{-/-} mice, the transplanted cells migrated into the ONL. Surprisingly, the ERG B-wave response was restored in these mice and the amplitude of the B-wave was correlated with the number of integrated rod photoreceptors. MacLaren et al. used *Nrl*-GFP⁺ postmitotic rod photoreceptors to demonstrate that the optimal donor stage of rod photoreceptor cells for integration into the host mouse retina was P3–6⁷³ (Figure 1C). Thus, cell type- and stage-specific selection of differentiated ES cell-derived cells will increase the efficiency of integration into the host retina⁷⁰. In addition to rod photoreceptor transplantation, cone photoreceptor transplantation appears promising. Lakowski et al. used *Crx*-GFP

photoreceptor precursors to demonstrate the feasibility of cone photoreceptor transplantation into the degenerating retinas of *Crb1*^{rd8/rd8} and *Gucy2e*^{-/-} mice, two models of Leber congenital amaurosis⁷⁴. It should be also noted that immune rejection reduces the survival of integrated cells in recipient retinas⁷⁵. Immune responses characterized by macrophages and T cells are prominent around the transplantation site. West et al. demonstrated that, if immune responses are modulated, correctly integrated transplanted photoreceptors can survive for extended periods of time in hosts with partially mismatched H-2 haplotypes⁷⁵. Although immune suppression may be effective for non-autologous donors, these findings suggest that autologous donor cells are optimal for photoreceptor transplantation. Human iPS cells can differentiate into photoreceptors^{58,59,76,77}, which could make it possible to transplant a patient's own retinal cells and eliminate the risk of immune rejection.

We also have shown that monkey ES cells can differentiate into RPE *in vitro* and that transplanted monkey ES cell-derived RPE can rescue the vision of RCS rats, a model of RPE degeneration^{78,79} (Figure 2C). Monkey ES cell-derived RPE phagocytized photoreceptor materials, enhanced the survival of host photoreceptors, and improved head-tracking behaviors. Human ES and iPS cells also can generate RPE *in vitro*^{56-59,80}. RPE derived from human ES and iPS cells has been shown to rescue photoreceptor degeneration after transplantation in RCS dystrophic rats⁸¹⁻⁸³. Transplantation of RPE derived from human ES cells has been approved by the FDA, making it the first therapy that uses human pluripotent stem cells.

One key issue in autologous transplantation for genetic disorders is that genetic defects due to mutations or deletion need to be repaired before transplantation. Sickle cell anemia was corrected by homologous recombination in mouse iPS cells for transplantation of hematopoietic stem cells⁸⁴. Homologous recombination in human ES cells and human iPS cells, however, is inefficient although gene correction efficiency varies considerably, depending on the genomic context of the target locus. Engineered zinc finger nucleases (ZFNs) that induce a sequence-specific double-strand break enhanced homologous recombination-mediated



gene targeting in human ES cells and human iPS cells^{85,86}). Helper-dependent adenoviral vectors which are modified by deletion of all viral genes from the vector genome, resulting in reduced cytotoxicity and an expanded cloning capacity provided high efficient homologous recombination in monkey ES cells and human ES cells⁸⁷). Interestingly, a mouse ES cell-like “naïve” state of human iPS cells facilitated efficient homologous recombination²⁷). Improvements of gene correction methods will be required for autologous cell transplantation therapy.

Human pluripotent stem cells can also be used for *in vitro* modeling of retinal degenerative diseases. *In vitro* systems using disease-specific iPS cells should yield important insights into disease pathogenesis, biomarkers, and new drugs. Several *in vitro* studies have demonstrated disease-related cellular phenotypes using patient-derived iPS cells. Cardiomyocytes derived from patient iPS cells have been used to model LEOPARD syndrome⁸⁸). Cardiac myocytes that differentiated from iPS cells of patients with long-QT syndrome type 1 were used to recapitulate the electrophysiologic features of the disorder⁸⁹). Patient iPS cells have also been used to model inherited metabolic disorders of the liver⁹⁰). Neurons that differentiated from iPS cells of patients with Rett syndrome had fewer synapses, reduced spine density, smaller soma size, altered calcium signaling and electrophysiological defects, and the synaptic defects were rescued by treatment with insulin-like growth factor 1 or gentamicin⁹¹). In retinal degenerative diseases, various mutations in genes required for the survival and functions of photoreceptors and RPE have been identified (see database in RetNet or Retinal International), yet the mechanisms of retinal degeneration are still unclear. We found that malfunctions in patient iPS cell-derived photoreceptors and RPE can be, at least in part, recapitulated *in vitro* using our established differentiation systems (manuscript in preparation). Furthermore, because differences among species are critical for drug discovery, human ES cells and iPS cells will be powerful tools to bridge the gap between experimental animals and humans. Moreover, drug effects, toxicity, and metabolic profiling could be predicted and analyzed using iPS cells from patients, permitting persona-

lized optimization of drug treatment.

Conclusion

iPS cells provide an avenue for progress not only in regenerative medicine and drug discovery but also in studies of pluripotency and nuclear reprogramming. A number of safety concerns must be overcome before cell transplantation therapies are used clinically, however. Using c-Myc as a reprogramming factor or mutagenesis owing to transgene insertion is likely to increase the risk of tumorigenesis from iPS cells. Recent studies have shown that c-Myc is not required for iPS cell generation and that nonviral reprogramming methods using membrane-permeable recombinant proteins or synthetic mRNA can generate iPS cells free of inserted transgenes⁹²⁻⁹⁵). To translate iPS technology into therapies, it is important to establish a GMP-compliant system that can be used to produce and maintain iPS cells. Of note, Rodriguez-Pizà et al. reported that human iPS cells can be generated and maintained under xeno-free conditions⁹⁶). Although personalized medicine would provide many advantages, a bank of iPS cells from individuals with homozygous HLA alleles may provide a source for regenerative medicine. Understanding the molecular underpinnings of reprogramming will certainly contribute to clinical applications of this technology.

Retinal regeneration and reacquisition of visual function after injury or disease require replication of the original structures. Evidence suggests that transplanted autologous photoreceptors at a specific ontogenetic stage could integrate within the recipient retina and restore some vision. Human ES cell-derived photoreceptors have therapeutic potential, but the number of integrated photoreceptors and the recovery of visual function are limited. Improving synapse and neural circuit formation by transplanted photoreceptors should enhance transplantation efficacy. On the other hand, RPE transplantation has been shown effective in rat models of RPE degeneration using both human ES and human iPS cells. The U.S. FDA has approved transplantation of human ES cell-derived RPE. To use iPS cells clinically, the safety and efficacy of this approach must first be evaluated using *in vivo* animal models. Larger animals that are more closely related to humans, such as rabbits, dogs, pigs,



and monkeys, may be useful for preclinical studies⁹⁷⁻¹⁰²). Because monkeys are closely related to humans, autografts and allografts of monkey iPS cell-derived cells will greatly advance our understanding of the safety and efficacy of transplantation, particularly the potential for immune responses. An integrated understanding of stem cells, embryogenesis, reprogramming, neural circuits, and disease pathogenesis will be essential to achieve effective retinal regeneration therapy.

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