Induction of the Differentiation of Lentoids from Primate Embryonic Stem Cells

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Purpose. To produce lens cells from primate embryonic stem (ES) cells in a reproducible, controlled manner.

Methods. Cynomologus monkey ES cells were induced to differentiate by stromal cell–derived inducing activity (SDIA). The lentoids produced by this treatment were processed for immunohistochemical and immunoblotting analysis. The effect of varying the concentration of fibroblast growth factor (FGF)-2 and the density of the ES colonies plated during the differentiation process were also examined.

Results. After a 2- to 3-week induction period, lentoids were produced by a subpopulation of ES colonies. Western blot analysis and immunohistochemistry revealed that these lentoids expressed αA-crystallin and Pax6. The number of lentoids resulting from treatment increased with increasing FGF-2 concentration and plated colony density.

Conclusions. The differentiation of primate ES cells into lentoids can be achieved by treatment with SIDA. ES cells can be used to facilitate a greater understanding of the mechanisms functioning in differentiation in vivo and in vitro. (*Invest Ophthalmol Vis Sci.* 2003;44:2689–2693) DOI:10.1167/iovs.02-1168

The ocular lens is a transparent organ functioning in the refraction, accommodation, and absorption of ultraviolet spectrum. The lens, derived from surface ectoderm, develops from the invagination of the lens placode into the concavity of the optic cup.

The transdifferentiation of iris and retinal pigment epithelial cells into the lens has been well characterized. Lens regeneration can also occur throughout life in some urodeles and fish,[1] the mechanism of which is the transdifferentiation of pigment epithelial cells from the dorsal iris.[2] The potential transdifferentiation of iris and retinal pigment epithelial cells into lens tissue in vitro is conserved throughout life without regard to the species. This activity is retained even in adult humans.[3] Recently, Kosaka et al.[4] established a culture system in which iris-pigment epithelial cells from newborn chicks transdifferentiated into lentoids. The mechanism of this transdifferentiation of pigment epithelial cells into lentoids, however, differs from the processes governing normal lens development.

Embryonic stem (ES) cells provide a valuable tool to study the in vivo mechanisms of cellular differentiation in vitro. ES cells can differentiate into a variety of cell types in vitro, including cardiomyocytes,[5] hematopoietic progenitors,[6] skeletal myocytes,[7] smooth muscle cells,[8] endothelial cells,[9] melanocytes,[10] glia,[11] neurons,[12] and pancreatic islet cells.[13] ES cells induced to differentiate in vitro are assumed to follow the developmental stages occurring within the normal embryo, generating mature, fully differentiated cell types through interactions with growth factors or genetic modification. Recently, Suemori et al.[14] established ES cell lines from the cynomolgus monkey, an animal widely used in experimental models. Because these animals are genetically similar to humans, studies using ES cells from cynomolgus monkeys may facilitate transitions to clinical applications for humans. We sought to manipulate ES cell differentiation systematically in this model to produce homogenous populations of differentiated cells.

Stromal cell-derived inducing activity (SDIA) induces the differentiation of mouse ES cells into neural cells, including midbrain tyrosine-hydroxylase–positive dopaminergic neurons.[15] SDIA also induces neural differentiation in primate ES cells,[16] producing populations containing not only tyrosine-hydroxylase–positive neurons but also pigmented epithelial cells of the eye. These results suggest the production of ocular cells can be induced by the SDIA treatment of primate ES cells.

In this report, we efficiently induced lentoid differentiation of ES cell lines from the cynomolgus monkey by using a modified SDIA method. This is the first report to demonstrate that lens cells can be consistently differentiated from primate ES cells in a controlled manner.

Materials and Methods

Maintenance of Primate ES Cells

ES cell lines were established from cynomolgus monkey blastocysts. Their pluripotential competence was confirmed as described elsewhere.[14] Undifferentiated ES cells were maintained on a feeder layer of mitomycin C–inactivated mouse embryonic fibroblasts (STO cells). STO cells were incubated with 10 μg/mL mitomycin C (Wako, Osaka, Japan) in a 37°C incubator with a 5% CO_2 environment for 2 hours and plated on a gelatin-coated dish at a cell density of 1.6×10^5 cells/mL. ES cells were cultured in Dulbecco's modified Eagle's medium-Ham's F12 (DMEM/F-12; Sigma, St. Louis, MO), supplemented with 0.1 mM 2-mercaptoethanol (Sigma), 1000 U/mL leukemia inhibitory factor (ESGRO; Chemicon, Temecula, CA), 20% knockout serum replacement (KSR; Gibco, Rockville, MD: KSR is used instead of fetal bovine serum to support the growth of undifferentiated ES cells), 0.1 mM nonessential amino acids (Gibco), and 8 ng/mL basic fibroblast growth factor-2 (FGF-2; Upstate Biotechnology, Lake Placid, NY). Medium was changed every day. ES cells were passaged after treatment with 0.25% trypsin in PBS with 1 mM $CaCl_2$ and 20% knockout serum replacement. Three to 4 days before plating on PA6 stromal cells (derived from mouse skull bone marrow[17]), FGF-2 was added at concentrations of 2, 4, or 8 ng/mL.

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Supported by a grant-in-aid from Ministry of Education, Culture, Sports, Science and Technology, Japan.

Submitted for publication November 15, 2002; revised January 16, 2003; accepted January 23, 2003.

Disclosure: **S. Ooto**, None; **M. Haruta**, None; **Y. Honda**, None; **H. Kawasaki**, None; **Y. Sasai**, None; **M. Takahashi**, None

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Induction of Lentoids by SDIA

As Kawasaki et al.[16] have described, SDIA is a method of inducing differentiation by the coculture of ES cells and PA6 cells in the differentiation medium without serum. PA6 cells were plated on gelatin-coated dishes for use as a feeder cell layer. After trypsinization, partially dissociated ES cell clumps (30–50 cells per clump) were seeded on a gelatin-coated dish in Glasgow minimal essential medium (GMEM; Gibco), supplemented with 10% FBS (Hyclone, Logan, UT). After a 30-minute incubation at 37°C, ES cells were dispersed by pipetting. Cell pellets collected by centrifugation were washed with ES differentiation medium (GMEM supplemented with 10% knockout serum replacement, 1 mM pyruvate [Sigma], 0.1 mM nonessential amino acids, and 0.1 mM 2-mercaptoethanol [Wako]). The cells, plated over PA6 feeder layers, were cultured in differentiation medium for at least 6 weeks. Medium was replaced every third day.

Immunohistochemistry

Cells were fixed in 4% paraformaldehyde (Wako) for 1 hour and then immersed in 25% sucrose-PBS. After washing in 0.1 M phosphate buffer (PB), specimens were incubated for 1 hour with 20% skim milk (Dainihon-Seiyaku, Osaka, Japan) in 0.1 M PB, containing 0.005% saponin (0.1 M PB-saponin; Merck, Darmstadt, Germany), to block nonspecific antibody binding. Specimens were incubated for 24 hours at 4°C with primary antibody diluted in 5% skim milk in 0.1 M PB-saponin. Rabbit polyclonal anti-αA-crystallin (1:1000; Stressgen, Victoria, British Columbia, Canada) and anti-Pax6 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) were used as the primary antibodies. The reactivity of the antibodies was confirmed by using rat lens as a positive control. After a wash in 0.1 M PB, specimens were incubated with the fluorescein-conjugated donkey anti-rabbit immunoglobulin (1:100; Amersham, Buckinghamshire, UK) secondary antibody diluted in 0.1 M PB-saponin with 5% skim milk for 1 hour at room temperature. After washes with 0.1 M PB, slides were mounted with glycerol-PBS (1:1) and observed by laser-scanning confocal microscope (Leica, Wetzlar, Germany).

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Cells, harvested by scraping, were lysed in 500 μL of lysis buffer (Laemmli sample buffer; Bio-Rad, Richmond, CA) with 5 mM 2-mercaptoethanol (Wako). Cell suspensions were homogenized on ice. Cell homogenates were stored at −80°C.

ES cell lysates were then subjected to electrophoresis, and the separated proteins were transferred to PVDF membrane (Immobilon-P; Millipore, Bedford, MA). Nonspecific antibody binding was blocked by incubation with 20% skim milk in 0.1 M PB for 1 hour. The blots were then incubated at room temperature with primary antibody diluted in 5% skim milk in 0.1 M PB for 1 hour. The rabbit polyclonal antibodies, anti-αA-crystallin (1:1000; Stressgen) and anti-Pax6 (1:200; Santa Cruz) were used as primary antibodies. The primary antibody binding was detected with a biotinylated anti-rabbit IgG conjugated to alkaline phosphatase (1:100; Vector Laboratories, Burlingame, CA) by the avidin-biotin complex (ABC) method. After washes in 0.1 M PB, the blots were developed with phosphatase substrate (Konica Immunostainning; Konica, Tokyo, Japan) according to the manufacturer's protocol.

Results

Induction of Lentoids

Culture for 2 to 3 weeks on PA6 stromal cells produced differentiating cells that continued to grow without pigmentation throughout the ES cell colonies. These cells eventually amassed to form transparent bodies of various sizes (Fig. 1A–C). These three-dimensional structures were characterized as lentoids by immunostaining, as demonstrated by the expression of αA-crystallin and Pax6 (Fig. 2).

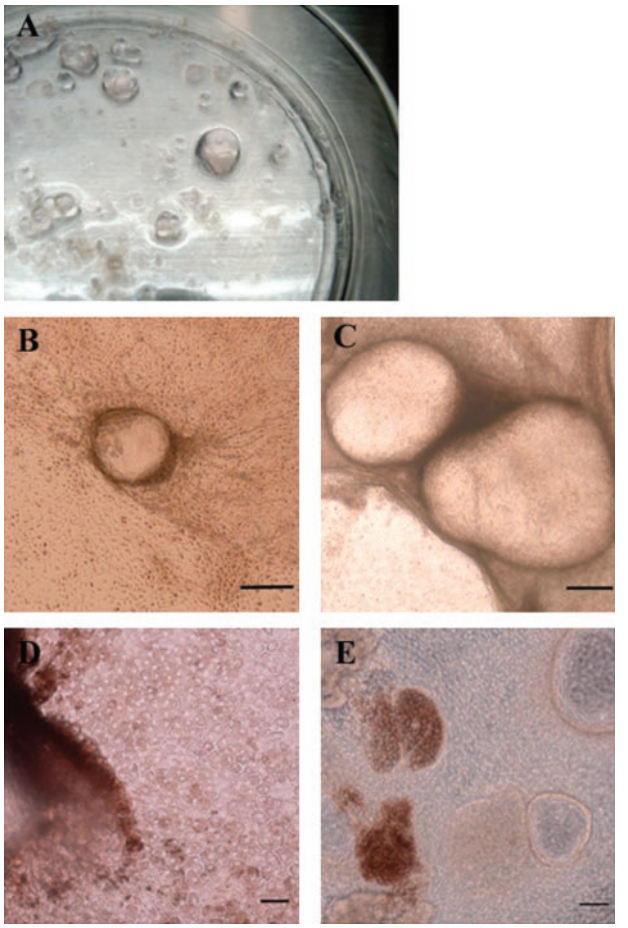

Figure 1. Morphology of the lentoids differentiated from ES cells by phase contrast microscopy. (**A**) Various sizes of lentoids were produced in a 10-cm dish after 23 days of induction (macro view). (**B**) A relatively small lentoid after 30 days of induction. (**C**) Large lentoids after 53 days of induction. (**D**) In some colonies, pigmented epithelial cells were observed. (**E**) Lentoids and pigmented epithelial cells were occasionally located within a single colony. Scale bar: (**B**, **C**) 300 μm; (**C**) 50 μm; (**E**) 100 μm.

Several colonies produced pigmented epithelial cells (Fig. 1D), most of which arose in independent colonies from those containing lentoids. Occasionally, the lentoids and pigment epithelial cells were situated within a single colony in the same configuration as that in the eye (Fig. 1E).

Lentoids first appeared after 14 to 16 days of induction. The proportion of colonies containing lentoids gradually increased after 20 days of induction. The number of lentoids peaked within 40 days of induction.

Western Blot Analysis

αA-crystallin expression by lentoids was further examined by Western blot analysis. The rabbit anti-αA-crystallin polyclonal antibody detects a 22-kDa protein. A single band representing αA-crystallin protein was detected in ES cell lysates, but not in the negative control (neural stem cells derived from adult rat hippocampus; Fig. 3A-1). In the positive control (rat lens protein), two bands were detected (22 and 25 kDa). The 25-kDa band may represent a minor, spliced version of αA-crystallin, which rodent lens could express. αA-crystallin protein was not expressed in PA6 cells, undifferentiated ES cells, and differentiated ES colonies which lentoids had been mechanically removed (Fig. 3B).

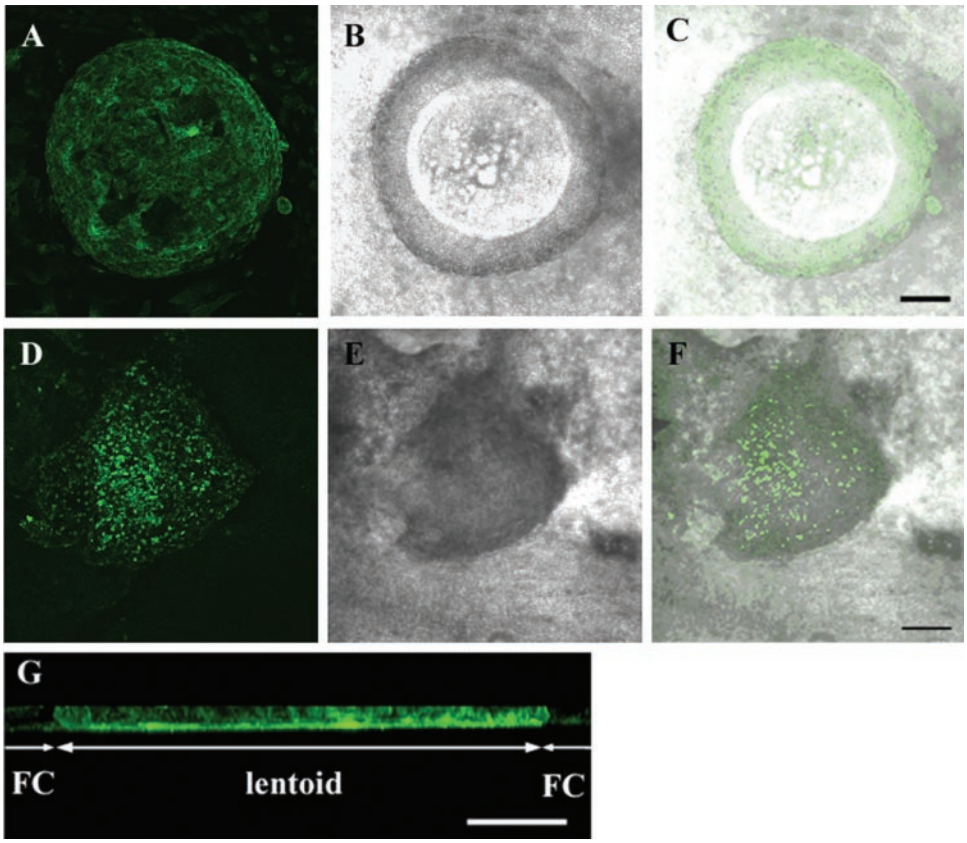

FIGURE 2. SDIA-induced lentoids expressed αA-crystallin and Pax6. (**A**) Immunostaining of SDIA-induced lentoids (after 3 weeks of induction) with anti-αA-crystallin antibody. (**B**) Bright-field view. (**C**) Merge of (**A**, **B**). (**D**) Immunostaining of SDIA-induced lentoids (after 3 weeks of induction) with anti-Pax6 antibody. (**E**) Bright-field view. (**F**) Merge of (**D**, **E**). (**G**) Three-dimensional digital image of lentoid expressing αA-crystallin. FC, feeder cell. Scale bar, 200 μm (**C**, **F**, **G**).

We also examined the expression of Pax6 (Fig. 3B). The rabbit anti Pax6 antibody detects the 48-kDa Pax6 protein. Pax6 was expressed in resultant lentoids, but not in the PA6 feeder cells or in undifferentiated ES cells.

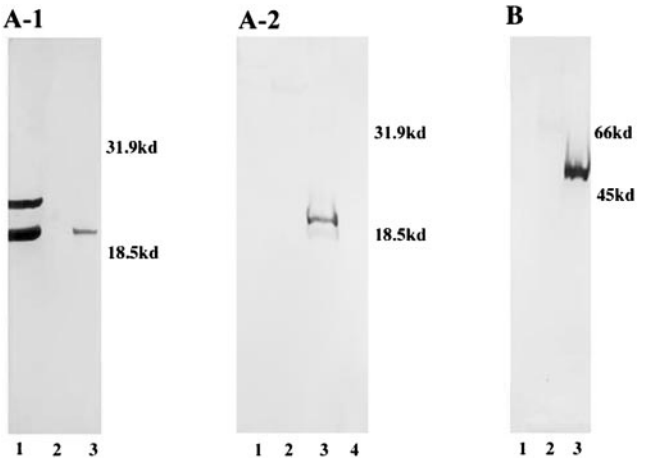

FIGURE 3. Detection of αA-crystallin and Pax6 proteins by Western blot analysis. Proteins of lentoids after 40 days of induction were transferred onto PVDF membrane after electrophoresis and probed with either anti-αA-crystallin or anti-Pax6 antibodies. (**A-1**) αA-crystallin expression. *Lane 1*: positive control (rat lens protein); *lane 2*: negative control (neural stem cells); *lane 3*: total SDIA-derived lentoid protein. (**A-2**) αA-crystallin expression; *lane 1*: protein of PA6 feeder cells; *lane 2*: total protein of undifferentiated ES cells; *lane 3*: total protein of SDIA-derived lentoid cells; and *lane 4*: total protein of differentiated ES cells from which lentoids were removed. (**B**) Pax6 expression. *Lane 1:* protein of PA6 feeder cells; *lane 2*: total protein of undifferentiated ES cells; *lane 3*: total protein of SDIA-derived lentoid cells.

Effects of Exogenous FGF-2 on Lentoid Induction

We evaluated the effect of FGF-2 concentration on the induction of lentoids from primate ES cells. Whereas at 20 days of induction, there were no significant difference between the different concentration, after 30 days, the percentage of colonies containing lentoids increased in a dose-dependent manner with increasing FGF-2 concentrations in maintenance undifferentiated ES cell cultures. At 40 days of induction, the proportion of colonies containing lentoids (% colonies with lentoids) were 14% ± 6% (n = 911) in 2 ng/mL FGF-2, 35% ± 2% (n = 980) in 4 ng/mL, and 38% ± 7% (n = 960) in 8 ng/mL (Fig. 4). We also attempted to add FGF-2 to the ES differentiation medium, but PA6 cells grew too rapidly to permit the maintenance of differentiating ES cells (data not shown).

Effects of the Colony Density on Lentoid Induction

The percentage of colonies containing lentoids increased in proportion to the density of ES colonies added at the start of the culture period (Fig. 5A). After 30 days of induction, the numbers of lentoids induced by ES cell differentiation in cultures plated at a high density on PA6 cells (200–300 colonies/10-cm dish) were greater than those at low density (70–150 colonies/10-cm dish) at each concentration of FGF-2 in maintenance undifferentiated ES cultures (Table 1). The same results were observed at 40 day of induction, although no significant differences were observable at 20 days (data not shown). The induction of pigmented epithelial cells also increased in high-colony-density cultures (Fig. 5B).

DISCUSSION

Recently, much attention has been paid to the potential applications of ES cells in both biology and medicine. ES cells

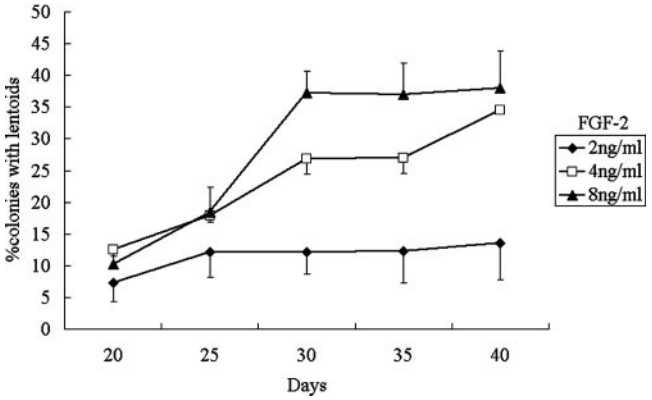

FIGURE 4. The effects of FGF-2 on lentoid induction. FGF-2 was added at a concentration of 2, 4, or 8 ng/mL during the maintenance of undifferentiated ES cells. After induction by SDIA treatment, lentoids were observed as a part of the ES colonies. The proportions of colonies containing lentoids (percentage of colonies with lentoids) after 20, 25, 30, 35, and 40 days of induction are shown. In all dishes, ES cells were plated on PA6 cells at a colony density of 180 to 250 colonies/10-cm dish. Mean ± SD obtained from five dishes.

provide a model to study basic embryology, a system in which to investigate the effect of growth factors and drugs, and a potential source of cells for transplantation. This is the first report to document the differentiation of lentoids from primate ES cells.

ES cells can be induced to differentiate through a variety of methods. On removal from culture on feeder layers and transfer to suspension culture, ES cells begin to differentiate into multicellular aggregates containing differentiated and undifferentiated cells, termed embryoid bodies. The coculture method also produces a variety of cell types.

In this study, we used the modified SDIA method to produce lentoids from primate ES cells. This method is highly reproducible. Differentiated lentoids were maintained in differentiation medium for at least 10 weeks. As described, SDIA treatment induces the production of both pigmented epithelium and dopaminergic neurons from primate ES cells.[16] Although the molecular mechanism governing differentiation in response to SDIA treatment remains to be elucidated, such a procedure may also induce the production of a variety of cell lineages.

The primate lens, derived from surface ectoderm, begins to develop during the third week of gestation. In a stage-13 to -15 embryo, the lens changes from a lens placode to a lens vesicle.

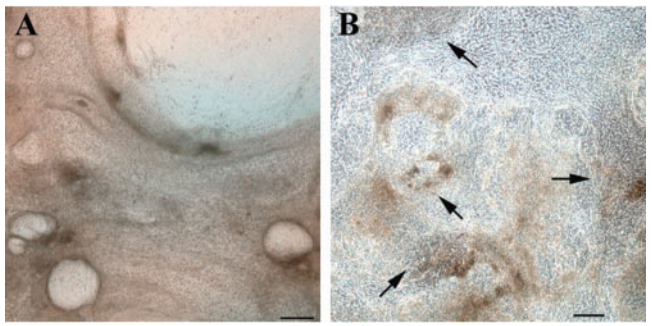

FIGURE 5. Effects of colony density on lentoid induction. (**A**) The proportion of lentoids in high-colony-density cultures is shown. Multiple lentoids were produced by several colonies. (**B**) The proportion of pigmented epithelial cells in high-colony-density cultures is shown. Pigmented epithelial cells (*arrows*) were produced by several colonies. Scale bar, 100 μm.

TABLE 1. Percentage of Colonies with Lentoids in Different Colony Densities

FGF-2 (ng/mL)	Day	Low-Density Dish (% Colonies)	High-Density Dish (% Colonies)
2	30	10 ± 2 (n = 442)	13 ± 2 (n = 704)
	40	10 ± 2	15 ± 3
4	30	14 ± 5 (n = 255)	31 ± 3 (n = 608)
	40	17 ± 9	36 ± 5
8	30	16 ± 7 (n = 219)	37 ± 3 (n = 788)
	40	19 ± 9	38 ± 6

The effects of colony density in concert with different concentrations of FGF-2 in maintenance undifferentiated ES cell cultures after 30 and 40 days of induction are shown. The percentage of colonies with lentoids induced by ES cell differentiation in cultures plated at a high density (200–300 colonies/10-cm dish) on PA6 cells were greater than those at low density (70–150 colonies/10-cm dish) at each concentration of FGF-2. Mean ± SD obtained from 3 to 4 dishes, n = counted colonies.

During embryonic stages 16 to 20, the lens vesicle gradually loses its contact with the surface ectoderm, allowing the obliteration of the lens cavity by the differentiating lens fibers of the deep wall. In stage-20 embryos, the lens exhibits numerous differentiating fibers.

The time course of lentoid differentiation by modified SDIA treatment is reminiscent of that observed in the developing primate lens. Lens cells are first detected by induction days 14 to 16. Given that ES cells behave in a manner similar to the inner cell mass (E5), the period required for lens induction in vitro correlates with that observed in the embryo.

Pax6 is a paired homeobox transcription factor with a highly conserved role in eye development.[18] Exogenous expression of Pax6 leads to ectopic eye formation in *Drosophila* and *Xenopus*,[19,20] whereas heterozygous Pax6 mutations result in the Small-eye phenotype.[21]

Several experiments demonstrate that Pax6 is essential for lens formation. Pax6 expression in the optic vesicle and the facial epithelium during their interaction leads to lens placode formation and invagination.[22] The deletion of Pax6 in the prospective lens results in a deficiency in lens formation.[23] In the current study, we observed the expression of Pax6 in lentoids produced from ES cell differentiation. Pax6 is likely to be a key factor functioning in both the formation of lentoids from ES cells and in normal development.

The induction of lentoids from ES cells offers an opportunity to study exogenous factors that may function in this process. Several studies have reported that FGF may play an important role in lens differentiation and development. In mammals, fibroblast growth factor (FGF) promotes lens fiber differentiation.[24] FGF-1 and -2 are expressed in the mouse neural retina and lens cells during development;[25] and FGF receptor (FGFR)-1 and -2 are also expressed in lens cells.[26] Additional reports suggest the importance of FGF and FGFR in the lens regeneration of the newt.[27] The induction of the differentiation of several cell lineages from ES cells can be enriched by growth factors, including FGF, nerve growth factor (NGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF), retinoic acid (RA), bone morphogenic protein (BMP-4), transforming growth factor (TGF)-β, and activin-A.[6,12,28] Human ES cells express FGF-2 and FGFR-1, and stimulation of these receptors with FGF-2 upregulates ectodermal and mesodermal markers.[28] These studies support the hypothesis that the induction of lentoids is related to stimulation of FGF-2. In this study, increasing concentrations of FGF-2 in the maintenance medium of undifferentiated ES cells influenced the differentiation of lentoids. FGF-2 may allow the undifferentiated ES cells to respond well to the differentiation factors,

possibly by upregulating the receptors of differentiation factors in SDIA medium.

The density of plated colonies also affected the quantity of lentoid induction. Cell-cell contact is important in the formation of lentoids by mouse lens epithelial cells.[29] Transdifferentiation of the chick neural retina into the lens occurs under crowded situations, in association with multilayering.[30] Thus, formation of lentoids is also likely to occur in a crowded state, and colony density could be an important differentiation factor. Colony density also affected the quantity of pigmented epithelial cell differentiation. These data suggest that ocular cells are more likely to be produced in crowded situations after SDIA treatment. Such studies of ES cells may increase our understanding of the early stages of development.

ES cells also may be a useful source of cells for transplantation. The production of lens cells by modified SDIA treatment may provide an alternative source of intraocular lens for cataract therapy. Today, intraocular lens is widely used for cataract therapy. The safety of this procedure is well established, but it remains difficult to solve the problem of accommodation. One advantage of using ES cells is that accommodation may be possible by the implantation of induced-lens cells into the lens capsule after lensectomy. ES-derived lentoids provide us with these new possibilities.

In conclusion, this study yielded fundamental information regarding the differentiation of primate ES cells. First, lentoids expressing αA-crystallin and Pax6 protein were generated from primate ES cells. Second, increasing FGF-2 concentration and colony density positively influenced the induction of lentoids. Further investigation is necessary to regulate precisely the differentiation of lentoids to allow a greater exploration of the possibilities for this technology in clinical applications and basic research.

Acknowledgments

The authors thank Hirofumi Suemori for providing the cynomolgus monkey ES cells, Megumi Sawamura and Noriaki Sasai for technical advice, and Noriyasu Murata and Tomoko Yokota for assistance with maintenance of the ES, STO, and PA6 cells.

References

1. Stone LS. An investigation recording all salamanders which can and cannot regenerate a lens from the dorsal iris. *J Exp Zool.* 1967; 164:87–103.
2. Eguchi G, Abe SI, Watanabe K. Differentiation of lens-like structures from newt iris epithelial cells in vitro. *Proc Natl Acad Sci USA.* 1974;71:5052–5056.
3. Eguchi G, Okada TS. Differentiation of lens tissue from the progeny of chick retinal pigment cells cultured in vitro: a demonstration of a switch of cell types in clonal cell culture. *Proc Natl Acad Sci USA.* 1973;70:1495–1499.
4. Kosaka M, Kodama R, Eguchi G. In vitro culture system for iris-pigmented epithelial cells for molecular analysis of transdifferentiation. *Exp Cell Res.* 1998;245:245–251.
5. Doetschman TC, Eistetter H, Katz M, Schmidt W, Kemler R. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J Embryol Exp Morphol.* 1985;87:27–45.
6. Wiles MV, Keller G. Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. *Development.* 1991;111: 259–267.
7. Rohwedel J, Maltsev V, Bober E, Arnold HH, Hescheler J, Wobus AM. Muscle cell differentiation of embryonic stem cells reflects myogenesis in vivo: developmentally regulated expression of myogenic determination genes and functional expression of ionic currents. *Dev Biol.* 1994;164:87–101.
8. Drab M, Haller H, Bychkov R, et al. From totipotent embryonic stem cells to spontaneously contracting smooth muscle cells: a retinoic acid and db-cAMP in vitro differentiation model. *FASEB J.* 1997;11:905–915.
9. Risau W, Sariola H, Zerwes HG, et al. Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies. *Development.* 1988;102:471–478.
10. Yamane T, Hayashi S, Mizoguchi M, Yamazaki H, Kunisada T. Derivation of melanocytes from embryonic stem cells in culture. *Dev Dyn.* 1999;216:450–458.
11. Brustle O, Jones KN, Learish RD, et al. Embryonic stem cell-derived glial precursors: a source of myelinating transplants. *Science.* 1999; 285:754–756.
12. Bain G, Kitchens D, Yao M, Huettner JE, Gottlieb DI. Embryonic stem cells express neuronal properties in vitro. *Dev Biol.* 1995; 168:342–357.
13. Soria B, Roche E, Berna G, Leon-Quinto T, Reig JA, Martin F. Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. *Diabetes.* 2000;49:157–162.
14. Suemori H, Tada T, Torii R, et al. Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. *Dev Dyn.* 2001;222:273–279.
15. Kawasaki H, Mizuseki K, Nishikawa S, et al. Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. *Neuron.* 2000;28:31–40.
16. Kawasaki H, Suemori H, Mizuseki K, et al. Generation of dopaminergic neurons and pigmented epithelia from primate ES cells by stromal cell-derived inducing activity. *Proc Natl Acad Sci USA.* 2002;99:1580–1585.
17. Kodama H, Hagiwara H, Sudo H, et al. MC3T3–G2/PA6 preadipocytes support in vitro proliferation of hemopoietic stem cells through a mechanism different from that of interleukin 3. *J Cell Physiol.* 1986;129:20–26.
18. Stoykova A, Fritsch R, Walther C, Gruss P. Forebrain patterning defects in Small eye mutant mice. *Development.* 1996;122:3453–3465.
19. Halder G, Callaerts P, Gehring WJ. Induction of ectopic eyes by targeted expression of the eyeless gene in Drosophila. *Science.* 1995;267:1788–1792.
20. Chow RL, Altmann CR, Lang RA, Hemmati-Brivanlou A. Pax6 induces ectopic eyes in a vertebrate. *Development.* 1999;126:4213–4222.
21. Hill RE, Favor J, Hogan BL, et al. Mouse small eye results from mutations in a paired-like homeobox-containing gene. *Nature.* 1991;354:522–525.
22. Furuta Y, Hogan BL. BMP4 is essential for lens induction in the mouse embryo. *Genes Dev.* 1998;12:3764–3775.
23. Ashery-Padan R, Marquardt T, Zhou X, Gruss P. Pax6 activity in the lens primordium is required for lens formation and for correct placement of a single retina in the eye. *Genes Dev.* 2000;14:2701–2711.
24. Chamberlain CG, McAvoy JW. Evidence that fibroblast growth factor promotes lens fibre differentiation. *Curr Eye Res.* 1987;6: 1165–1169.
25. de Iongh R, McAvoy JW. Spatio-temporal distribution of acidic and basic FGF indicates a role for FGF in rat lens morphogenesis. *Dev Dyn.* 1993;198:190–202.
26. Orr-Urtreger A, Givol D, Yayon A, Yarden Y, Lonai P. Developmental expression of two murine fibroblast growth factor receptors, flg and bek. *Development.* 1991;113:1419–1434.
27. Del Rio-Tsonis K, Jung JC, Chiu IM, Tsonis PA. Conservation of fibroblast growth factor function in lens regeneration. *Proc Natl Acad Sci USA.* 1997;94:13701–13706.
28. Schuldiner M, Yanuka O, Itskovitz-Eldor J, Melton DA, Benvenisty N. From the cover: effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc Natl Acad Sci USA.* 2000;97:11307–11312.
29. Rohrbach DH, Russell P, Church RL. In vitro production of basement membrane collagen by a clonal line of mouse lens epithelial cells. *Curr Eye Res.* 1981;1:267–273.
30. Pritchard DJ, Clayton RM, de Pomerai DI. 'Transdifferentiation' of chicken neural retina into lens and pigment epithelium in culture: controlling influences. *J Embryol Exp Morphol.* 1978;48:1–21.