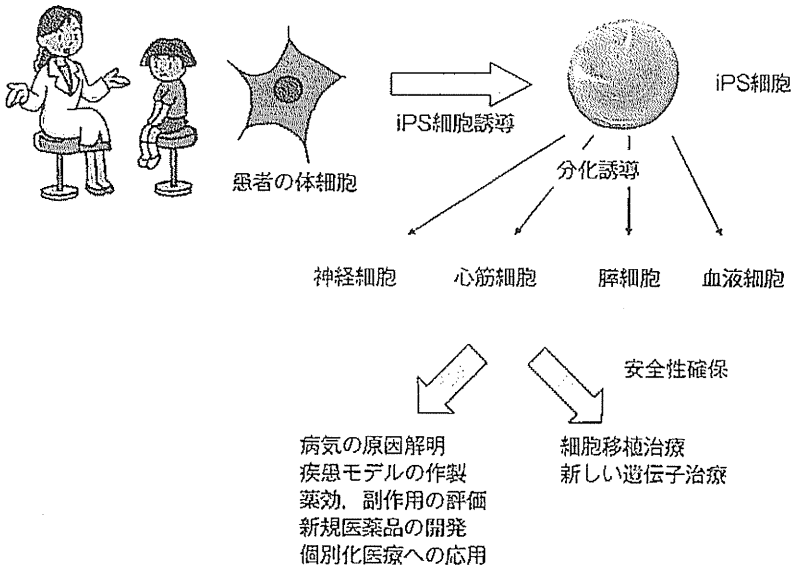

図4　疾患特異的iPS細胞を用いた新しい医療の開発

Mycの代わりにL-Mycが用いられるようになった[3]．また，遺伝子導入にはレトロウイルスベクターが用いられていたが，染色体に組み込まれないアデノウイルスベクター，センダイウイルスベクターやepisomal plasmid vectorsなどが用いられるようになってきている[4]．

2010年11月1日に改正された「ヒト幹細胞を用いる臨床研究に関する指針」ではヒトiPS細胞を用いた再生医療も指針の中に含まれている．iPS細胞を用いた再生医療はそう遠くない将来わが国でも始まるであろう．iPS細胞を用いた遺伝子治療についても現在見直しが始まろうとしている．

Ⅱ 疾患特異的iPS細胞

iPS細胞の持つ意義として，基礎研究への貢献，再生医療や創薬への応用などさまざまな面がいわれているが，わが国では再生医療への応用を目指した研究を中心に研究費が投下されてきた．一方，われわれは当初から，患者の細胞から疾患特異的iPS細胞を樹立し（図3），それを用いた疾患の病因，病態解析，遺伝子治療への応用を中心に研究してきた．

iPS細胞の持つもっとも画期的な臨床的側面は，さまざまな疾患の患者から皮膚などの組織を用いて疾患特異的iPS細胞を樹立できることである．この細胞を用いて，疾患の病態解析，新規治療法の開発，新規薬剤の有効性・毒性の検定などに応用されると考えられる．将来的には患者iPS細胞は異常遺伝子を修復した遺伝子治療と複合した再生医療につながるものと期待される（図4）．

1．疾患特異的iPS細胞の診断・病態解析への応用

疾患特異的iPS細胞を用いることによりさまざまな研究が進展すると予想される．その中でも，iPS細胞を用いた診断，病態解析，疾患モデルの作製など患者の治療に直結する成果が期待されている．

a．診断への応用

iPS細胞から生検が困難な組織の細胞を作り出し，それを用いた疾患の診断が期待される．患者皮膚などから樹立したiPS細胞を生検困難

な大脳，小脳，脊髄などの中枢神経組織に分化させ，それを使った診断や病態解析が考えられる．心筋，軟骨，肺，膵臓などの組織は生検可能だが，大量の組織を繰り返し採取することはできないので，そのような場合も対象となりえるだろう．

b．病気の発症機序の解明

患者からつくったiPS細胞を患部と同じ組織に分化させることができれば，病気発症機序を詳細に解析できる可能性がある．健常人と患者のiPS細胞を同じように分化させて，各分化段階の細胞を回収し比較することにより，いままでとはまったくちがう手法で病気の本態に迫ることができるかもしれない．

また，Kostmann症候群の一部では，好中球減少と中枢神経の異常を，Shwachman症候群では膵外分泌の異常と造血障害を合併することが知られているが，どうみても関係のない2つの異常がなぜ合併するのかまったくわかっていない．これらの患者から疾患特異的iPS細胞を樹立し，その細胞から血液（好中球），神経，膵臓に分化させ，その過程を正常と比較することにより新たな知見が得られるのではないかと考えている．

c．疾患モデルの構築

iPS細胞技術は，今までモデル動物のなかった疾患に対して新しいモデルを提供すると考えられる．たとえば脊髄性筋萎縮症（spinal muscular atrophy：SMA）と筋萎縮性側索硬化症（amyotrophic lateral sclerosis：ALS）は筋組織を支配している運動ニューロンが選択的に変性，死滅し，その結果筋力低下，筋萎縮が生じ，不幸な転機をとる疾患である．しかし，なぜ運動ニューロンが選択的に変性，死滅してしまうのかまったくわかっていない．ともによい動物モデルがないことから病態解明が進まず，治療法の開発のためには新しいモデルの開発が待たれている．iPS細胞技術を使うことで，SMA，ALS患者の皮膚細胞から疾患特異的iPS細胞を樹立し，この細胞からSMAやALS患者と同じ遺伝子セットをもった運動ニューロン細胞を大量に作り出すことができるようになった．この細胞を使うことで，なぜ患者の運動ニューロンは正常iPS細胞由来運動ニューロンに比べ壊れやすいのかの解明が可能となり，これら疾患の病態解明や薬剤探索のスタートラインに立つことができるようになった．

d．時空を超えて病気の本態に迫る

Duchenne型筋ジストロフィー患者は一般に10歳頃に歩けなくなり，20歳ぐらいで人工呼吸器が必要となる．たとえば10歳の患者から筋生検をして診断する際，皮膚または筋肉の細胞からiPS細胞を樹立し，その細胞から分化させた骨格筋組織は，いわば生まれたての筋肉の細胞と同じ状態であることが予想される．つまり10年間という時間を過去に遡って，そのときの筋肉の状態と現在の筋組織を比較することが可能となると考えられる．このような手法は，今までには考えられなかったことであり，iPS細胞が持つ強力なインパクトではないかと思っている．

Ⅲ 疾患特異的iPS細胞の遺伝子治療・個別化医療への応用

1．遺伝子治療への応用

遺伝子治療は疾病の治療を目的として遺伝子または遺伝子を導入した細胞をヒトに投与することにより疾病の治療を直接の目的とするものを指すが，遺伝子マーキングのような新しい治療法の開発や従来の治療法の検証に使われるものも広義の遺伝子治療に含める場合もある．しかし，アンチセンスを用いた治療は一般には遺伝子治療には含めない．各種疾患の遺伝子異常を修復し，正常の遺伝子に戻すことが究極の目標であるが，現在そのための相同組み換えの技術はヒトの遺伝子治療では確立されたとはいえない．そのため，子孫に影響が残る可能性がない体細胞を標的として，欠陥遺伝子の補充

(gene complimentation)，あるいは正常の遺伝子の付加（gene addition）の形で遺伝子治療が行われている．

遺伝子の投与法は *in vivo* 法と *ex vivo* 法に分けられる．前者は治療用の遺伝子を直接患者に投与する方法で簡便ではあるが，目的とする標的細胞へ選択的に遺伝子を導入することが難しく，安全性のチェックも容易でない．後者は治療の標的となる細胞を一旦体外に取り出し，遺伝子導入を行った後患者に戻す方法である．この方法は特定の細胞だけに遺伝子を導入できより安全な方法といえるが，対象が体外に取り出せる造血幹細胞などの細胞に限られていた．また，この造血幹細胞にしても確実に遺伝子が付加あるいは補充できている細胞だけを取り出してそれを治療に用いることはできなかった．さらに造血幹細胞への遺伝子導入は容易ではなく，加えて造血幹細胞の持つ自己複製能は限定的なことから遺伝子治療後導入細胞が時間とともに消滅していくこともしばしばみられた．

このような従来の遺伝子治療の抱える問題点を解決してくれそうなのがiPS細胞を用いた遺伝子治療であり，大きな期待が寄せられている．その主な理由は患者皮膚や血液からiPS細胞を作製すれば，この細胞は患者が侵されている臓器を含むすべての細胞に分化できることから，従来の方法では細胞を体外に取り出すことができないために対象とならなかった疾患にも応用できること，iPS細胞に遺伝子を導入し確実に遺伝子が付加あるいは補充できているiPS細胞クローンを選別し，これを増やすことができるようになることなどである．さらに従来は不可能と考えられていたヒトにおける相同組み換え技術を用いた究極的な遺伝子修復も，iPS細胞を用いれば可能になるのではないかと期待されている．

すでにマウスでは，鎌状赤血球のモデルマウスの尻尾から採取した細胞に山中4因子を導入して作製したiPS細胞の異常遺伝子を修復し，修復されたiPS細胞から作られた造血前駆細胞を用いた移植実験で，鎌状赤血球マウスの貧血が良くなったモデルが報告されている（図5）[5]．また，さまざまな致死的代謝異常症に対する新しい治療として，羊水検査で得られた細胞からあらかじめiPS細胞を作製し，この細胞の遺伝子を修復したiPS細胞を用意しておき，出生直後にこの細胞を用いて治療するモデルも提唱されている（図6）[6]．将来の遺伝子治療は，iPS細胞を使って行う治療法が主となっていくのではないかと筆者は考えている．

②．個別化医療への応用

a．患者自身の細胞を使った薬物試験

新薬の開発に当たっては，一般的に前臨床試験として動物実験を行うが，動物実験の結果が必ずしもヒトに当てはまるわけではない．次に健常人で薬物試験を行い，ある程度安全性を確認してから患者での臨床試験に移ることが多い．しかし，健常人には異常がなくても，患者では重篤な副作用が出現することがありしばしば問題となる．実際薬を投与されるのは患者なのだから，患者の細胞そのものを使って毒性試験や有効性試験をするほうがよいはずであり，疾患特異的iPS細胞から目的とする細胞に分化させ，その細胞を用いて毒性試験や有効試験をする時代が始まろうとしている．

b．個別化医療への応用

最近米国では，QT延長症候群のiPS細胞樹立が盛んに行われている．QT延長症候群は8つ以上のタイプに分かれ，タイプによって使用する薬が違うため，タイプを決めるのに薬物負荷試験をする必要がある．ところがこの薬物負荷試験が非常に危険な検査で，致死的な不整脈が出現することがあり，除細動器を横に置き，循環器専門医が慎重に薬物負担を行い診断している．そこでQT延長症候群の患者の皮膚から作製したiPS細胞を *in vitro* で心筋に分化させ，この細胞を用いて薬物負荷試験が行われている．従来患者で行っていた負荷試験を，iPS

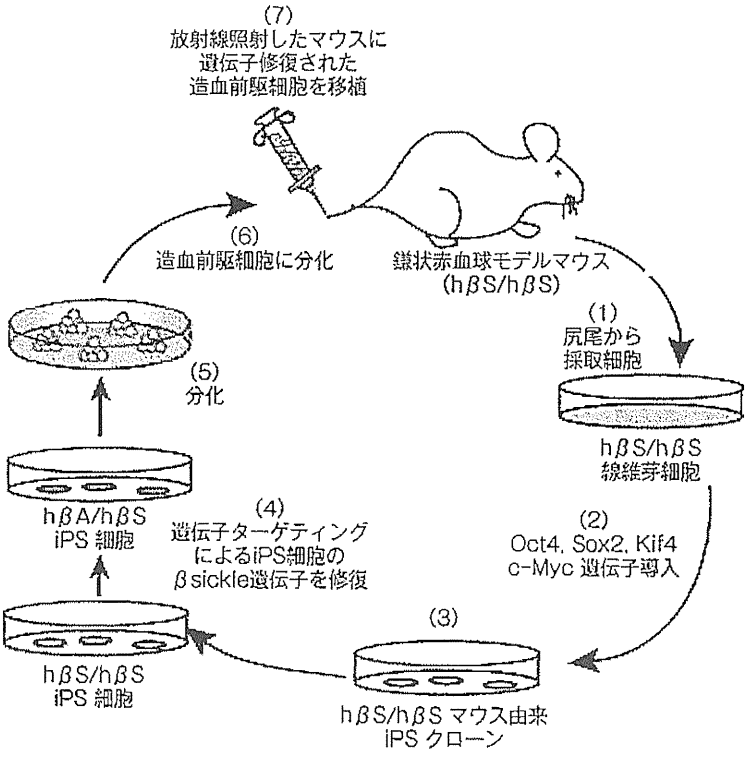

図5　iPS 細胞を用いた遺伝子治療の可能性：鎌状赤血球症マウスの例（Hanna J et al, 2007[5]より改変）

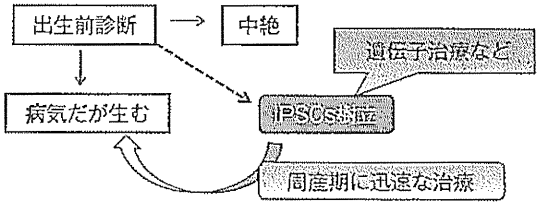

図6　出生前診断を遺伝病治療に応用できるよう，羊水検査や絨毛検査で得られた細胞から iPS 細胞を樹立（Ye L et al, 2009[6]より改変）

細胞で代用できるようになるかもしれない．このような iPS 細胞から分化させた細胞を用いてさまざまな負荷試験を行う時代がくることも考えられる．

おわりに

遺伝的疾患では iPS 化（初期化：リセット）した細胞を再び分化させることで，疾患特異的な現象を再現できる可能性が高いと考えられる．いままでとは違ったアプローチにより病気の本体に迫っていけるかもしれない．疾患特異的 iPS 細胞は，今まで考えられなかった新しい研究手段を提供しており，患者に還元される多くの知見が得られることが期待される．疾患特異的 iPS 細胞は疾患の病因，病態の解明に有用であるとともに，遺伝子治療や個別化医療などの新規治療法の開発へ結び付くことを考えると，iPS 細胞技術は小児科（子ども）のために開発してくれたのではないかと思ってしまう．この分野の研究の発展に期待したい．

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Stepwise differentiation of pluripotent stem cells into retinal cells

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Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of blastocyst-stage embryos. They can maintain an undifferentiated state indefinitely and can differentiate into derivatives of all three germ layers, namely ectoderm, endoderm and mesoderm. Although much progress has been made in the propagation and differentiation of ES cells, induction of photoreceptors has generally required coculture with or transplantation into developing retinal tissue. Here, we describe a protocol for generating retinal cells from ES cells by stepwise treatment with defined factors. This method preferentially induces photoreceptor and retinal pigment epithelium (RPE) cells from mouse and human ES cells. In our protocol, differentiation of RPE and photoreceptors from mouse ES cells requires 28 d and the differentiation of human ES cells into mature RPE and photoreceptors requires 120 and 150 d, respectively. This differentiation system and the resulting pluripotent stem cell-derived retinal cells will facilitate the development of transplantation therapies for retinal diseases, drug testing and *in vitro* disease modeling. It will also improve our understanding of the development of the central nervous system, especially the eye.

INTRODUCTION

Embryonic stem cells are pluripotent cells derived from the inner cell mass of blastocyst-stage embryos. They can grow indefinitely and can differentiate into derivatives of all three germ layers, namely ectoderm, endoderm and mesoderm[1]. These properties make ES cells powerful tools for modeling development and disease, as well as for developing cell replacement therapies. As functional impairment results from cell loss in most central nervous system (CNS) diseases, recovery of lost cells is an important treatment strategy. Neurogenesis occurs in two regions of the adult mammalian brain[2]. In the adult retina, Müller glia serve as endogenous retinal progenitors in response to injury[3,4] (**Fig. 1a,b**). However, the CNS has poor potential for regeneration to compensate for cell loss. Thus, transplantation of ES cell-derived neurons into damaged or diseased CNS is a promising treatment approach for neurodegenerative disorders, including Parkinson's disease, Huntington's disease, spinal cord injury and stroke[5].

Most retinal degeneration diseases in humans are caused by the impairment of the neural retina, which causes irreversible blindness. For instance, in retinitis pigmentosa, photoreceptors are selectively lost owing to genetic mutations[6]. In age-related macular degeneration, degeneration of the RPE is followed by the loss of photoreceptors[7]. Thus, transplantation of photoreceptor or RPE cells may permit recovery of visual function.

Accumulating evidence indicates that RPE cells derived from monkey and human ES cells can restore visual function in a retinal degeneration model, the Royal College of Surgeons (RCS) rat[8,9]. On postnatal days (P) 3–6, postmitotic rod photoreceptors are able to form functional synaptic connections with host bipolar cells and to improve visual function when transplanted into the normal or degenerating adult mouse retina[10]. It is of note that the stage of differentiation of donor photoreceptors is important for successful transplantation. As somatic progenitors derived from the adult ciliary body[11] or the iris[12] are limited in both differentiation potential and proliferation capacity (**Fig. 1a,c**), ES cells and induced pluripotent stem cells[13–15] represent a promising donor source for transplantation, if indeed P3–6 photoreceptors can be induced from them. A reliable and efficient *in vitro* method of

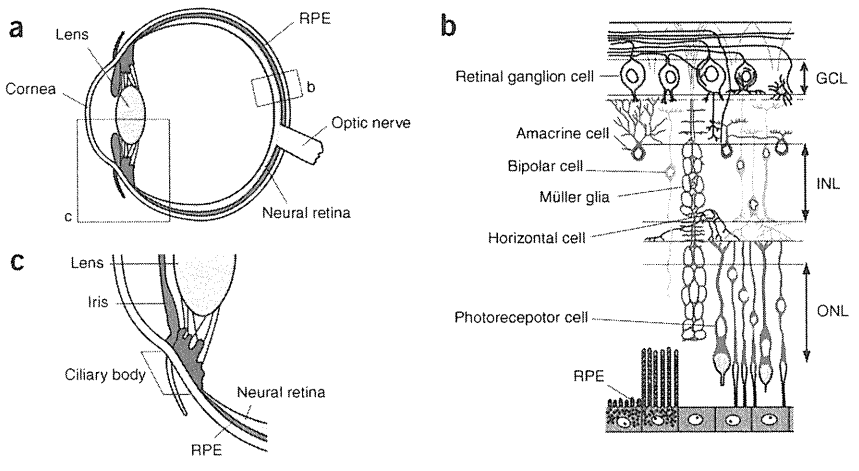

Figure 1 | Localization of neural stem/progenitor cells in adult eye tissue. Schematic diagram of a section of an adult mammalian eye (**a**). Magnified views of boxed region in panel **a** (b and c). Müller glia act as retinal progenitors in response to injury (**b**, purple). Retinal progenitors are present in the ciliary body (**a,c**: red). Iris-derived cells display neural stem/progenitor cell properties (**a,c**: orange). RPE, retinal pigment epithelium; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. (Reproduced with permission from ref. 23)

generating retinal cells from ES cells will greatly contribute to medical and pharmaceutical research aimed at finding treatments for retinal diseases[16–18].

In vitro differentiation of ES cells mimics, at least in part, the patterning and differentiation events that occur during embryogenesis[19–21]. During development of the neural plate, the anterior neurepithelium evaginates to give rise to the optic vesicle, then invaginates along with the overlying surface ectoderm to form a bilayered optic cup[22,23]. The outer layer of the optic cup differentiates into the RPE, whereas the inner neurepithelial layer differentiates into the neural retina. Within the neural retina, seven types of cells differentiate from a common set of progenitors in the following temporal sequence: retinal ganglion cells, cone photoreceptors, amacrine cells, horizontal cells, rod photoreceptors, bipolar cells and Müller glia[24]. As is the case for *in vivo* development, ES cells differentiate into retinal progenitors in response to patterning signals and subsequently differentiate into retinal cells in a stepwise fashion (**Fig. 2**). It is of note that ES cells have never been observed to differentiate directly into retinal cells[25,26].

Since the establishment of mouse ES cells in 1981 (see ref. 1) and human ES cells in 1998 (see ref. 27), much progress has been made in ES cell propagation and differentiation techniques. Over the last decade, several methods have been reported to control the differentiation of ES cells into neural cells[19,21,28–33]. Each method has its own advantages and disadvantages, depending on the type of neural cells desired, and can induce differentiation of neural tissues with distinct regional identities within the CNS[19–21]. ES cells can be differentiated into floating aggregates known as embryoid bodies, which are cultured in the presence of serum and contain cells derived from all three germ layers. In contrast, our method is based on a serum-free, feeder-free suspension culture, which can induce selective differentiation toward the ectoderm[21].

RPE cells can be generated from primate ES cells cocultured with PA6 stromal cells[34] and from human ES cells after spontaneous differentiation at a low efficiency with $<1\%$ of the embryoid bodies containing pigmented cells[35]. Retinal progenitors can also be generated from ES cells *in vitro*, but they rarely differentiate into photoreceptors unless cocultured with or transplanted into developing retinal tissues[36–38]. Factors involved in *in vivo* retinal development are critical for the *in vitro* differentiation of ES cells into retinal cells. On the basis of our knowledge of embryonic development, we have established a defined culture method for generating photoreceptors and RPE from mouse and human ES cells *in vitro*[25,26] (**Fig. 3**). This differentiation system and the resulting human ES cell-derived cells will substantially facilitate the development of human ES cell-based transplantation therapies for retinal diseases, the discovery of therapeutic drugs and the investigation of CNS development and disease mechanisms[39]. In addition, induced pluripotent stem cell technology will likely allow us to make progress on *in vitro* modeling and patient-specific therapy[40–42]. Here, we introduce our step-by-step protocol for inducing retinal progenitors, RPE cells and photoreceptors *in vitro* (**Figs. 2** and **3**).

Although the ES cell-derived photoreceptors we describe here express all the appropriate markers, we have not yet determined whether they can function normally. The function of photoreceptors has generally been tested by electrophysiology in retinal cells freshly prepared from animals, because the crucial outer segment of the cell is lost during cell culture. Thus, to test whether ES

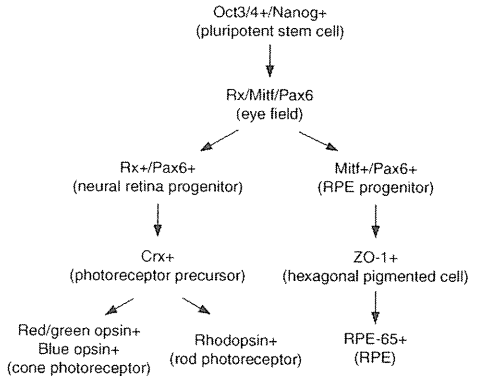

Figure 2 | Multistep commitment in the development of retinal cells. Markers for the differentiation steps are shown in bold font. ES cells differentiate into retinal cells in a stepwise manner along the developmental time course.

cell-derived photoreceptors are functional, we must transplant them into the developing and adult retina and analyze their function within retinal tissue. Transplantation studies will require large numbers of differentiated retinal cells. Although retinal cells can be obtained from mouse and human ES cells, the differentiation efficiency is low. The pluripotency of ES cells permits the use of large numbers of undifferentiated ES cells, but improvement of the differentiation efficiency is important for *in vitro* modeling of development and disease, drug screening and transplantation studies.

Experimental design

Mouse ES cells. Mouse ES cells should be passaged before reaching confluence (**Fig. 4a**), because overconfluent ES cells differentiate even under conditions that normally maintain pluripotency. We use mouse ES cells carrying a blasticidin-S resistance gene in the

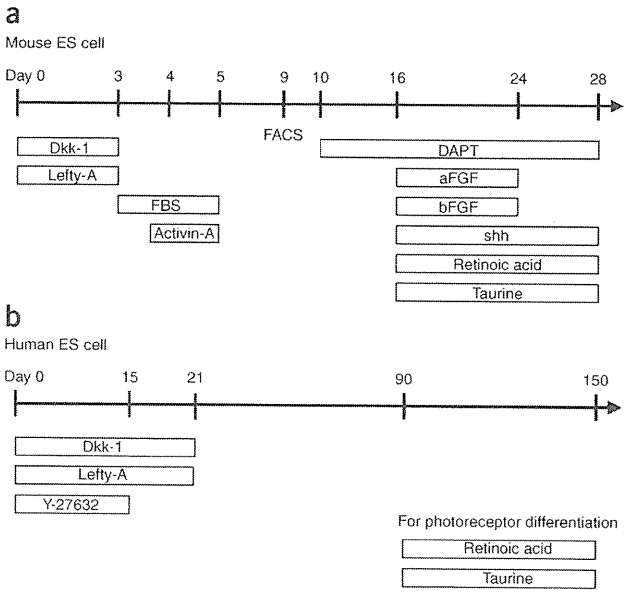

Figure 3 | Schematic diagram of mouse and human ES cell differentiation into retinal cells. Differentiation of mouse ES cells into retinal cells (**a**). Differentiation of human ES cells into retinal cells (**b**).

Oct-3/4 locus to select Oct-3/4+ undifferentiated pluripotent cells (**Fig. 4a**). Our mouse ES cells also contain a green fluorescent protein (GFP) reporter gene knocked into the Rx locus to aid in later purification[26]. To induce differentiation of mouse ES cells, we dissociate undifferentiated ES cell colonies into single cells and plate the cell suspension onto a Petri dish as a floating culture. Floating mouse ES cells spontaneously form aggregates within 1 d under these conditions (**Fig. 4b,c**). As shown in **Figure 3a**, we add Dkk-1, Lefty-A, fetal bovine serum (FBS) and Activin-A to the media to induce retinal specification of ES cells[25,26]. After the addition of Dkk-1 and Lefty-A on differentiation day 0, we replace half of the media with the media that do not contain Dkk-1 and Lefty-A on day 3. Thus, during days 3–5, Dkk-1 and Lefty-A are present at half concentration. We confirmed that the differentiation efficiency is the same at half concentration as at full concentration. Therefore, we have modified the earlier reported protocol. After the addition of FBS on day 3, cell aggregates become adherent. Thus, to avoid adhesion of floating cells to the culture dish, we re-plate the cell aggregates onto a 2-methacryloyloxyethyl phosphorylcholine (MPC)-treated non-adhesive dish. As the cells form aggregates, it is difficult to judge by cell morphology alone whether the protocol is carried out well. Thus, retinal marker expression should be examined by immunostaining or reverse transcriptase-PCR analysis. The transcription factor Rx plays an essential role in the specification of the retinal primordium and is an early marker of the eye field (**Fig. 2**). Rx-GFP expression peaks on differentiation day 9 and thereafter declines. Thus, we purify retinal progenitors using fluorescent-activated cell sorting (FACS) on day 9 (**Fig. 4d**). Without FACS, multiple cell types are present after differentiation, which may mask patterning signals for retinal specification. After FACS, we steer purified cells toward mature retinal phenotypes by modifying the reaggregation pellet culture reported by Watanabe and Raff[43] (**Fig. 5a**). *N*-[*N*-(3,5-Difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT) is added to increase the number of Crx+ photoreceptor precursors (**Fig. 2**), then fibroblast growth factors (FGFs), Sonic hedgehog N-terminal peptide (Shh), retinoic acid (RA) and taurine are added to promote rod differentiation, because these factors promote rod genesis from retinal progenitors *in vitro*[26,44] (**Figs. 5a–c** and **6a,b**). After addition of these factors, marked morphological changes are not observed immediately, as differentiation progresses slowly. We recommend monitoring differentiation by examining the expression of differentiation markers observed during eye development (**Figs. 2** and **6**) at various steps rather than by microscopy alone.

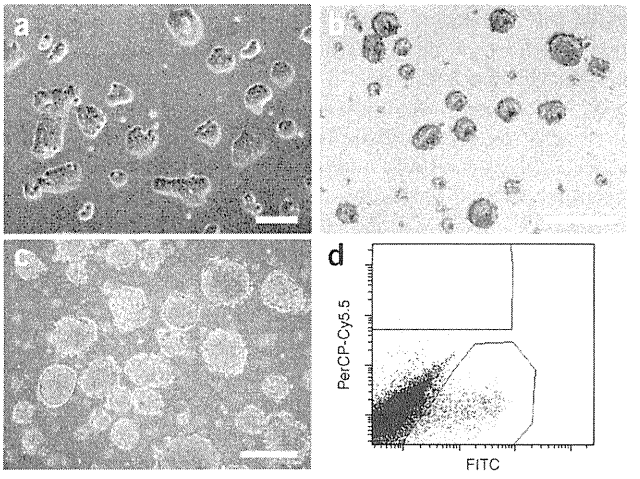

Figure 4 | Undifferentiated mouse ES cells, formed aggregates and FACS analysis. Typical morphology of undifferentiated mouse ES cell colonies cultured under feeder-free conditions (**a**). The shape of formed aggregates after 3 d (**b**) and 8 d (**c**) in the SFEB/DLFA culture. Typical data for FACS analysis (**d**). Rx-GFP+ mouse ES cells (green) are selected by flow cytometry. Scale bars, 300 μm.

Human ES cells. Human ES cells require more careful handling compared with mouse ES cells. For instance, although mouse ES cells can be dissociated into single cells, a similar treatment of human ES cells causes cell death and abnormal karyotypes. We follow the method described by Suemori *et al.*[45] and maintain human ES cells at 37 °C in a humidified atmosphere of 2% CO_2 and 98% air and use a mild enzymatic technique that enables bulk passaging of human ES cells and retention of normal karyotypes after more than 100 passages (**Fig. 7**). In addition, to improve cell survival during differentiation, the Rho kinase inhibitor, Y-27632, is added 1 h before dissociation and is maintained in the culture media during the first 15 days of floating culture, because Y-27632 prevents dissociation-induced cell death in human ES cells[46]. In our protocol for differentiation, ES cell colonies are dissociated into 5–10 cells per clump. It should be noted that efficiency of neural induction is low if ES clumps are too large. Furthermore, to avoid cell damage by centrifugation, we permit large aggregates to sink down to the bottom of the tube by gravity and use centrifugation only with small aggregates.

Unlike mouse ES cells, human ES cells require mouse embryonic fibroblast (MEF) feeder layers for maintenance. To prevent contamination with MEFs, we plate the cell suspension containing both human ES cells and MEFs onto gelatin-coated dishes before differentiation. MEFs adhere more quickly than do human ES cells, so incubating the dish for 1 h allows MEFs to adhere to the dish, while ES cells remain floating. To establish defined conditions for human ES cell culture, FBS is not used. For differentiation (**Fig. 3b**), human ES cells are incubated in the presence of Dkk-1 and Lefty-A for 21 d in a floating culture (**Fig. 8**) and are plated onto a fibronectin-, laminin- and poly-D-lysine-coated dish[26], which supports adhesion of cell aggregates and subsequent neural

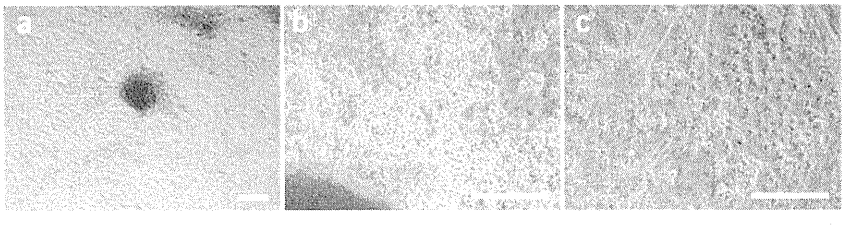

Figure 5 | Morphology of differentiated pelleted mouse ES cells after sorting. After FACS sorting and pellet formation, differentiated cells migrate out from the pellet in a radial manner (**a**). Round cells tend to be observed around the pellet (**b**). Neural- and epithelial-like cells tend to be observed far from the pellet (**c**). Scale bars, 100 μm.

differentiation. Excessively high plating density is not suitable for long-term culture, because cell growth is impeded. In adherent culture, differentiating cells migrate out from the aggregates (**Fig. 9**). Although cells with neurite-like processes are observed under the microscope, it is difficult to judge without staining whether these neural cells differentiate into retinal cells.

Retinal progenitors positive for Rx, Mitf and Pax6 are induced between differentiation days 30–40. Approximately 40 d after differentiation, pigmented cells can be observed under a light microscope. At this time, pigmented cells rarely have a polygonal shape, but long-term culture (> 60 d) results in increased pigmentation and polygonal morphology (**Figs. 10** and **11**). Finally, treatment with RA and taurine, both of which are important for photoreceptor genesis, induces photoreceptor differentiation (**Figs. 12** and **13**).

Our method preferentially induces the differentiation of RPE and photoreceptors from ES cells. When the procedure is carried out smoothly, we observe pigmented colonies without having to perform any cell staining, unless the ES cells are derived from albino mice. Retinal cells differentiate only from colonies committed to retinal fate, whereas colonies not committed to the retinal fate fail to generate retinal cells, including pigmented cells. Thus, if pigmented cells are observed, other types of differentiated retinal cells should also be present.

Mouse and human ES cells differ in both their maintenance and differentiation conditions. Mouse ES cells can be maintained in a pluripotent state without a feeder layer when grown in the presence of LIF. In contrast, human ES cells require a feeder layer and addition of the growth factor, FGF-2, but not LIF. The condition of the MEF feeder layer is very important for maintaining undifferentiated human ES cells, and the preparation of MEF feeders is described in **Box 1**. Only early passage MEFs (up to passage 2) should be used to prepare feeders for human ES cell culture, and feeders should be used within 3 d of preparation.

Mouse ES cells can be genetically engineered more easily than human ES cells. We use mouse ES cells carrying internal ribosome entry site (IRES)-blasticidin-S deaminase in the Oct-3/4 locus, which allows for the selection of Oct-3/4+ undifferentiated pluripotent cells[26,47]. Purification methods must be devised to isolate target cells for transplantation, to prevent tumorigenesis resulting from undifferentiated ES cells, to establish *in vitro* models of development and disease and to facilitate drug screening[39,48]. To purify retinal progenitors, our mouse ES cells also contain a GFP reporter gene knocked into the Rx locus[26].

Differentiation of human ES cells takes a longer duration than that of mouse ES cells. Differentiation of mouse ES cells into RPE and photoreceptors requires ~1 month, whereas generation of mature RPE from human ES cells takes ~4 months, and photoreceptors require ~5 months. Even after 2 weeks of human ES cell differentiation, a small number of cells still express the undifferentiated ES cell markers, Oct3/4 and Nanog. It is possible that this difference in differentiation time between human and mouse ES cells is because of the large difference in time required for overall embryonic development. Further improvements to the differentiation protocol might enable faster generation of retinal cells from human ES cells.

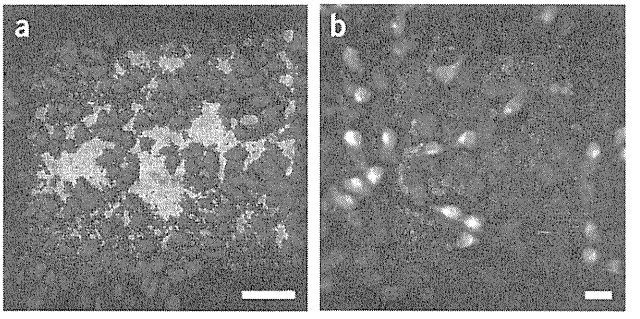

Figure 6 | Immunocytochemical analyses of the differentiated cells from mouse ES cells. The sorted Rx-GFP+ cells treated with DAPT express Crx on day 20 (**a**). The sorted cells treated with DAPT, aFGF, bFGF, shh, taurine and RA express rhodopsin and recoverin on day 28 (**b**). Scale bar, 20 μm (**a**) and 10 μm (**b**). (Reproduced with permission from ref. 26.)

Evaluation of ES cell differentiation. Evaluating the effectiveness of retinal differentiation can be performed using FACS analysis to quantify the differentiated cells. Knock-in ES cells expressing a fluorescent protein under the control of a specific promoter and antibody detection of specific cell surface antigens are both useful for FACS. Moreover, differentiation can be evaluated by immunocytochemistry. Antibodies that we have tested by staining appropriate tissues (frozen sections of embryonic, postnatal and adult eyes) are shown in **Table 1**. ES cells express the undifferentiated cell markers, Oct3/4, Nanog, TRA-1-60 and TRA-1-81 (**Fig. 2**). Staining for Rx, Mitf, Pax6 or Chx10 individually does not provide a good index for retinal differentiation, because these markers are also expressed in other CNS regions. For example, Rx is also expressed in the ventral diencephalon, Pax6 in the telencephalon and Mitf in melanocytes. However, co-immunostaining for Rx, Mitf, Pax6 and Chx10 in combination marks cells that have undergone retinal specification (**Fig. 2**). Quantitative PCR analysis can be used not only to examine the expression of markers for retinal differentiation but also to detect genes normally expressed in a specific developing brain region along the rostral–caudal and dorsal–ventral axes. This provides us with positional information of differentiated cells[20,21,33]. Finally, although analysis of human embryos is not possible because of ethical concerns, analysis of mouse embryonic development will help us learn molecular

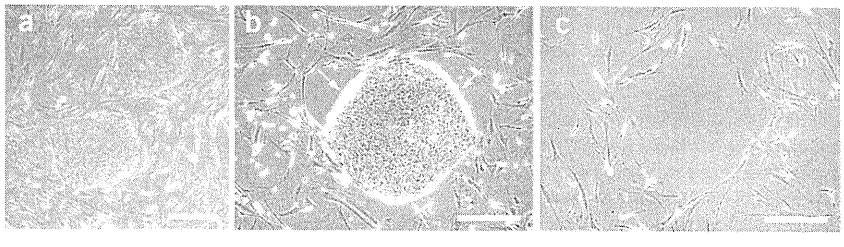

Figure 7 | Morphology of undifferentiated human ES cells and detachment of ES cell colonies. Typical morphology of undifferentiated human ES cell colonies cultured on MEF feeders (**a**). ES cell colonies have partially detached from the substratum (**b**). Arrows indicate the detached edge of human ES cell colonies. Human ES cell colonies are preferentially detached, whereas MEF feeders remain on the dish (**c**). Scale bar, 500 μm (**a**) and 300 μm (**b**,**c**).

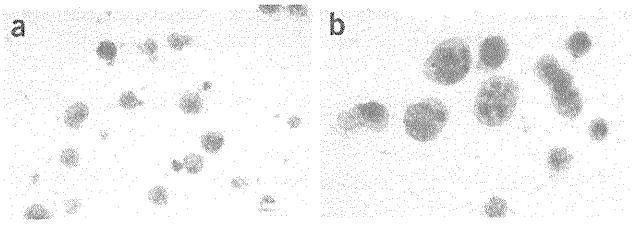

Figure 8 | Morphology of floating aggregates formed from human ES cells. The shape of aggregates formed after 6 d of culture (**a**) or 21 d of culture (**b**) in the SFEB/DLFA culture. Scale bars, 300 μm.

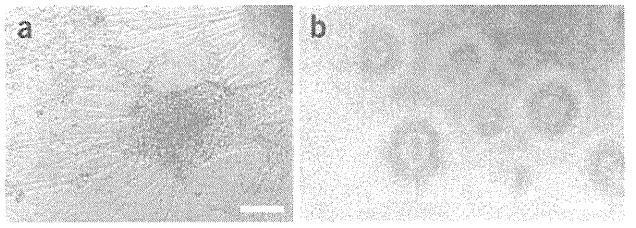

Figure 9 | Morphology of human ES cell aggregates in adherent culture. In adherent culture on day 30, differentiated cells extend from the aggregates in a radial manner (**a**). Neurite formation is observed (**a**). Some of the aggregates form neural rosettes on day 30 (**b**). Scale bars, 100 μm.

and cellular controls in the differentiation of ES cells toward various types of cells.

The variability of Knockout Serum Replacement (KSR) and FBS from one lot to another is a factor that affects the maintenance and differentiation of ES cells. For instance, a suboptimal lot of KSR or FBS can decrease the speed of colony growth and cause colony collapse and the loss of undifferentiated marker expression during maintenance. It can also inhibit neural or retinal differentiation. Therefore, we always test several lots of KSR and FBS on ES cells and measure the expression of various markers of differentiation by immunocytochemistry, reverse transcriptase-PCR or FACS analysis.

MATERIALS

REAGENTS

- Mouse ES cell: Rx-KI ES cell lines 116-18 and 20-10 (see refs. 26,47)
- Human ES cell: khES-1, khES-2 and khES-3 (see ref. 45)
- MEF (Kitayama Rabesu, cat. no. KBL9284600)
- Glasgow MEM (GMEM) (GIBCO, cat. no. 11710-035)
- DMEM/F12 (Sigma, cat. no. D6421)
- DMEM (Sigma, cat. no. D5796)
- KSR (GIBCO, cat. no. 10828-028)
- FBS (JRH Biosciences, cat. no. 12103-78P), heat-inactivated at 56 °C for 30 min
- Minimum essential media Eagle, HEPES modification (Sigma, cat. no. M7278)
- Hank's balanced salt solution (GIBCO, cat. no. 24020-117)
- PBS (GIBCO, cat. no. 10010-023)
- dH_2O (GIBCO, cat. no. 15230-162)
- Dimethylsulfoxide (DMSO) (Nacalai, cat. no. 13408-64)
- Gelatin Type A (Sigma, cat. no. G2500) for mouse ES cells
- Gelatin Type B (Sigma, cat. no. G9391) for human ES cells
- Non-essential amino acid solution (Sigma, cat. no. M7145)
- Pyruvate, 100 mM solution (Sigma, cat. no. S8636)
- 2-Mercaptoethanol (2-ME) (Sigma, cat. no. M7522) ! **CAUTION** 2-ME is toxic. When used, avoid inhalation and skin contact.
- N2 supplement (GIBCO, cat. no. 17502-048)
- Glucose (Nacalai, cat. no. 16805-35)
- L-Glutamine (Sigma, cat. no. G7513)
- Penicillin/streptomycin (GIBCO, cat. no. 15070-063)
- 0.25% Trypsin–EDTA (GIBCO, cat. no. 25200-056)
- 2.5% (wt/vol) Trypsin (GIBCO, cat. no. 15090-046)
- Collagenase type IV (GIBCO, cat. no. 17104-019)
- Trypan blue stain (GIBCO, cat. no. 1520-061)
- Propidium iodide (PI) (Sigma, cat. no. P4170)
- Bovine serum albumin (BSA) (Sigma, cat. no. A2058)
- Blasticidin (Blas) (Funakoshi, cat. no. KK-400)
- LIF (ESGRO) (Chemicon, cat. no. R-ESG1107)
- Mitomycin C (Wako, cat. no. 134-07911)
- Dkk-1 (R&D Systems, cat. no. 1096-DK-010)
- Lefty-A (R&D Systems, cat. no. 746-LF-025)
- Activin-A (R&D Systems, cat. no. 338-AC-025)
- DAPT (Calbiochem, cat. no. 565770)
- Acidic FGF (aFGF) (R&D Systems, cat. no. 232-FA-025)
- Basic FGF (bFGF) (Upstate, cat. no. 01-106)
- Shh (R&D Systems, cat. no. 1314-SH-025)
- Taurine (Sigma, cat. no. T-8691)
- All-*trans* RA (Sigma, cat. no. R2625) ▲ **CRITICAL** Store in light-proof vials because RA is light sensitive.
- Laminin (BD Biosciences, cat. no. 35-4239)
- Fibronectin (GIBCO, cat. no. 33016-015)
- Y-27632 (Calbiochem, cat. no. 688001)

EQUIPMENT

- Water bath
- Centrifuge

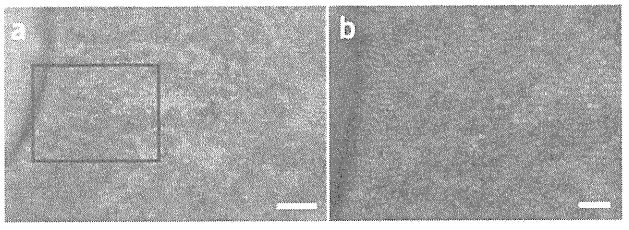

Figure 10 | Morphology of RPE cells differentiated from human ES cells. Pigmented cells begin to form ~40 d after initiation of differentiation. On day 120, pigmented cells form a single layer on the culture dish (**a**). Polygonal cells at various pigmentation stages are observed. Magnified image of pigmented cells in the boxed region of panel a (**b**). Scale bar, 100 μm (**a**) or 30 μm (**b**).

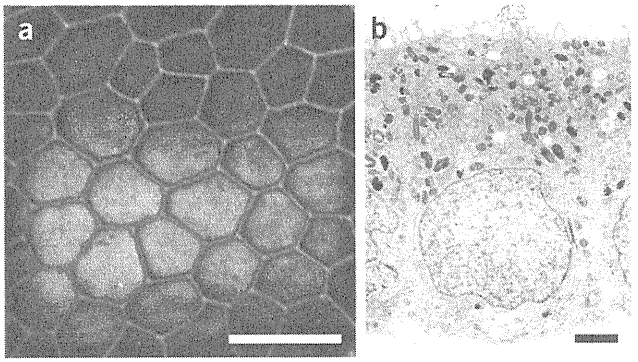

Figure 11 | RPE derived from human ES cells. Formation of tight junctions positive for ZO-1 on day 120 (**a**). Electron micrograph of human ES cell-derived pigmented cells on day 120 (**b**). Scale bar, 20 μm (**a**) or 100 nm (**b**). (Reproduced with permission from ref. 26).

- 100-mm dish (Falcon, cat. no. 35-3003)
- Petri dish (BIOBIK, cat. no. I-90)
- Low cell binding MPC-treated dish (NUNC, cat. no. 145401)
- Tubes (15 ml, 50 ml) (Falcon, cat. nos. 352095 and 352070)
- Disposable sterile filter system (0.22 μm) (Millipore, cat. no. SCGPU05RE)
- Plastic disposable transfer pipettes (5, 10, 25 and 50 ml) (Corning Costar, cat. nos. 4487, 4488, 4489 and 4490, respectively)
- Glass Pasteur pipette (IWAKI, cat. no. IK-PAS-9P)
- Eight-well chamber slide pre-coated with poly-D-lysine (BD BioCoat, cat. no. 354632)
- Tube with cell strainer (BD Biosciences, cat. no. 352235)
- Phase contrast microscope (Carl Zeiss)
- FACS machine (BD Biosciences)

REAGENT SETUP

Gelatin-coated dish

(1) Dissolve 500 mg of gelatin in 500 ml of dH_2O and autoclave at 121 °C for 20 min. The gelatin solution can be stored at room temperature (20–25 °C) for several months.
(2) For preparation of gelatin-coated dish, add 7 ml of gelatin solution to a 100-mm dish and let sit for at least 1 h.
(3) Dishes containing gelatin solution can be stored in a humidified incubator for several days. Storage for a longer period induces evaporation of gelatin solution even in a humidified incubator.
(4) Before use, aspirate the gelatin solution from the dish.

Poly-D-lysine/fibronectin/laminin-coated dish For the preparation of dishes coated with poly-D-lysine/fibronectin/laminin, we use poly-D-lysine-coated eight-well chamber slides.

(1) Mix 800 μl of 60 μg ml^{-1} laminin and 8 μl of 1 mg ml^{-1} fibronectin.
(2) Apply 100 μl of the mixture to each well of poly-D-lysine-coated eight-well chamber slide.
(3) Incubate the eight-well slide at 37 °C overnight.
(4) Add 500 μl of PBS to each well of the coated slide. Slides containing PBS can be stored in a humidified incubator for several days.
(5) Before use, aspirate the PBS from the wells.

The media for culturing mouse ES cells are shown in the tables below:

- Media for mouse ES cell maintenance

Component	Volume (ml)	Final concentration
GMEM	500	
Non-essential amino acid solution	5.8	0.1 mM
Pyruvate	5.8	1 mM
2-ME solution	0.58	0.1 mM
KSR	58	10%
FBS	5.8	1%

- Media for differentiation of mouse ES cells into retinal progenitors

Component	Volume (ml)	Final concentration
GMEM	500	
Non-essential amino acid solution	5.1	0.1 mM
Pyruvate	5.1	1 mM
2-ME solution	0.51	0.1 mM
KSR	28	5%

- Media for differentiation of mouse ES cells into retinal cells

Component	Volume (ml)	Final concentration
MEM-E HEPES	130	66%
HBSS	25	33%
Glucose solution	40	5.75 mg ml^{-1}
L-Glutamine	0.2	200 μM
N2 supplement	2	1%
FBS	2	1%
Penicillin/streptomycin	1	25 U ml^{-1}/25 mg ml^{-1}

Filter each medium using a 0.22-μm filter. Store the media at 4 °C for up to a week or in small aliquots (e.g., in a 50-ml tube) at −20 °C for several months. ▲ **CRITICAL** Maintenance of pluripotency, proliferation and differentiation efficiency depends on KSR and FBS. We recommend testing several lots beforehand and using the best lot. Old media result in low differentiation efficiency.

The media for culturing human ES cells are shown in the table below:

- Media for human ES cell maintenance

Component	Volume (ml)	Final concentration
DMEM/F12	500	
Non-essential amino acid solution	6.3	0.1 mM
L-Glutamine	6.3	2 mM
2-ME	0.005	0.1 mM
KSR	125	20%

- Media for human ES cell differentiation (20% KSR)

Component	Volume (ml)	Final concentration
GMEM	500	
Non-essential amino acid solution	6.6	0.1 mM
Pyruvate	6.6	1 mM
2-ME solution	0.66	0.1 mM
KSR	127	20%
Penicillin/streptomycin	5.0	50 U ml^{-1}/50 mg ml^{-1}

- Media for human ES cell differentiation (15% KSR)

Component	Volume (ml)	Final concentration
GMEM	500	
Non-essential amino acid solution	6.2	0.1 mM
Pyruvate	6.2	1 mM
2-ME solution	0.64	0.1 mM
KSR	93	15%
Penicillin/streptomycin	5.0	50 U ml^{-1}/50 mg ml^{-1}

- Media for human ES cell differentiation (10% KSR)

Component	Volume (ml)	Final concentration
GMEM	500	
Non-essential amino acid solution	5.5	0.1 mM
Pyruvate	5.5	1 mM
2-ME solution	0.58	0.1 mM
KSR	58.4	10%
Penicillin/streptomycin	5.0	50 U ml^{-1}/50 mg ml^{-1}

- Media for differentiation of human ES cells into photoreceptors

Component	Volume (ml)	Final concentration
GMEM	500	
Non-essential amino acid solution	5.5	0.1 mM
Pyruvate	5.5	1 mM
2-ME solution	0.56	0.1 mM
KSR	28	5%
N2 supplement	5.6	1%
Penicillin/streptomycin	5.0	50 U ml^{-1}/50 mg ml^{-1}

- Media for MEF

Component	Volume (ml)	Final concentration
DMEM	500	
FBS	55.5	10%

Filter each media using a 0.22-μm filter. Store at 4 °C for up to a week or in small aliquots (e.g., in a 50-ml tube) at −20 °C for several months. ▲ **CRITICAL** Maintenance of pluripotency, proliferation and differentiation efficiency depends on KSR. We recommend testing several lots beforehand and using the best lot. Old media result in low differentiation efficiency.

Human ES cell dissociation solution Dissolve collagenase IV in PBS at 10 mg ml^{-1} to produce the collagenase solution. Dissolve $CaCl_2/2H_2O$ in PBS at 100 mM to produce the $CaCl_2$ solution. Mix 10 ml of 2.5% (wt/vol) trypsin, 10 ml of collagenase solution, 1 ml of $CaCl_2$ solution, 20 ml of KSR and 59 ml of PBS, and filter using a 0.22-μm filter. Store small aliquots at −20 °C for several months.

2-ME solution Mix 14.1 ml of PBS and 0.1 ml of 2-ME. Prepare freshly before the preparation of media.

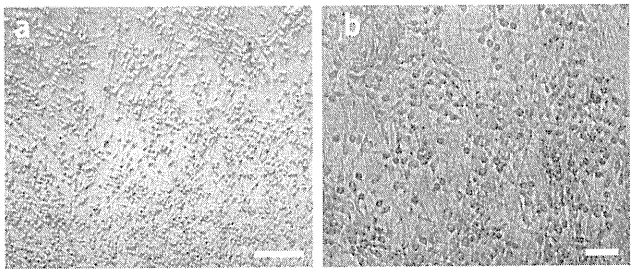

Figure 12 | Morphology of putative photoreceptors differentiated from human ES cells. Many neuron-like cells are observed on day 150 (**a**), although photoreceptors cannot be identified without staining. Magnified image of neuron-like cells (**b**). Scale bar, 100 μm (**a**) or 30 μm (**b**).

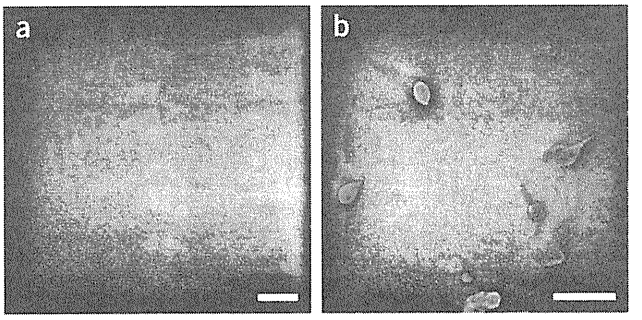

Figure 13 | Photoreceptors derived from human ES cells. Rhodopsin+ cells (red) are observed on day 150 (**a**). Rhodopsin+ cells (red) coexpress recoverin (green) on day 150 (**b**). Scale bar, 20 μm. (Reproduced with permission from ref. 26.)

Glucose solution Dissolve 1 g of glucose in 40 ml of HBSS in a 50-ml test tube. Prepare freshly before the preparation of media.
Blasticidin solution Dissolve blasticidin in dH_2O to a final concentration of 10 mg ml^{-1} and filter using a 0.22-μm filter. Store at 4 °C for up to several weeks or −80 °C for 6 months.
LIF solution Add 50 μl of LIF to 450 μl of PBS.
0.1% (wt/vol) BSA–PBS solution Dissolve 200 mg of BSA in 20 ml of PBS and filter using a 0.22-μm filter to produce a 1% (wt/vol) BSA–PBS. Mix 1 ml of 1% (wt/vol) BSA–PBS and 9 ml of PBS and filter using a 0.22-μm filter. Store at 4 °C for several weeks.
Dkk-1 solution Dissolve 10 μg (one vial) of Dkk-1 in 100 μl of 0.1% (wt/vol) BSA–PBS to produce a 100 μg ml^{-1} solution. Store at 4 °C for up to 1 week or −80 °C for several months.
Lefty-A solution Dissolve 25 μg (one vial) of Lefty-A in 100 μl of 0.1% (wt/vol) BSA–PBS to produce a 250 μg ml^{-1} solution. Store at 4 °C for up to 1 week or −80 °C for several months.
Activin-A solution Dissolve 25 μg (one vial) of Activin-A in 500 μl of 0.1% (wt/vol) BSA–PBS to produce a 50 μg ml^{-1} solution. Store at 4 °C for up to 1 week or −80 °C for several months.
aFGF solution Dissolve 25 μg (one vial) of aFGF in 500 μl of 0.1% (wt/vol) BSA–PBS to produce a 50 μg ml^{-1} solution. Store at 4 °C for up to 1 week or −80 °C for several months.
bFGF solution For mouse ES cells, mix 5 μl of 1 mg ml^{-1} bFGF and 500 μl of 0.1% (wt/vol) BSA–PBS to produce a 10 μg ml^{-1} solution. Store at 4 °C for up to 1 week or −80 °C for several months.
bFGF solution For human ES cells, mix 2.5 μl of 1 mg ml^{-1} bFGF and 500 μl of human ES cell maintenance media to produce a 5 μg ml^{-1} solution. Store at 4 °C for up to 1 week or −80 °C for several months.
Shh solution Dissolve 25 μg (one vial) of Shh in 500 μl of 0.1% (wt/vol) BSA–PBS to produce a 2.5 μM solution. Store at 4 °C for up to 1 week or −80 °C for several months.
Taurine solution Prepare a 50 mM solution in dH_2O and filter using a 0.22-μm filter. Store at 4 °C for up to 1 week or −80 °C for several months.
RA solution Prepare a 100 mM solution in DMSO. Store at 4 °C for up to 1 week or −80 °C for several months. Before use, dilute 100 mM RA solution with differentiation media to produce 100 μM solution.
DAPT solution Prepare a 10 mM solution in DMSO. Store at 4 °C for up to 1 week or −80 °C for several months.
Y-27632 solution Prepare a 10 mM solution in dH_2O and filter using a 0.22-μm filter. Store at 4 °C for up to 1 week or at −80 °C for several months.
Mitomycin C solution Prepare a 1 mg ml^{-1} solution in dH_2O and filter using a 0.22-μm filter. Store at −80 °C for several months.
PI solution Dissolve PI in ethanol at 10 mg ml^{-1}. Store at 4 °C up to 1 week or at −80 °C for several months.
MEF freezing solution Mix 8 ml of DMEM, 1 ml of FBS and 1 ml of DMSO. Prepare freshly before use.

EQUIPMENT SETUP

FACS In our laboratory, the FACSAria (Becton Dickinson) was used for analysis, counting and sorting of GFP-expressing cells. The entire process of cell sorting should be performed at room temperature. Excitation of GFP should be performed at a wavelength of 488 nm and fluorescence emission should be detected using a band pass filter of 530 nm (FITC). Cells should be sorted at low speed (<4,000 cells per s) using a wide nozzle (100 μm). Cell aggregates and PI-positive dead cells should be gated out using forward scatter and side scatter parameters and a band pass filter of 695 nm (PerCP-Cy5.5). Data analysis was performed using FACSDiva software (BD Biosciences).

PROCEDURE

1| Obtain mouse or human ES cells as described earlier[26,45,47].

2| Differentiation of ES cells to retinal cells can be performed using option A for mouse ES cells or option B for human ES cells.

(A) Retinal differentiation from mouse Rx-GFP ES cells (day 0: maintenance and passaging of cells ● TIMING 1.5 h)

(i) To maintain and passage ES cells, aspirate the media from a confluent 100 mm dish of mouse ES cells, then add 9 ml of pre-warmed PBS (37 °C). Remove the PBS, and add 1 ml of pre-warmed (37 °C) 0.25% (wt/vol) trypsin-EDTA and incubate the dish at 37 °C for 5 min.

(ii) Add 2 ml of retinal progenitor differentiation media to the dish, and dissociate the ES colonies into single cells by pipetting with a P1000 Pipetman. Transfer the cell suspension to a 15-ml conical tube.

(iii) To collect the remaining cells in the dish, add 2 ml of retinal progenitor differentiation media to the dish, and transfer the cell suspension into the 15-ml conical tube.

(iv) Centrifuge the 15-ml tube for 3 min at 180*g* at room temperature.

(v) Aspirate the supernatant and add 2 ml of retinal progenitor differentiation media to resuspend the cells.
▲ **CRITICAL STEP** Do not use the maintenance media to resuspend cells for differentiation experiments because the maintenance media contain FBS, which inhibits neural differentiation.

(vi) Mix 50 μl of the cell suspension and 50 μl of 0.4% (wt/vol) Trypan blue and count the number of cells using a cell counter.

BOX 1 | PREPARATION OF MEF FEEDERS

For maintenance of human ES cells, we use freeze-thawed MEF feeders. We treat MEFs with mitomycin C and prepare frozen stocks of the MEFs. One to two days before passage of human ES cells, frozen stocks of mitomycin C-treated MEF are thawed as feeders and should be used within 3 d.

Cyropresevation of mitomycin C-treated MEF feeders

1. Aspirate the media from a confluent 150-mm dish of MEFs. Add 25 ml of MEF media containing mitomycin C (final concentration: 10 μg ml^{-1}), and incubate the dish at 37 °C for 2 h.
2. Wash the dish with 15 ml of pre-warmed (37 °C) DMEM three times.
3. Add 25 ml of MEF media without mitomycin C, and incubate the dish overnight.
4. Aspirate the media, wash the dish with 20 ml of PBS add 5 ml of pre-warmed 0.25% (wt/vol) trypsin-EDTA and incubate the dish at 37 °C for 4 min.
5. Add 5 ml of MEF media into the dish, and dissociate the MEFs into single cells by pipetting. Transfer the cell suspension to a 50-ml conical tube.
6. To collect the remaining cells in the dish, add 10 ml of MEF media to the dish, and transfer the cell suspension into the 50-ml conical tube from Step 5.
7. Mix 50 μl of the cell suspension and 50 μl of 0.4% (wt/vol) Trypan blue, and calculate the total number of cells in the 50 ml tube
8. Centrifuge the 50 ml tube for 3 min at 180*g* at room temperature.
9. Aspirate the supernatant carefully and adjust the cell concentrations to 4.8×10^6 cells per ml with pre-cooled (4 °C) freezing solution.
10. Add 250 μl of the cell suspension to a cryovial.
11. Place the cryovials in a cell-freezing container and transfer the freezing container directly into −80 °C freezer.
12. The next day, transfer the cryovials into −150 °C freezer or liquid nitrogen.

Thawing of MEF feeders

13. 1–2 d before passage of human ES cells, thaw the cryovial in a 37 °C water bath with gentle agitation until most of the ice disappears.
14. Transfer the content of the cryovial to a 50-ml tube containing 20 ml of pre-warmed (37 °C) MEF media.
15. To collect the remaining cells in the cryovial, add 1 ml of MEF media to the cryovial with a P1000 micropipette, and transfer the cell suspension into the 50 ml conical tube from Step 14.
16. After gentle pipetting, centrifuge the 50 ml tube for 3 min at 180*g* at room temperature.
17. Aspirate the supernatant and suspend all the cells with 10 ml of pre-warmed MEF media.
18. Plate the cell suspension onto a gelatin-coated 100 mm dish.
19. Before passage of human ES cells, wash the MEF feeders twice with pre-warmed DMEF/F12 (at Step 2Bviii).

▲ **CRITICAL STEP** Freezing solutions contain DMSO. Higher concentrations of DMSO are toxic to cells. Freezing and thawing of cells should be as quickly as possible.

(vii) Adjust the concentration to 4.7×10^4 cells per ml with maintenance media containing LIF (final concentration: 2.0×10^3 unit ml^{-1}) and Blas (final concentration: 20 μg ml^{-1}).

(viii) Seed 9 ml of the cell suspension onto a gelatin-coated 100-mm dish (4.2×10^5 cells/100 ml dish), and incubate the dish at 37 °C in a humidified atmosphere of 5% CO_2.

(ix) After 2 d, the cells become confluent. Passage the cells using the same procedure described above in Steps A(i–viii).

(x) **Day 0: Start of ES cell differentiation.** Repeat Steps A(i–vi).

(xi) Adjust the cell concentration to 5.6×10^4 cells per ml with retinal progenitor differentiation media containing Dkk-1 (final concentration: 100 ng ml^{-1}) and Lefty-A (final concentration: 500 ng ml^{-1}), and seed 9 ml of the cell suspension onto a Petri dish (5.0×10^5 cells/100 mm dish).

(xii) Incubate the dish at 37 °C in a humidified atmosphere of 5% CO_2 for 72 h.

▲ **CRITICAL STEP** We recommend that the pH of the differentiation media be checked using the color of the phenol red. Old media turn pink or red because of the loss of CO_2, which causes low differentiation efficiency. We recommend using fresh media for differentiation.

? TROUBLESHOOTING

(xiii) **Day 3: Addition of factor and media change.** Observe the dish and confirm the formation of ES cell aggregates (**Fig. 4b**). Collect the media containing floating aggregates into a 15-ml tube.

(xiv) Leave the tube until the aggregates sink down to the bottom of the tube (~15 min).

(xv) Remove half of the media (4.5 ml), and add 4 ml of fresh differentiation media and 450 μl of FBS (final concentration: 5% (vol/vol)).

TABLE 1 | List of useful antibodies and reagents for staining.

Protein	Expression	Maker	No.	Host	Dilution
Oct3/4	Undifferentiated ES cells	BD	611202	Mouse	1:300
Nanog	Undifferentiated ES cells (mouse)	ReproCell	RCAB0001P	Rabbit	1:1,000
	Undifferentiated ES cells (human)	R&D	AF1997	Goat	1:20
TRA-1-60	Undifferentiated ES cells	Chemicon	MAB4360	Mouse (IgM)	1:300
TRA-1-81	Undifferentiated ES cells	Chemicon	MAB438	Mouse (IgM)	1:300
Nestin	Neural progenitors (mouse)	BD	556309	Mouse	1:1,000
	Neural progenitors (human)	Covance	PRB-570C	Rabbit	1:1,000
NCAM	Neurons	Chemicon	AB5032	Rabbit	1:200
βIII-Tubulin	Neurons	Sigma	T8660	Mouse	1:500
	Neurons	Covance	PRB-435P	Rabbit	1:600
Rx	Retinal progenitors	Gift	Dr. Sasai (RIKEN)	Rabbit	1:200
Mitf	Retinal progenitors	Abcam	ab2384	Mouse	1:30
Pax6	Retinal progenitors	DSHB		Mouse	1:200
	Retinal progenitors	Covance	PRB-278P	Rabbit	1:500
Chx10	Retinal progenitors	Exalpha	X1180P	Sheep	1:1,000
Crx	Photoreceptor precursors	Gift	Dr. Sasai (RIKEN)	Rat	1:200
Blue opsin	Cone photoreceptors	Chemicon	AB5407	Rabbit	1:500
Red/green opsin	Cone photoreceptors	Chemicon	AB5405	Rabbit	1:500
Rhodopsin	Rod photoreceptors	Sigma	04886	Mouse	1:2,000
Recoverin	Rod and cone photoreceptors	Chemicon	AB5585	Rabbit	1:2,000
ZO-1	Tight junctions	Zymed	61-7300	Rabbit	1:100
Phalloidin (Alexa488)	F-actin (polygonal morphology)	Invitrogen	A-12379		5 U ml^{-1}
Phalloidin (Alexa546)	F-actin (polygonal morphology)	Invitrogen	A-22283		5 U ml^{-1}

(xvi) Seed the cell suspension onto an MPC-treated dish and place in a 37 °C incubator with 5% CO_2. Culture the cells for 24 h.
▲ **CRITICAL STEP** After addition of FBS, cells become adherent. Therefore, MPC-treated dishes are recommended for floating culture.

(xvii) **Day 4: Addition of factor.** Add 18 μl of Activin-A (final concentration: 10 ng ml^{-1}) to the media and incubate at 37 °C in a humidified atmosphere of 5% CO_2. Culture the cells for 24 h.

(xviii) **Day 5: Media change.** Repeat Steps A(xiii) and (xiv).

(xix) Aspirate the media, and add 9 ml of fresh retinal progenitor differentiation media.

(xx) Plate the cell suspension onto a new MPC-treated dish and place in a 37 °C incubator with 5% CO_2. Culture the cells for 48 h.

(xxi) **Day 7: Media change.** Repeat Steps A(xviii) to (xx) (**Fig. 4c**).

(xxii) **Day 9: FACS analysis.** Repeat Steps A(xiii) and (xiv).

(xxiii) Aspirate the media, add 1 ml of 0.25% (vol/vol) trypsin-EDTA, collect the cells and centrifuge for 3 min at 180*g* at room temperature.

(xxiv) Aspirate the supernatant, add 1 ml of 0.25% (vol/vol) trypsin-EDTA, place the tube at 37 °C for 5 min. Mix the contents gently every 2 min.
▲ **CRITICAL STEP** KSR in the culture media prevent trypsin reaction. Before reaction with 0.25% (vol/vol) trypsin-EDTA, the cells are washed with 0.25% (vol/vol) trypsin-EDTA once to remove KSR.

(xxv) Pipette gently ~20 times to dissociate aggregates into single cells.

(xxvi) Transfer the cell suspension to a 1.5-ml tube, and add 500 μl of DMEM containing 10% (vol/vol) FBS and 6 μl of DNase I (10 μg ml^{-1}). Pipette gently ~10 times.

(xxvii) Centrifuge at for 3 min at 200*g* at 4 °C.

(xxviii) Aspirate the supernatant and add 800 μl of 0.5 μg ml^{-1} PI-containing buffer (9 ml PBS, 1 ml of 1% (wt/vol) BSA–PBS and 5 μl of PI solution).

(xxix) Pass the cell suspension through a cell strainer (35-μm nylon mesh) to remove cell aggregates and DNA debris. Place the tube on ice until FACS analysis.

(xxx) Collect GFP+ cells using a FACSAria (**Fig. 4d**). Count Rx-GFP+ cells with GFP fluorescence and sort using a 530-nm band pass filter (FITC). Exclude cell aggregates and PI-positive cells by adjusting gating parameters for forward scatter and side scatter and using a 695-nm band pass filter (PerCP-Cy5.5). Analyze data using FACSDiva software.

(xxxi) Transfer 2–3 × 10^4 sorted cells to a 1.5-ml tube and centrifuge at for 3 min at 200*g* at 4 °C.
▲ **CRITICAL STEP** The number of reaggregated pellets obtained depends on the differentiation efficiency. Higher efficiency requires preparation of more 1.5-ml tubes of cells.
? TROUBLESHOOTING

(xxxii) Aspirate the supernatant and resuspend the cells in 100 µl of retinal cell differentiation media containing 10% (vol/vol) FBS, and centrifuge for 10 min at 800*g* at room temperature to produce a reaggregation pellet.

(xxxiii) Let the tube sit at 37 °C in a humidified atmosphere of 5% CO_2 for 1 h.
▲ **CRITICAL STEP** Treat the tube gently to avoid the collapse of reaggregation pellets. Do not shake the tube.

(xxxiv) Plate 3–5 reaggregated pellets into one well of an eight-well chamber slide coated with poly-D-lysine, laminin and fibronectin using a wide-tip pipette. Incubate the pellets in retinal cell differentiation media containing 10% (vol/vol) FBS.
▲ **CRITICAL STEP** Use wide-tip pipettes, as narrow-tip pipettes collapse the reaggregation pellets. Survival of single cells is low.

(xxxv) Incubate the slide at 37 °C in a humidified atmosphere of 5% CO_2. Culture for 24 h.

(xxxvi) **Days 10, 12 and 14: Media change.** Aspirate the media gently and add 500 µl of retinal cell differentiation media containing DAPT (10 µM) but not FBS.

(xxxvii) Incubate the slide at 37 °C in a humidified atmosphere of 5% CO_2. Culture for 48 h.

(xxxviii) Repeat Steps A(xxxvi) and (xxxvii).

(xxxix) Repeat Steps A(xxxvi) and (xxxvii).

(xl) **Days 16, 18, 20 and 22: Media change.** Aspirate the media gently and add 500 µl of retinal cell differentiation media containing DAPT (10 µM), aFGF (50 ng ml^{-1}), bFGF (10 ng ml^{-1}), Shh (3 nM), RA (500 nM) and taurine (100 µM).

(xli) Incubate the slide at 37 °C in a humidified atmosphere of 5% CO_2. Culture for 48 h.

(xlii) **Days 24, 26 and 28: Media change.** Aspirate the media gently and add retinal cell differentiation media containing DAPT (10 µM), Shh (3 nM), RA (500 nM) and taurine (100 µM).

(xliii) Incubate the slide at 37 °C in a humidified atmosphere of 5% CO_2. Culture for 48 h.

(xliv) Repeat Steps A(xlii) and (xliii) three times to change the media every other day (**Fig. 5**). Culture for a total of 28 d (**Fig. 6**).
? TROUBLESHOOTING

(B) Retinal differentiation of human ES cells (day 0: maintenance and passaging of cells ● TIMING 1.5 h)

(i) Prepare mitomycin C-treated MEFs as feeder layers for the culture of human ES cells (**Box 1**).

(ii) Aspirate the media from a 100-mm dish of human ES cells and wash with 9 ml of pre-warmed PBS (37 °C). Aspirate the PBS, and add 2 ml of pre-warmed human ES cell dissociation solution.

(iii) Incubate at 37 °C for 5–7 min and tap the dish every 2 min.

(iv) Examine the cells by inverted microscopy to confirm that most ES cell colonies have partially detached from the substratum (**Fig. 7**). Add 2 ml of human ES cell maintenance media to detach ES colonies with gentle pipetting, and transfer the ES cell suspension into a 15-ml tube.

(v) Add 2 ml of human ES cell maintenance media to the dish, detach remaining ES colonies with gentle pipetting and transfer the ES cell suspension into the same 15-ml tube.

(vi) Dissociate the ES colonies into clusters of ~50 cells with gentle pipetting.

(vii) Centrifuge for 5 min at 180*g* at room temperature.

(viii) Meanwhile, prepare three new dishes of MEF feeders for a 1:3 split. Wash the feeders twice with DMEM/F12, add 8 ml of maintenance media and apply 10 µl of bFGF solution (final concentration 5 ng ml^{-1}) to each dish.

(ix) Aspirate the supernatant of the tube of cells from Step B(vii), and add 6 ml of maintenance media to resuspend the ES cells for the 1:3 split.

(x) Dispense 2 ml of the cell suspension into each of the three maintenance media-containing feeder dishes prepared in Step B(viii) (total volume of media in the dish is 10 ml).

(xi) Incubate the dish at 37 °C in a humidified atmosphere of 2% CO_2 and 98% air.

(xii) Change the media daily. Human ES cells should be passaged every 3–4 d.

(xiii) **Day 0: Start of ES cell differentiation.** To a confluent dish of ES cells, apply 10 µl of Y-27632 (final concentration: 10 µM), and incubate the dish at 37 °C for 1 h in a humidified atmosphere of 2% CO_2.

(xiv) Aspirate the media from the dish and add 9 ml of pre-warmed PBS. Aspirate the PBS and add 2 ml of pre-warmed human ES cell dissociation solution. Incubate the dish at 37 °C for 5–7 min.

(xv) Examine the cells by inverted microscopy, and confirm that most ES cell colonies have partially detached from the substratum. Add 2 ml of maintenance media and detach the ES colonies with gentle pipetting. Transfer the ES cell suspension into a 15-ml tube.

(xvi) Dissociate the ES colonies into clusters of ~30–40 cells with gentle pipetting.

(xvii) Add 2 ml of maintenance media to detach the remaining ES colonies, and collect the ES colonies in the same 15-ml tube.

(xviii) Centrifuge for 5 min at 180*g* at room temperature.

(xix) Aspirate the supernatant and resuspend with 9 ml of human ES cell maintenance media containing Y-27632 (10 μM).

(xx) Dissociate the colonies into 3–10 cells by gentle pipetting.

▲ **CRITICAL STEP** Dissociation of human ES cells to single cells markedly decreases cell viability, although Y-27632 protects from dissociation-induced cell death. Pipette gently, as excessive, forceful and repetitive pipetting or formation of air bubbles during pipetting decreases cell viability.

(xxi) Plate the cell suspension onto gelatin-coated dishes to remove the MEFs, and incubate the dish at 37 °C for 1 h.

▲ **CRITICAL STEP** MEFs adhere more quickly to the dish than do human ES cells, so incubating the dish at 37 °C for 1 h will allow the MEFs to settle, while ES cells remain floating.

(xxii) Collect the supernatant with the floating ES cells into a 50-ml tube, and centrifuge for 3 min at 180*g* at room temperature.

(xxiii) Aspirate the supernatant and add 2 ml of maintenance media.

(xxiv) Seed the 50 μl of the cell suspension onto the dish, and count the total number of clumps in 50 μl of the cell suspension without Trypan blue staining.

(xxv) Seed the cells onto MPC-treated non-adhesive dishes at a density of 6,000 cells in 9 ml of media containing Y-27632 (10 μM), Dkk-1 (100 ng ml^{-1}) and Lefty-A (500 ng ml^{-1}). Incubate the dish at 37 °C in a humidified atmosphere of 5% CO_2.

▲ **CRITICAL STEP** We recommend that the pH of the differentiation media be checked on the basis of the color of the phenol red. Old media turn pink or red because of the loss of CO_2, which causes low differentiation efficiency. We recommend using fresh media for differentiation.

? TROUBLESHOOTING

(xxvi) **Day 3: Media change with 20% (vol/vol) KSR differentiation media.** Collect the supernatant in a 50-ml tube and centrifuge for 3 min at 180*g* at room temperature.

(xxvii) Discard the upper half of the media and add 4.5 ml of 20% (vol/vol) KSR differentiation media containing Y-27632 (10 μM), Dkk-1 (100 ng ml^{-1}) and Lefty-A (500 ng ml^{-1}).

(xxviii) Plate the cell suspension onto a new MPC-treated dish and incubate the dish at 37 °C in a humidified atmosphere of 5% CO_2. Culture the cells for 72 h (**Fig. 8a**).

▲ **CRITICAL STEP** We recommend that the pH of the differentiation media be checked on the basis of the color of the phenol red. Old media turn pink or red because of the loss of CO_2, which causes low differentiation efficiency. We recommend using fresh media for differentiation.

? TROUBLESHOOTING

(xxix) **Days 6, 9 and 12: Media change with 15% KSR differentiation media.** Collect the supernatant in a 15-ml tube and centrifuge for 3 min at 180*g* at room temperature.

(xxx) Discard the upper half of the media and add 4.5 ml of 15% (vol/vol) KSR differentiation media containing Y-27632 (10 μM), Dkk-1 (100 ng ml^{-1}) and Lefty-A (500 ng ml^{-1}).

(xxxi) Plate the cell suspension in a new MPC-treated dish and incubate the dish at 37 °C in a humidified atmosphere of 5% CO_2. Culture the cells for 72 h.

(xxxii) Repeat Steps B(xxix–xxxi) twice.

? TROUBLESHOOTING

(xxxiii) **Days 15 and 18: Media change with 10% KSR differentiation media.** Collect the supernatant in a 50-ml tube and let the tube stand for 20 min.

▲ **CRITICAL STEP** Confirm that the aggregates sink down to the bottom of the tube.

(xxxiv) Discard the upper half of the media and add 4.5 ml of 10% (vol/vol) KSR differentiation media containing Dkk-1 (100 ng ml^{-1}) and Lefty-A (500 ng ml^{-1}).

(xxxv) Plate the cell suspension onto a new MPC-treated dish and incubate the dish at 37 °C in a humidified atmosphere of 5% CO_2. Culture the cells for 72 h (**Fig. 8b**).

(xxxvi) Repeat Steps B(xxxiii–xxxv).

? TROUBLESHOOTING

(xxxvii) **Day 21: Seed the aggregates onto poly-D-lysine/laminin/fibronectin-coated dishes.** Repeat Steps B(xxxiii) and (xxxiv).

(xxxviii) Transfer the aggregates to poly-D-lysine/laminin/fibronectin-coated chamber slides at 10–20 aggregates per well.

(xxxix) Incubate the slides at 37 °C in a humidified atmosphere of 5% CO_2. Culture the cells for 72 h.

(xl) **Days 24–40: Media change every other day.** Remove the media gently.
(xli) Gently add 500 µl of fresh 10% (vol/vol) KSR differentiation media to each well.
(xlii) Incubate the dish at 37 °C in a humidified atmosphere of 5% CO_2 for 48 h (**Fig. 9**).
(xliii) Repeat Steps B(xl–xlii) nine times so that the cells are cultured for a total of 40 d.
? TROUBLESHOOTING
(xliv) **Days 41–90: Media change every day.** Gently remove the media from the 40 d ES cells, and add 500 µl of fresh 10% KSR differentiation media to each well.
(xlv) Incubate the dish at 37 °C in a humidified atmosphere of 5% CO_2.
(xlvi) Repeat Steps B(xliv) and (xlv) every 24 h so that the cells have been cultured for another 50 d (total 90 d of culture).
? TROUBLESHOOTING

3| Human ES Cells can be differentiated into RPE cells using option A or into photoreceptor cells using option B.

(A) Differentiation into RPE cells (days 91–120: media change every day ● TIMING 1 h plus 30 d of culture)
(i) Gently remove the media from the 91 d ES cells and add 500 µl of fresh 10% KSR differentiation media to each well.
(ii) Incubate the dish at 37 °C in a humidified atmosphere of 5% CO_2.
(iii) In order to obtain mature RPE cells, repeat Steps 3A(i) and (ii) every 24 h so that the cells have been cultured for another 30 d (total 120 d of culture) (**Figs. 10** and **11**).
? TROUBLESHOOTING

(B) Differentiation into photoreceptor cells (days 91–150: Media change every day ● TIMING 1 h plus 60 d of culture)
(i) Gently remove the media from the 91 d ES cells and add 500 µl of photoreceptor differentiation media containing retinoic acid (100 nM) and taurine (100 M).
(ii) Incubate the dish at 37 °C in a humidified atmosphere of 5% CO_2.
(iii) Repeat Steps 3B(i) and (ii) every day for another 60 d (total 150 d of culture) (**Figs. 12** and **13**).
? TROUBLESHOOTING

● TIMING
Step 2(A) Differentiation of mouse ES cells: 28 d
Step 2(B) Differentiation of human ES cells: 90 d
Step 3(A) Differentiation of human ES cells into RPE cells: 30 d (total 120 d)
Step 3(B) Differentiation of human ES cells into photoreceptor cells: 60 d (total 150 d)

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2.**

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Possible solution
2A(xii)	Mouse ES cells do not form aggregates	Use of wrong media and reagents. Initially, ES cells were not normal and did not retain pluripotency	Ensure proper preparation of media and reagents. Media, KSR and supplements must be fresh. Check the pluripotency by RT-PCR for pluripotency factors or by immunostaining for Oct3/4 and Nanog. Use a new frozen stock of ES cells at an early passage number
2A(xxxi)	The number of the FACS-sorted cells is low	Differentiation efficiency is low	Ensure the media and reagents. Media, KSR and supplements must be fresh. Check the pluripotency by RT-PCR for pluripotency factors or by immunostaining for Oct3/4 and Nanog. Use a new frozen stock of ES cells at early passage number. Try a different ES cell line
		Cell viability is low. Repetitive pipetting force is too high during dissociation. Formation of air bubbles during repetitive pipetting. Cell viability gradually decreases with increased sorting time	Pipetting should be gentle. Sorting duration should be <4 h

(continued)

TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reason	Possible solution
2B(xxv), (xxviii), (xxxii) and (xxxvi)	Human ES cells do not form aggregates Low survival in floating cultures	Repetitive pipetting force is too high during dissociation. Formation of air bubbles during repetitive pipetting. Too many cells or too many colonies plated	Ensure the preparation of media and reagents. Pipetting should be gentle. Add Y-27632 at a higher concentration and/or for a longer period of time. Optimize the density by serial dilution of cells or colonies
2B(xliii), (xlvi), 3A(iii) and 3B(iii)	Low survival in cell cultures after replating	Culture surface is not adequate. Too many colonies plated	Use cell culture grade glass slides and coat them with poly-D-lysine, laminin and fibronectin. Ensure preparation of media and regents. Optimize the density by serial dilution of colonies
2A(xliv), 3A(iii) and 3B(iii)	ES cells do not differentiate into retinal cells	Initially, ES cells were not normal and did not retain pluripotency. Use of old media and reagents	Check for the generation of retinal progenitors. Expression of retinal progenitor markers should be examined on days 8–10 and days 30–40 in mouse and human ES cells, respectively Ensure that the media and reagents support self-renewal. Media, KSR and supplements must be fresh. Check the pluripotency by immunostaining for Oct3/4, Nanog, TRA-1-60 and TRA-1-81, or by RT-PCR for pluripotency factors Use a new frozen stock of ES cells at early passage number. Try a different ES cell line

ANTICIPATED RESULTS

When differentiating mouse ES cells (**Fig. 3a**), under optimal conditions, 10–20% of cells become Rx-GFP+, as evaluated by FACS (**Fig. 4**). Expression of the retinal progenitor markers Rx, Mitf and Pax6 are detected between days 8 and 12, with 20–30% of colonies positive for Rx/Pax6 (neural retina progenitors) or Mitf/Pax6 (RPE progenitors) (**Fig. 2**). For mature phenotypes, 25–35% of sorted cells are positive for rhodopsin, red/green opsin or blue opsin in mouse ES cell cultures between days 28 and 40 (**Figs. 2** and **6**). Under the same conditions, Mitf+ RPE cells are observed in 20–30% of sorted cells.

For human ES cells (**Fig. 3b**), expression of Rx, Mitf and Pax6 is detected between days 30 and 45, with 20–30% of colonies positive for Rx/Pax6 or Mitf/Pax6 (**Figs. 2, 8** and **9**). Prolonged culture generates mature RPE cells at 25–35% of total colonies between days 50 and 120, as determined by staining for Mitf, ZO-1 and F-actin. Pigmentation is observed starting at ~40 d. More mature RPE cells with a polygonal shape require longer culture (more than 90 d) (**Figs. 2, 10** and **11**). For photoreceptor differentiation, addition of RA and taurine induces 10–20% of cells to become positive for rhodopsin, red/green opsin or blue opsin between days 120 and 200 (**Figs. 2, 12** and **13**). These cells express genes responsible for phototransduction in rods and/or cones.

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Generation of retinal cells from mouse and human induced pluripotent stem cells

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ABSTRACT

We previously reported a technique for generating retinal pigment epithelia (RPE) and putative photoreceptors from embryonic stem (ES) cells. Here we tested whether our procedure can promote retinal differentiation of mouse and human induced pluripotent stem cells (iPSCs). Treating iPSCs with Wnt and Nodal antagonists in suspension culture induced expression of markers of retinal progenitor cells and generated RPE cells. Subsequently, treatment with retinoic acid and taurine generated cells positive for photoreceptor markers in all but one human cell lines. We propose that iPSCs can be induced to differentiate into retinal cells which have a possibility to be used as patient-specific donor cells for transplantation therapies.

Retinal degenerative diseases cause photoreceptor cell death and severely impair vision. Transplantation of retinal photoreceptors or RPE cells is a potential treatment to restore visual function. Although embryonic stem (ES) cells are potential donor cells for cell transplantation, clinical applications for these cells have drawbacks such as immune rejection. Induced pluripotent stem cells (iPSCs) are an alternative source of the donor cells. These ES-like cells are generated by reprogramming somatic cells through the retroviral activation of ES cell specific factors such as the four factors Oct3/4, Sox2, Klf4, and c-Myc and raise the possibility of treating patients with their own iPS cell-derived retinal cells which may resolve the problem of immune rejection [12,15,16].

Previously, we generated putative photoreceptors and RPE cells from rodent and primate ES cells using step-wise induction with defined factors [6,13]. During development of the vertebrate eye, the transcription factor gene Rx is expressed in progenitors of the neural retina [4,9], whereas the basic-helix-loop-helix-zipper transcription factor gene Mitf is expressed in the presumptive RPE [11]. At a later stage of differentiation in the neural retina, photoreceptor precursors express the homeobox gene Crx [2,5]. Finally, mature photoreceptors express recoverin and rhodopsin. ES cells differentiating into retinal cells *in vitro* also express this progression of retinal markers [6,13]. Here we tested whether iPSCs can be differentiated into retinal progenitors, RPE and photoreceptors with the same procedure used for ES cell differentiation.

All experiments in this study were conducted using mouse Nanog-iPS cell line iPS-MEF-Ng-20D-17 which were induced from mouse embryonic fibroblasts by retroviral transfection of Oct3/4, Sox2, Klf4, and c-Myc [12], human iPS cell lines 201B6 and 201B7 which were induced from human dermal fibroblasts by the transfection of OCT3/4, SOX2, KLF4 and c-MYC [16] and 253G1 which were induced from human dermal fibroblasts by the transfection of OCT3/4, SOX2 and KLF4 [10]. Cell culture of iPSCs was according to previously described methods [12,15,16].

Differentiation of iPSCs by serum-free embryoid body-like (SFEB) culture was performed according to previously described method [6,13,18]. Briefly, 5×10^4 cells/ml of iPSCs were cultured in 100 mm low cell binding dishes (Nalge NUNC international, Rochester, NY) with ES differentiation medium [G-MEM (GIBCO) containing 5% KSR, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, 50 units/ml penicillin, and 50 μg/ml streptomycin]. Recombinant Dkk-1, a Wnt antagonist, (100 ng/ml, R&D Systems, Minneapolis, MN) and recombinant Lefty A, a Nodal antagonist (500 ng/ml, R&D Systems) were added to the medium during the suspension culture. Floating cells that formed aggregates were plated on poly-D-lysine, laminin and fibronectin-coated slides. Cells were then harvested for subsequent analysis. To compare mouse ES and iPS cells, the mouse ES cell line RF8 was maintained and differentiated in cultures identical to those described above. After determining the method for induction of $Rx^+/Pax6^+$ retinal progenitors, the duration of adherent culture

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