Mini Review

Control of neural differentiation from pluripotent stem cells

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Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of blastocyst stage embryos that can maintain an undifferentiated state indefinitely and differentiate into derivatives of all three germ layers: the ectoderm, endoderm and mesoderm. In addition, induced pluripotent stem (iPS) cells are generated by reprogramming somatic cells through the retroviral gene transfer of four factors (Oct3/4, Sox2, Klf4, and c-Myc). Here we summarize *in vitro* neural differentiation of pluripotent stem cells and their differentiated progeny, with a special emphasis on extracellular patterning signals for regional specification in the developing central nervous system. We have reported two methods that potently induce neural differentiation from ES cells: stromal cell-derived inducing activity (SDIA) method and serum-free floating culture of embryoid body-like aggregates (SFEB) method. SDIA- or SFEB-treated ES cells generate naive precursors that are competent to differentiate into neuroectodermal derivatives along the rostral-caudal and dorsal-ventral axes in response to patterning signals. By modifying the SFEB method with patterning factors, we have induced retinal cells from ES cells. These findings indicate the potential of the pluripotent stem cell culture system to be used for basic and medical researches.

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Introduction

The first differentiation event in human embryos occurs at approximately five days after fertilization, when the outer layer of future placental cells, the trophectoderm, separates from the inner cell mass (ICM). ICM cells have the potential to generate any cell type of the body, but after implantation, they are quickly depleted as they differentiate into other cell types with more limited developmental potential. However, if ICM cells are removed from their normal embryonic environment and cultured under appropriate conditions, the resulting embryonic stem (ES) cells can continue to proliferate indefinitely while maintaining their pluripotency[1,2].

The pluripotency of ES cells may permit their widespread use for replacing or restoring tissues damaged by disease or injury[3].

Some important human diseases are caused by the death or dysfunction of one or a few cell types, such as insulin-producing cells in diabetes, dopaminergic neurons in Parkinson's disease, and photoreceptor cells in retinitis pigmentosa. The treatment strategy of cell replacement therapy is not new; dopaminergic neurons and retinal cells derived from fetal tissues have been transplanted into the adult striatum and eye, but because fetal tissues are limited in supply and ethically problematic, many efforts have been made to find alternative cell sources. Recent progress in the *in vitro* culture and differentiation of human ES cells has raised the possibility of using ES cell derivatives for cell transplantation therapy.

In addition, the differentiation of ES cells provides a model system for studying early events in human development[4,5]. Due to possible harm to the resulting child, experimental manipulation of post-implantation human embryos is ethically unacceptable. Moreover, since human embryos differ significantly from mouse embryos, particularly in the formation, structure, and function of the fetal membranes and placenta and the formation of an embryonic disc instead of an egg cylinder[6], mice can serve in only a limited capacity as a model system for understanding the developmental events that support the initiation and maintenance of human pregnancy. Human ES cells provide a new *in vitro* model that will improve our understanding of the differentiation of human tissues, and thus provide important insights into such phenomena as infertility, pregnancy loss, and birth defects. In addition, ES cells may be used to study the mechanisms of neural differentiation and the genetic and environmental signals that direct the specialization of precursor cells into particular cell types[7].

ES cell culture system as an *in vitro* model for CNS development

During vertebrate embryogenesis, nervous tissue arises from uncommitted ectoderm during gastrulation. Subsequently, the central nervous system (CNS) anlage is patterned to acquire regional specification along the rostral-caudal and dorsal-ventral axes. At an early phase of rostral-caudal specification, the neural tube is subdivided into the forebrain (telencephalon and diencephalon), midbrain, hindbrain, and spinal cord. Classical experiments in amphibian embryology suggest a two-step model for rostral-caudal specification in which naive CNS progenitors acquire rostral identities following neural induction and then receive secondary "caudalizing" signals. Recent molecular studies in Xenopus have also provided evidence supporting this model[8-10]. Neural inducers, such as the soluble factors Chordin and Noggin, cause differentiation of naive animal cap ectodermal cells into neural tissues with rostral character[8,9,11], and treatment of neuralized animal caps with retinoic acid, FGF, and Wnt cause induction of caudal CNS markers.

However, comparatively little is known about the mechanisms of neural induction and CNS regional specification in mammals, in part because good experimental systems for *in vitro* neural differentiation comparable to the animal cap assay commonly used in Xenopus studies are still lacking in mice. Disruption of the *Wnt3* gene in mice causes defects in posterior body structures[12], while embryos null for the Wnt antagonist *Dkk-1* exhibit anterior truncations[13]. In these cases, however, it is not possible to distinguish direct effects on CNS development from secondary effects caused by mesodermal defects.

Mammalian ES cells can differentiate into all embryonic cell types when injected into blastocyst-stage embryos[14]. This pluripotency can be recapitulated *in vitro* by floating culture of ES cell aggregates, or embryoid bodies (EBs)[15]. EBs frequently contain ectodermal, mesodermal, and ectodermal derivatives, but must undergo selective differentiation to generate neurons. We have reported two efficient *in vitro* systems for neural differentiation of ES cells: the stromal cell-derived inducing activity (SDIA) method[16] and the serum-free floating culture of embryoid body-like aggregates (SFEB) method[17]. In these methods, ES cells differentiate into neural precursors at $>90\%$ efficiency, while the rest of cells are mostly E-cadherin$^+$, non-neural ectodermal cells. Differentiation of SDIA- or SFEB-treated ES cells *in vitro* reasonably mimics the natural course of *in vivo* neurogenesis as judged by the temporal expression of molecular markers and the temporal requirement of patterning signals. Importantly, no significant generation of mesodermal cells is detected in these cultures. These observations indicates that SDIA and SFEB culture serve as an *in vitro* model system to analyze neural induction, CNS patterning, and neural differentiation in mammals.

Neural induction of ES cells

In isolated Xenopus ectoderm (animal caps), molecules such as Noggin, Chordin, and Follistatin induce neural differentiation by binding to and inactivating bone morphogenetic protein 4 (BMP4), which suppresses neural differentiation and ventralizes mesoderm. However, neither transfection of pCMV-Chordin plasmid nor addition of neutralizing BMPR-Fc antibody to culture medium causes significant neural differentiation of mouse ES cells, indicating that attenuation of BMP signaling is not sufficient to induce neural differentiation in mouse ES cells[16]. On

the other hand, exogenous application of BMP4 protein efficiently suppresses *in vitro* neural differentiation of mouse ES cells. These results indicate that blockade of BMP signaling is required but not sufficient for neural differentiation of undifferentiated mouse ES cells.

By using a co-culture system, we have established an efficient method of inducing selective neural differentiation of ES cells under serum-free, retinoic acid-free conditions[16]. In the SDIA system, PA6 stromal feeder cells promote neural differentiation of ES cells, and are particularly efficient at producing midbrain TH+ dopaminergic neurons[16,18]. This induction does not require embryoid body formation or retinoic acid treatment[19,20]. When BMP4 is added at an early stage of SDIA culture, ES cells differentiate into non-neural epidermal ectoderm. Importantly, mesodermal induction does not occur in these conditions. A possible function of SDIA is to promote differentiation of ES cells into ectodermal cells that adopt a default neural fate unless they receive a considerable level of BMP signaling. The molecular nature of SDIA remains to be elucidated, but it would be interesting to discover whether SDIA-related activities play a role in neural induction *in vivo*.

Positional identity of ES cell-derived neural cells

After neural induction, the CNS anlage acquires regional specification along the rostral-caudal and dorsal-ventral axes. Thus, we examined the positional identity of ES cell-derived neural cells[21-23]. RT-PCR analyses with rostral-caudal CNS markers showed that SDIA-treated ES cells express the forebrain marker Otx2, the midbrain-hindbrain border marker En2, and the rostral hindbrain marker Gbx2. In contrast, little expression was detected for the spinal cord markers Hoxb4, Hoxb9, and HB9. The rostral-caudal specification of SDIA-induced neural cells could be modified by adding the caudalizing factor retinoic acid. Treatment with retinoic acid (0.2 μM, days 4-9) promotes the expression of caudal CNS markers such as the hindbrain marker Gbx2 and the spinal cord markers Hoxb4, Hoxb9, and HB9, whereas the forebrain marker Otx2 was suppressed. In addition, we examined positional markers along the dorsal-ventral axis. SDIA treatment induces both dorsal (Pax7 and Dbx1) and ventral (Irx3 and HNF3β) neural tube markers. The ventral-most neural tube markers Nkx6.1, Nkx2.2, and HNF3β are also expressed in SDIA-treated cells. Thus, SDIA-induced neural precursors differentiate into a wide range of CNS cell types that correlate with their positions along the dorsal-ventral and rostral-caudal axes.

The neural crest arises from the juncture of the dorsal CNS and nonneural ectoderm[24], where a number of BMP family members are expressed. Although BMP signals inhibit neural induction at the early gastrula stage, the same signals promote neural crest formation when applied at later developmental stages[24,25]. Consistent with these *in vivo* events, late BMP4 exposure after the fourth day of SDIA treatment causes differentiation of neural crest cells and dorsal-most CNS cells, with autonomic nervous tissue preferentially induced by high BMP4 concentrations and sensory lineages by low BMP4 concentrations. Moreover, early exposure of SDIA-treated ES cells to BMP4 suppresses neural differentiation and promotes formation of epidermal cells. In contrast, sonic hedgehog (shh) suppresses the development of dorsal tissues and promotes the differentiation of ventral CNS tissue *in vivo*[25]. Consistent with this activity, shh suppresses differentiation of dorsal cells, including AP2+/NCAM+ neural crest cells, and increases the number of ventral cells, including Nkx2.2+ cells, HNF3β+ floor plate cells, and motor neurons.

We have also established a serum-free, feeder-free culture system called SFEB that induces efficient neural differentiation from ES cells[17]. In the presence of Wnt antagonist (Dkk-1), SFEB efficiently induces the formation of Bf1+ telencephalic precursors (Fig.1). Subregional specification of the telencephalon can be reproduced *in vitro* using embryologically relevant patterning molecules. Wnt inhibits neural differentiation and forebrain development at earlier stages[13,26,27], but positively regulates pallial telencephalic specification at later developmental stages[28-30]. In contrast, shh has been implicated in the ventral specification of the forebrain[31-33]. Consistent with these activities, treatment with Wnt3a or shh during late SFEB culture increases differentiation into the pallial (Pax6+, Bf1+) or basal (Nkx2.1+, Islet1/2+, Bf1+) telencephalic population, respectively (Fig.1). Moreover, caudal CNS tissues such as Math1+ cerebellar neurons are induced from ES cells by SFEB culture followed by BMP4/Wnt3a treatment[34]. The induced Math1+ cells are mitotically active and express markers characteristic of granule cell precursors (Pax6, Zic1, and Zipro1). L7+/Calbindin-D28K+ Purkinje cells are also induced under similar culture conditions. These observations indicate that SDIA- or SFEB-treated ES cells generate naive precursors that are competent to differentiate into the rostral-caudal and dorsal-ventral ranges of neuroectodermal derivatives in response to patterning signals.

Eye development

The eye is derived from three types of tissue during embryogenesis: the neural ectoderm gives rise to the retina and the reti-

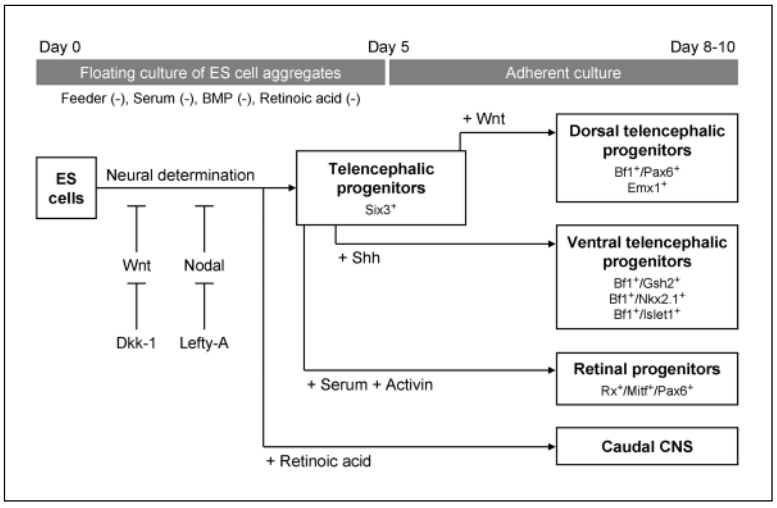

Fig.1 Directed differentiation of ES cells into various neural progenitors in response to patterning signals

ES cells differentiate into the rostral-caudal and dorsal-ventral ranges of neuroectodermal derivatives in response to patterning signals. SFEB and soluble factor treatment induce telencephalic, retinal, cerebellar and caudal neural progenitors.

nal pigment epithelium (RPE), the mesoderm produces the cornea and sclera, and the lens originates from the surface ectoderm. During embryogenesis, the eye develops as a result of interactions between the surface ectoderm and the optic vesicle, an evagination of the diencephalon (forebrain) (Fig.2A). The optic vesicle is connected to the developing CNS by a stalk that later becomes the optic nerve. Upon contacting the surface ectoderm, the optic vesicle epithelium forms a lens placode (Fig.2B), which subsequently invaginates, pinches off, eventually and becomes the lens. During these events, the optic vesicle folds inward to form a bilayered optic cup (Fig.2C,D). The outer layer of the optic cup differentiates into the RPE, whereas the inner layer differentiates into the neural retina (Fig.2E). The iris and ciliary body develop from the peripheral edges of the retina. The sclera is derived from mesenchymal cells of neural crest origin, which migrate to form the cornea and trabecular meshwork of the anterior chamber of the eye (Fig.2F,G).

Within the neural retina, seven types of retinal cells are differentiated from common progenitors in the following temporal sequence: retinal ganglion cells, cone photoreceptors, amacrine cells, and horizontal cells, followed by rod photoreceptors, bipolar cells, and Müller glia (Fig.3). These cells comprise three cell layers: rod and cone photoreceptors in the outer nuclear layer; horizontal, bipolar, and amacrine cells and Müller glia in the inner nuclear layer; and ganglion and displaced amacrine cells in the ganglion cell layer (Fig.2H). During retinal development, the retinal progenitors change their competency under the control of intrinsic regulators (such as transcription factors) and extrinsic regulators (such as neurotrophic factors)[35,36]. Fate-committed cells migrate to fixed positions throughout the laminated retina and establish synaptic connections to other neurons. Synapse formation proceeds in a centrifugal manner, from the inner to the outer retina. It occurs first in horizontal connections within the plexiform layers, followed by vertical connections between layers.

Retinal specification of ES cells

As discussed above, ES cells differentiate into a variety of neural cells with specific spatio-temporal identities. In response to exogenous patterning signals such as Wnt, shh, BMP4, and RA, ES cell-derived neural progenitors differentiate into a wide range of neural cells that correlate with their positions along the dorsal-ventral and rostral-caudal axes. We therefore asked whether ES cells differentiate into retinal cells by a combination of exogenous patterning factors[37].

In the early embryogenesis, the retinal primordia form within the rostral-most diencephalic region expressing Six3[38]. The transcription factor Rx, an early marker of the eye field, plays an essential role in the specification of the retinal primordium within the Six3+ rostral CNS[39,40]. During early embryogenesis (E10.5), Rx expression coincides with Pax6 expression in neural retinal progenitors, whereas Rx+ cells in the floor of the ventral diencephalon are Pax6+ (Fig.4A-C). The retinal pigment epithelium (RPE) is Rx- and Pax6+ (Fig.4A-C). Thus, the neural retinal lineage during early development is characterized by Rx/Pax6 coexpression.

To test the suitability of the SFEB-based approach for the induction of retinal tissues from ES cells, we first examined the expression of the rostral-most CNS marker Six3[38]. On culture day 5, strong expression of Six3 was found in SFEB-treated ES cell aggregates cultured in the absence or presence of Dkk-1 (100 ng/ml) plus Lefty-A (500 ng/ml) (in 82% or 87% of aggregates,

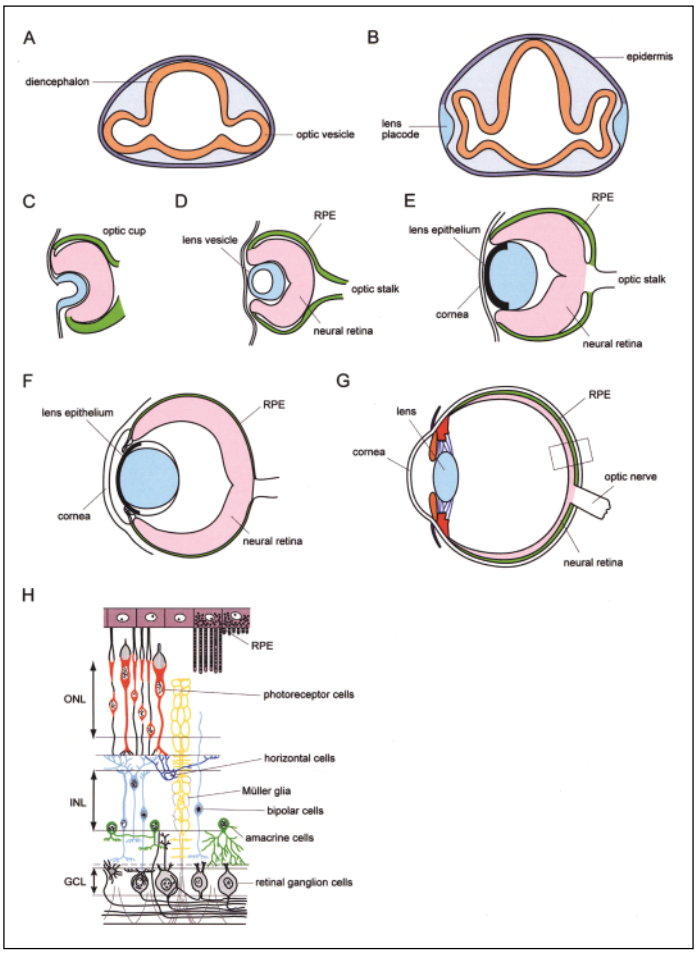

Fig.2 Development of the eye

A-F: Mouse embryos at E9.5 (A), E10.0 (B), E10.5 (C), E11.5 (D), E13.5 (E) and E18.5 (F). G: Adult eyes. H: Cell types and layers in the adult retina. RPE: retinal pigment epithelium, ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer. Modified from Figure 1 of Experimental Medicine, 24: 256-262, 2006. (Osakada F and Takahashi M).

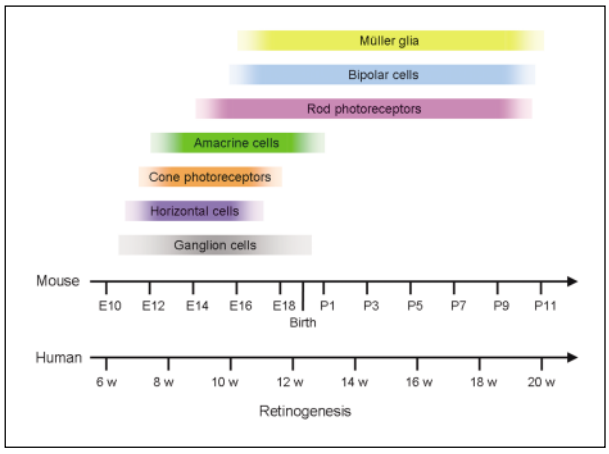

Fig.3 Genesis of seven types of retinal cells during development

Retinal ganglion cells and horizontal cells differentiate first, followed by cone photoreceptors, amacrine cells, rod photoreceptors, bipolar cells and, finally Müller glia, with overlap in the appearance of these different cell types. The sequence of cell genesis in the vertebrate retina is highly conserved among many species.

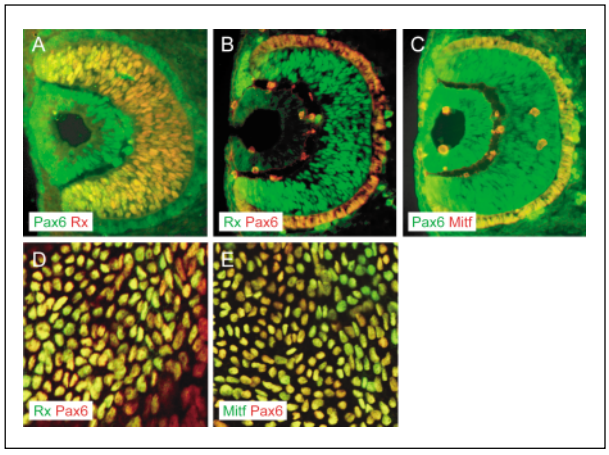

Fig.4 Differentiation of ES cells into retinal progenitors in the optic cup

A, B, C: E10.5 mouse embryos immunostained with anti-Rx, anti-Mitf and anti-Pax6 antibodies. D: Generation of Rx+/Pax6+ neural retina progenitors from ES cells. E: Generation of Mitf+/Pax6 RPE progenitors from ES cells.

respectively), but not in those cultured with the caudalizing factor RA (0.2 μM, during days 3-5). Because retinal progenitors arise from the Six3+ rostral CNS tissue, we attempted to induce Rx+/Pax6+ cells from SFEB-induced neural precursors by modifying the culture conditions. Because the extracellular patterning signals that determine the induction of retinal primordia in the embryo have not yet been elucidated, we experimentally searched for soluble factors that induce Rx/Pax6 expression by testing a number of candidate factors for such activities. The strongest enhancement was seen when cells were treated with 5% fetal bovine serum (FBS) during days 3-5. Furthermore, Activin-A treatment (100 ng/ml) during days 4-6 increased the induction (28.3 + 5.0% of colonies) when combined with Dkk-1, Lefty-A, and FBS treatment. This positive effect may be relevant to the essential role of Smad2 in eye development reported previously[41]. The signaling mechanism of FBS and Activin-A and their *in vivo* relevance to the induction of the retinal primordia should be interesting in future investigation. We also tested

the effects of shh, Wnt, BMP4, Nodal (without Lefty-A), IGF, FGF-1, FGF-2, and FGF antagonists during days 3-6, but observed only marginal effects, if any, on Rx induction in this culture system. Hereafter, cells treated with SFEB and Dkk-1, Lefty-A, FBS and Activin-A are referred to as SFEB/DLFA cells.

Next, we further analyzed the nature of the SFEB/DLFA-induced Rx+ cells by examining the expression of multiple eye markers. Consistent with the *in vivo* profile, the induced Rx+ cells coexpressed Pax6 (Fig.4D). In the embryo, Otx2 is expressed in both layers of the optic cup; however, Otx2 is coexpressed with Rx in the embryonic neural retina, whereas the RPE is Rx-/Otx2+. Consistent with the *in vivo* coexpression, nearly all Rx+ cells in SFEB/DLFA cells were Otx2+. In the early neural retina (E10.5), most Rx+ cells are proliferating progenitors, which are positive for the mitotic marker Ki67. Similarly, Rx+ cells in SFEB/DLFA cells were Ki67+, and positive for BrdU uptake, indicating that Rx+ cells produced by SFEB/DLFA are mitotically active. Collectively, a high proportion of Rx+ cells produced by SFEB/DLFA exhibit characteristics consistent with those of progenitors in the developing neural retina.

On the other hand, another component of the retina, the RPE (the outer layer of the optic cup) expresses Mitf, Pax6 and Otx2, but not Rx, during retinal development (Fig.4B,C)[42-44]. We therefore asked whether differentiation of the RPE was observed in the SFEB/DLFA culture, and examined the expression of the early RPE marker Mitf. In the SFEB/DLFA culture, Mitf+ cell aggregates were observed (17.1 + 3.7% of colonies), 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+ (Fig.4E). Rx+ cells were frequently found in the close vicinity of Mitf+ cell clusters. None of the Mitf+ cells on day 12 coexpressed Rx. These findings show that the SFEB/DLFA treatment preferentially induces differentiation of retinal progenitors from ES cells (Fig.1).

Perspectives

Several methods of controlling differentiation of ES cells into neural cells have been developed over the past few years[16,17,20,23,45-48]. The different methods induce the differentiation of neural tissues with distinct characteristics, with regard to their regional identities in the CNS[17,21,23,45]. Given the potential clinical application of stem cell therapy, xenogenic material-free methods are desired. Indeed, human ES cells cultured on mouse feeder cells express an immunogenic non-human sialic acid on their surfaces[49]. Thus, we need to establish a method of generating retinal cells under chemically defined culture conditions, without contamination from other animal products. Moreover, recent research has succeeded in producing induced pluripotent stem (iPS) cells from somatic cells[50-53]. Four transcription factors (Oct4, Sox2, Klf4 and c-Myc) reprogram fibroblasts into pluripotent cells equivalent to ES cells. In addition to their similarity in pluripotency, differentiation methods for ES cells are also applicable to iPS cells. These findings raise the possibility of treating patients using their own iPS cell-derived retinal cells, as well as of the discovery of therapeutic drugs[54]. Finally, to use cell transplantation therapy to treat retinal degeneration, we must purify retinal cells derived from human ES cells or iPS cells, transplant them in animal models closely resembling human diseases, and determine their efficacy and safety[55,56].

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