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Biochemical and Biophysical Research Communications 332 (2005) 241–247
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Photoreceptor cells from mouse ES cells by co-culture with chick embryonic retina

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Received 30 March 2005
Available online 30 April 2005

Abstract

Degeneration of photoreceptors is a consistent and common endpoint in retinal diseases. Herein, we report the efficient induction of photoreceptor-like cells from mouse embryonic stem (ES) cells using chick embryonic retina tissue. Undifferentiated mouse ES cells were initially cultured in a preferential condition into a neural lineage, and ES cells were then co-cultured with chick embryonic day 6 (E6) retina tissues. After a 10-day co-culture, approximately 20% of the mouse ES derivatives became immuno-positive for rhodopsin. RT-PCR analysis demonstrated expression of the transcription factor crx and a distinct increase of rod photoreceptor-specific markers, IRBP and recoverin, after the start of the co-culture. These results indicate that co-culture of ES cells with chick embryonic retina tissue is a useful and efficient method for the induction of photoreceptor-like cells.

Keywords: ES cells; Photoreceptor cells; Chick embryonic retina; Differentiation

Degeneration of photoreceptors is a consistent and common endpoint in retinal diseases such as retinitis pigmentosa (RP) and age-related macular degeneration (AMD) [1,2]. Currently, no effective medical therapy is available for RP and AMD patients, though one of the treatment strategies proposed for retinal regeneration is to re-supply the type of cells that were damaged or lost with progenitor or stem cells. Although there are several candidate cell sources for such cell therapy, including neural stem cells (NSCs) in adult brains [3–5] and mesenchymal stem cells in bone marrow and other organs [6–8], an alternative source is embryonic stem (ES) cells.

Mouse ES cells have the potential to generate cells of all three types of embryonic germ layers [9,10]. A number of recent studies have shown that ES cells differentiate into a variety of different cell types in vitro, including muscle cells [11], adipocytes [12], osteocytes [13], hepatocytes [14], and insulin-secreting pancreatic β-like cells [15], as well as those composing dopaminergic [16], serotonergic [17], and GABAergic [18] neurons. However, there are only a few reports documenting the differentiation of ES cells into retinal neurons [19–21]. In the present study, we attempted a co-culture of mouse ES-derived NSCs with chick embryonic retina tissue that was harvested on embryonic day 6 (E6), and observed an efficient induction of rhodopsin immuno-positive photoreceptor-like cells from the ES cells. Our results show that avian embryonic retina tissue provides a conducive environment for the differentiation of ES cells into photoreceptor-like cells and suggest that the present system may be useful in cell replacement therapies to treat retinal degeneration diseases.

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doi:10.1016/j.bbrc.2005.04.125

Materials and methods

Murine ES cell lines. We utilized the mouse ES cell line EB3 (129/SvJ mouse ES cells, a kind gift from Dr. Hitoshi Niwa, RIKEN Center for Developmental Biology, Kobe, Japan) [22], a subline derived from E14tg2a ES cells [23] that carries the blasticidin S-resistant selection marker gene driven by the Oct-3/4 promoter (active under undifferentiated status). Undifferentiated EB3 cells were maintained on gelatin-coated dishes without feeder cells in maintenance medium, which was knockout-DMEM (Gibco/BRL) supplemented with 10% fetal bovine serum (FBS; Gibco/BRL), 0.1 mmol/L of 2-mercaptoethanol (Sigma), 10 mmol/L of non-essential amino acids (Gibco/BRL), L-glutamine, and 1000 U/mL of leukemia inhibitory factor (LIF; Gibco/BRL).

Neural induction of ES cells. We used both an embryoid body (EB)-based multistep protocol [24] and a retinoic acid (RA) protocol [25], with minor modifications. Briefly, for both protocols, undifferentiated ES cells (stage 1) were dissociated into single-cell suspensions and then cultured in hanging drops to induce EB formation at an initial cell density of 500 cells per drop (20 μl) of ES cell growth medium in the absence of LIF for 4 days (stage 2). In the EB-based multistep culture, EBs were plated on a gelatin-coated tissue culture surface for 24 h to promote adherence, after which the selection of nestin-positive cells was initiated by replacing the ES cell medium with serum-free insulin/transferrin/selenium/fibronectin (ITSFn) medium (Gibco/BRL, Grand Island, NY) for 7 days (stage 3). Cell expansion was initiated in N2 medium supplemented with 1 μg/ml of laminin and 5 ng/ml of basic fibroblast growth factor (bFGF) (RD Systems, Minneapolis, MN) for 6 days (stage 4). ES cells were then co-cultured with E6 chick retina tissue across a 0.4-μm Millicell CM membrane (Millipore) for 9 days (stage 5) in retinal culture medium, as previously described [21]. In the cultures using RA, EBs were plated on a gelatin-coated tissue culture surface and cultured for 4 days in the presence of all-*trans* RA (5×10^{-7} M, Sigma). ES cells were then grown together with E6 chick retina tissue in retinal culture medium for 10 days. For an immunocytochemistry study, RA-treated EBs were dissociated into single cells by trypsinization, and then re-plated onto laminin and poly-ornithine-coated culture dishes. After 24 h, the immunoreactivity against nestin and pax6 was examined.

RNA extraction and RT-PCR analysis. RNA was isolated from the cells and cDNA synthesis was performed as previously described [14,15]. One microliter of cDNA was amplified using gene-specific primers, with the following step cycles: denaturation for 60 s at 94 °C, annealing for 30 s at 55–64 °C, and extension for 30 s at 72 °C for 30 cycles. The primers used for the PCR assays are described in Table 1. PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining.

In vitro immunofluorescence analysis. Immunofluorescence analysis of plated ES cells was carried out using standard protocols. Briefly, the cells were fixed in 4% paraformaldehyde and incubated with cell-specific marker antibodies in blocking serum at 4 °C overnight. After incubation in species-specific IgG conjugated with FITC or RITC, the cells were washed with PBS and examined under a microscope. All nuclei were stained with DAPI (Dojindo). The primary antibodies and dilutions used were as follows: neural precursors (goat polyclonal anti-nestin, 1:100, Santa Cruz), immature neurons (goat polyclonal anti-NFL, 1:100, Santa Cruz or rabbit polyclonal anti-Map2, 1:2000, Chemicon), retinal progenitors (rabbit polyclonal anti-Pax6, 1:200, Santa Cruz), horizontal and amacrine cells (goat polyclonal anti-calbindin, 1:100, Santa Cruz), and photoreceptors (rabbit polyclonal anti-rhodopsin, 1:500, LSL).

Results

RT-PCR analysis

To investigate the potential of ES cells to differentiate into neural retina tissue, we first examined the expression of nestin and pax6, both of which are thought to be expressed on retinal and neural progenitor cells [26] (Fig. 1), and found that their expression patterns in the culture stages were very similar. No expression was observed in undifferentiated ES cells (stage 1, lane S1) or EBs (stage 2, lane S2), while it was faintly detected in stage 3 (lane S3) and clearly in stage 4 (lane S4). Crx, a transcription factor specific for photoreceptor cells, was not detected until stage 5 (lane S5), at which time the ES-NSCs were co-cultured with the chick embryonic retina tissue. Other markers for photoreceptor cells such as IRBP and recoverin were also detected in the ES derivatives at stage 5.

As for gene expression in the culture of RA-treated ES cells, a distinct expression of nestin and pax6, and

Table 1
List of primers and their respective sequences used for RT-PCR analyses

Genes	Primer sequences	Product size (bp)	GenBank Accession No.	Temperature (°C)
β-Actin	Forward: 5′ATGGATGACGATATCGCTG3′ Reverse: 5′ATGAGGTAGTCTGTCAGGT3′	569	NM007393	54
Nestin	Forward: 5′GGTCTCCCTCGAATCTCTC3′ Reverse: 5′GATCCAGGCAGCTCCCATT3′	1037	NM0167	58
Pax6	Forward: 5′TTTAACCAAGGGCGGTGAGCAG3′ Reverse: 5′TCTCGGATTTCCCAAGCAAAGATG3′	428	NM013627	60
Chx10	Forward: 5′TTCTACACACAGCCCACCTT3′ Reverse: 5′CGACTTTTTGTGCATCCCCA3′	518	L34808	60
Crx	Forward: 5′CCCAATGTGGACCTGATGCACC3′ Reverse: 5′GGGCTGTAAGAATCTGAGATGCC3′	371	U77615	62
SyntaxinI	Forward: 5′AAGAGCATTGAGCAGAGCATC3′ Reverse: 5′CATGGCCATGTCCATGAACAT3′	342	NM016801	62
IRBP	Forward: 5′CCCTCCCCAGAAGTCTTT3′ Reverse: 5′CAGCCTCTTCATGATGTA3′	427	NM015745	55
Recoverin	Forward: 5′ACACCAAGTTCACAGA GGAG3′ Reverse: 5′CCGCTTTTCTGGGGTGTTTT3′	394	NM009038	60
GFAP	Forward: 5′AAGCTCCAAGATGAAACCAACCTGA3′ Reverse: 5′CTCACCATAGCCAGATTCAAACG3′	278	NM010277	57

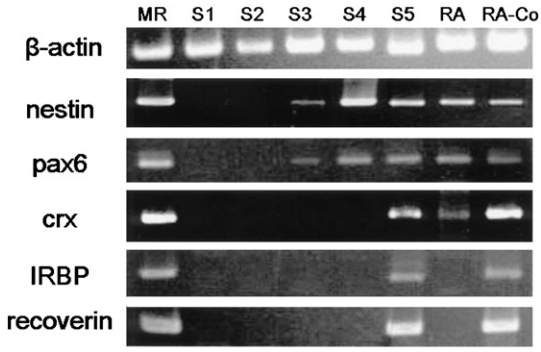

Fig. 1. mRNA expressions of nestin, pax6, and rod photoreceptor cell-related genes. RNA samples were obtained from mouse embryonic retina (lane MR), undifferentiated ES cells (stage 1, lane S1), EBs (stage 2, lane S2), differentiating ES cells at stages 3, 4, and 5 (lanes S3, S4, and S5, respectively), RA-treated ES cells (lane RA), and RA-treated ES cells co-cultured with chick E6 retina tissue (lane RA-Co). β-Actin transcripts are shown as an internal reference for amplification of cDNA.

a faint expression of Crx, were observed before the start of co-culture with chick retina tissue (lane RA). The expression of nestin and pax6 remained after 10 days (lane RA-co), while that of Crx was also firmly expressed, along with that of recoverin and IRBP.

Emergence of rhodopsin-positive cells in co-culture with chick E6 retina tissue

Prior to beginning the co-culture with chick embryonic retina tissue, we investigated the immuno-characteristics of stage 4 ES cells and RA-treated ES cells. More than 90% of the ES cells at stage 4 were immuno-positive for nestin and pax6, while about 75% of the RA-treated ES cells were immuno-positive for those factors.

Rhodopsin is one of the characteristic proteins of photoreceptor cells. No immunoreactivity against rhodopsin was detected in undifferentiated ES cells (stage 1), or in EBs (stage 2), selected (stage 3), or expanded (stage 4) ES cells. Rhodopsin-positive cells appeared after the initiation of co-culture with chick embryonic retina tissue (stage 5). Representative microscopic images of ES cells at stage 5 on days 1, 4, 7, and 10 after immuno-staining for rhodopsin are shown in Figs. 2A–D, respectively. After the start of co-culture with chick embryonic retina tissue, an increased frequency of rhodopsin immuno-positive cells was observed as the co-culture period extended. The frequency of rhodopsin immuno-positive cells was estimated by counting the numbers of those cells in three different microscopic

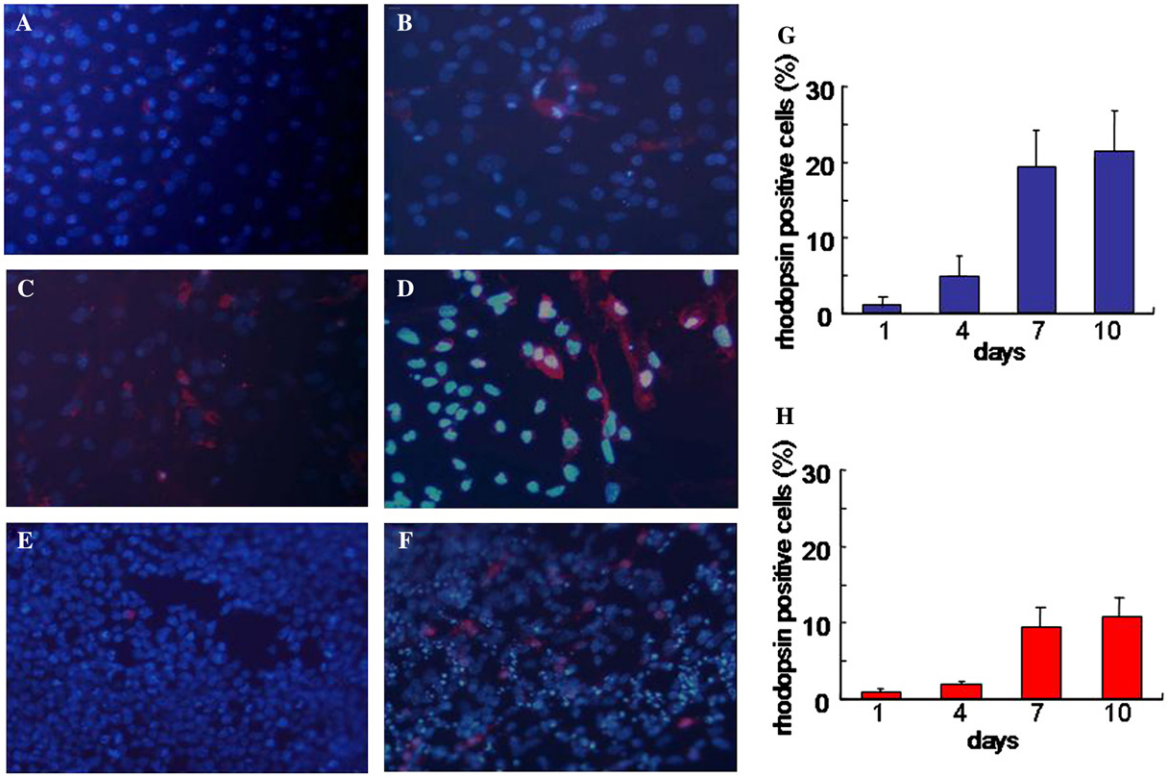

Fig. 2. Immunofluorescence analysis of rhodopsin. Representative microscopic images of ES cells at stage 5 on days 1, 4, 7, and 10 are shown in (A)–(D), respectively, and the proportion of rhodopsin immuno-positive cells during stage 5 is shown in (G). Representative microscopic images of RA-treated ES cells on days 1 and 10 after the start of co-culture with chick E6 retina tissue are shown in (E) and (F), respectively, and the proportion of rhodopsin immuno-positive cells during the co-culture of RA-treated ES cells with chick E6 retina tissue is shown in (H). Data are presented as means ± SD.

fields. Approximately 1.3%, 4.8%, 19.4%, and 21.5% of the cultured cells at stage 5 were positive for rhodopsin on days 1, 4, 7, and 10, respectively (Fig. 2G). When cells at stage 4 were cultured without chick embryonic retina tissue, rhodopsin immuno-positive cells did not appear during the 10-day culture period.

Similarly, the number of rhodopsin immuno-positive cells was increased during the co-culture of RA-treated ES cells with chick E6 retina tissue (Fig. 2H) and finally composed about 10% of the differentiating ES cell population at the end of the 10-day co-culture period (Fig. 2F), whereas very few of those cells were found at the start of co-culture (Fig. 2E).

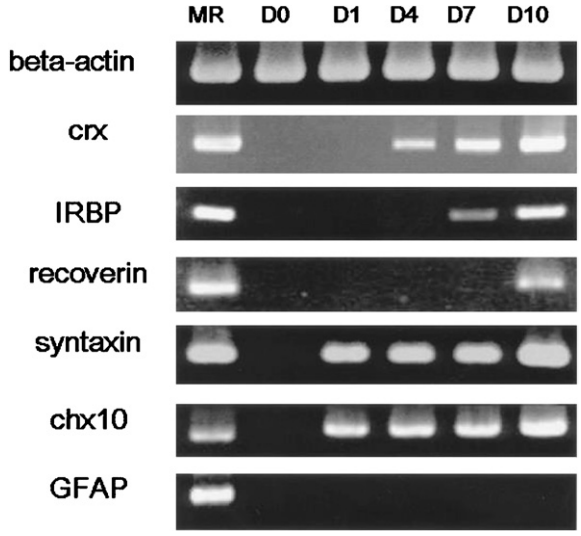

Fig. 3. mRNA expression of syntaxin I, chx10, and rod photoreceptor cell-related genes during the co-culture with chick E6 retina tissue. RNA samples were obtained from mouse embryonic retina tissue (lane MR), as well as differentiating ES cells at the start of co-culture with chick E6 retina tissue (lane D0) and on days 1, 4, 7, and 10 (lanes D1, D4, D7, and D10, respectively).

Prompt induction of syntaxin mRNA and calbindin immuno-positive cells after the start of co-culture with chick E6 retina tissue

We investigated the gene expression of syntaxin I, chx 10, and photoreceptor-related genes in ES cells at stage 5 during the co-culture with E6 chick retina tissue (Fig. 3). Syntaxin I is a marker for amacrine cells, while Chx 10 is a marker for bipolar cells and also expressed on retinal progenitor cells [27]. mRNA expression for both syntaxin I and Chx 10 was observed as early as 24 h after starting the co-culture (day 1, lane D1), whereas the expression of crx, IRBP, and recoverin was not detected at that time. Crx mRNA was first detected on day 4 (lane D4) and that of IRBP on day 7 (lane D5). Only recoverin was detected in the ES derivatives co-cultured with chick E6 retina tissue for the full 10-day period (lane D10).

Calbindin is a marker for horizontal cells. Immuno-positivity for calbindin was absent in the ES cells before starting the co-culture with chick E6 retina tissue. However, immuno-positivity for calbindin appeared as early as 1 day after starting the co-culture (Fig. 4A), though no drastic increase was observed thereafter or throughout the remaining period of stage 5 (Fig. 4E). Representative microscopic images of ES cells at stage 5 on days 4, 7, and 10 after immuno-staining for calbindin are shown in Figs. 4B–D, respectively.

Discussion

Differentiation of ES cells into various cell types including neurons has been investigated in a number of studies, though there are few reports documenting the differentiation of those cells into retinal neurons [19–21]. In the present study, we investigated the poten-

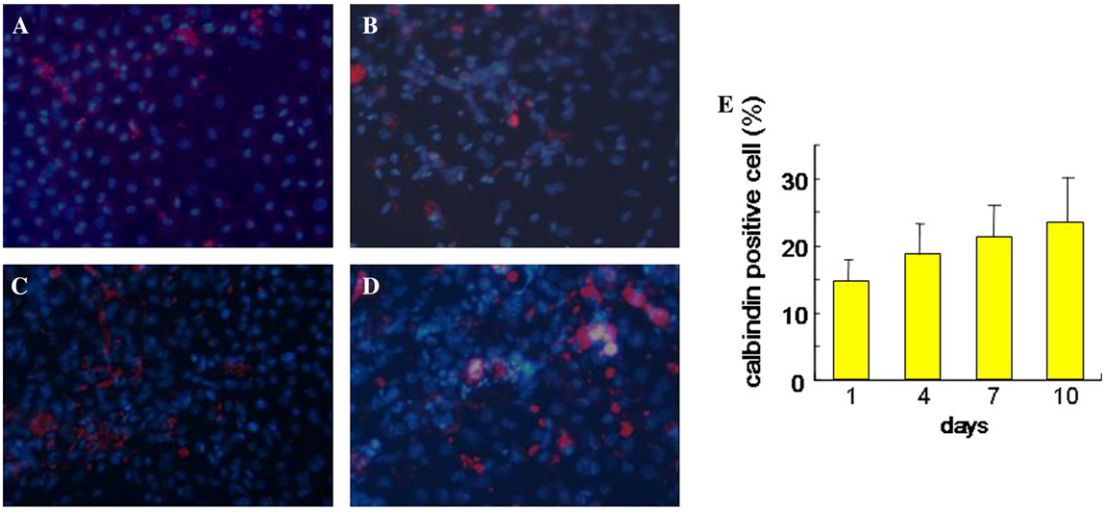

Fig. 4. Immunofluorescence analysis of calbindin. Representative microscopic images of ES cells at stage 5 on days 1, 4, 7, and 10 are shown in Fig. 2, A–D, respectively. The proportion of calbindin immuno-positive cells during stage 5 is shown in (E). Data are presented as means ± SD.

tial of ES cells to differentiate into a photoreceptor lineage during co-culture with chick embryonic retina tissue.

Although an induction method for retinal progenitors or retinal stem cells (RSCs) from undifferentiated ES cells has not been established, differentiation into NSCs and neural progenitor cells has been reported [17,28]. We adopted an EB-based multistep differentiation method using ITSFn medium [24] and an RA induction technique [25] for the initial induction of undifferentiated ES cells along neuronal lineages, which was basically the same as the NSC induction methods reported by Lee et al. [17] and Bibel et al. [28]. More than 90% of the stage 4 cells and 75% of the RA-treated ES cells in our EB-based multistep differentiation experiment were immuno-positive for nestin and pax 6, suggesting successful induction of NSCs from undifferentiated ES cells. However, it is still difficult to induce differentiating ES cells or NSCs toward retinal neurons spontaneously or using defined growth factors alone. It is known that embryonic and postnatal retina tissues provide a favorable environment for retinal progenitors to differentiate into retinal neurons [29–31], thus we used developing retinal tissue to provide epigenetic cues for the in vitro differentiation of ES cells toward retinal neurons. We co-cultured differentiating ES cells with chick embryonic retina tissue and generated photoreceptor-like cells, which were confirmed by rhodopsin expression. To selectively assess successful differentiation of the ES cells, chick E6 retina tissues were cultured in a chamber separated from the ES cells by a 0.4-μm Millicell CM membrane, which permitted the transfer of diffusible factors.

Rhodopsin immuno-positive cells did not appear in the differentiation culture without chick E6 retina tissue, indicating that the differentiation of ES cells into retinal neurons is a very rare event in the spontaneous differentiation of ES cells and even in neuronal lineage-oriented differentiation. However, rhodopsin immuno-positive cells emerged and increased in the co-culture with chick E6 retina tissue for 10 days, at which time more than 20% of the mouse ES derivatives were immuno-positive for rhodopsin. In addition, RT-PCR analysis demonstrated expression of the transcription factor crx, as well as a distinct increase of IRBP and recoverin, other markers specific to rod photoreceptors, after beginning the co-culture. These results indicate that co-culture of ES cells with chick embryonic retina tissue is a useful and efficient method for the induction of photoreceptor-like cells.

It has been reported that the generation of retinal neurons follows an evolutionary conserved temporal sequence [32,33]. In mice, ganglion cells, cone photoreceptors, amacrine cells, and horizontal cells develop during the early stage of histogenesis between E10.5 and E16, whereas rod photoreceptors, bipolar cells, and Muller glial cells develop during the late stage of histogenesis between E18 and postnatal day 6. However, in chick retina tissue, retinal neurogenesis is not clearly divided into early and late phases, while a spatial gradient from the central area to the periphery also exists [34]. According to an in ovo labelling study of chick embryo retinas with [^{3}H]thymidine [34], ganglion cells first appeared on E2-E3, followed by amacrine cells, horizontal cells, photoreceptors, and muller glial cells on E4-E5, and finally bipolar cells on E5. The maximal amount of accumulation of most types of cells including photoreceptor cells is in the dorso-central area of the developing retina on E6. Therefore, we utilized E6 chick retina tissue in the present co-culture system, as we expected epigenetic assistance for the ES-NSCs to differentiate into photoreceptor cells.

We speculated that chick E6 retina tissue would induce differentiation of ES cells into not only rod photoreceptor cells, but also amacrine and horizontal cells, because it has been reported that the accumulation of amacrine and horizontal cells was maximal on E6, as in the case of rod photoreceptor cells [34]. Syntaxin mRNA expression, a marker of amacrine cells, and immunoreactivity toward calbindin, a marker of horizontal cells, were detected in the ES cells co-cultured with chick E6 retina tissue. In addition, mRNA from Chx10, which is expressed on retinal progenitor cells and a marker of bipolar cells, was also detected as early as 24 h after beginning the co-culture (day 1, stage 5). These results suggest that a co-culture with chick E6 retina tissues provides favorable conditions to generate early-type neurons and retinal progenitor cells from mouse ES cells. Interestingly, the markers of early-born neurons and progenitor cells appeared earlier than rhodopsin immunoreactivity in stage 5, which seemed to be in accordance with the mode of retinal development in mice.

There are only a few reports documenting the differentiation of ES cells into a retinal cell-lineage in vitro [19–21]. Hirano et al. [20] reported the generation of eye-like structures, composed of a lens and neural retina, from mouse ES cells that were cultured with PA6 stromal feeder cells. Further, Zhao et al. [21] recently found that mouse ES cells differentiated into retinal neurons, including bipolar-like cells and photoreceptor-like cells, using rat postnatal retinal cells. In both of those reports, specific cellular components were utilized for the induction of retinal neurons from ES cells. PA6 cells are known to support neural differentiation of undifferentiated ES cells, including differentiation into a dopaminergic lineage [16], and rat postnatal retinal cells have been reported to support the differentiation of retinal progenitor cells, not only into retinal neurons but also into non-retinal neurons [35]. In the present study, eye-like structures did not appear in the cultures of differentiating ES cells co-cultured with chick E6 retina tissue. However, it was evident that ES cells differentiated

along rod photoreceptor, amacrine, and horizontal cell lineages.

Chick embryo tissue has been recognized as useful to evaluate the potentiality of mouse embryonic tissues [36,37]. Further, it was recently demonstrated that mouse ES cells are able to differentiate appropriately and generate neurons in a co-culture with chick embryo tissue in response to local cues [38]. In the present study, mouse ES cells were co-cultured with chick E6 retina tissue, which provided epigenetic cues, and more than 20% of the cultured ES cells were immuno-positive for rhodopsin after 10 days. Although the ES cells did not have direct cellular contact with the chick embryonic retina tissue in the present method, diffusible factors from that tissue were sufficient to induce rod photoreceptor cells from ES cells.

In conclusion, the present results demonstrated that mouse ES cells can be induced into rhodopsin immuno-positive photoreceptor-like cells using a co-culture method with chick embryonic retina tissue. This co-culture system is an efficient and useful method to obtain photoreceptor-like cells, and may be useful in cell replacement therapy to treat retinal degeneration diseases.

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