Parthenogenetic activation

Activation stimulation of the *in vitro* mature oocytes was examined with electric stimulation or strontium chloride. For activation by electric stimulation, two electrical pulses of 150 V/mm, lasting for 50 μsec each, were applied for three cycles to the oocytes every 20 min in Zimmerman's cell fusion medium (Wolfe and Kraemer, 1992). Next, the oocytes were cultured in modified Whitten's medium containing 5 g/mL cytochalasin B (CB) or modified Whitten's medium including 5 g/mL CB and 2 mM 6-dimethylaminopurine (DMAP) for 4 h. For activation by strontium chloride, the oocytes were cultured in calcium-free modified Whitten's medium containing 5 g/mL CB and 10 mM strontium chloride for 6 h. Six and 24 h later, the effects of these activation treatments were evaluated by pronuclear formation and cleavage of the eggs, respectively.

As mentioned above, diploid parthenogenetic embryos were derived from the activated oocytes, preventing a second polar body emission by CB. When tetraploid parthenogenetic embryos were produced, each blastomere of the two-cell stage parthenogenetic embryos was fused by stimulation of two electrical pulses of 150 V/mm, lasting for 50 μsec.

Preparation of donor cells

As the nuclear donor, marmoset bone marrow mononuclear cells (MBMMNCs) from male adult and embryonic fibroblast cells from a female fetus at around 60–70 days of gestation were used. MBMMNCs were isolated from the femoral bone marrow by Ficoll-Paque density gradient centrifugation (Hibino et al., 1999). Collected cells were cultured in DMEM (Gibco, 11885-084) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Culture medium was changed every 3 days, and floating cells were removed while changing the culture medium. About 2 weeks later, adhesive MBMMNCs were split to new plates. For the preparation of fetal fibroblasts, small pieces of fetus depleted of the internal organs, head, feet, and arms were placed in the DMEM supplemented as mentioned above, and outgrowth cells were transplanted to new plates. To confirm the contribution of the donor nuclei to *in vitro* development of clone embryos, MBMMNCs were used as the nuclear donor, where the EGFP gene driven by CAG promoter was transduced using self-inactivating (SIN) lentiviral vectors based on the human immunodeficiency virus type 1 (HIV-1) vector (Miyoshi et al., 1998). An EGFP-expressing cell line was established by fluorescence-activated cell sorting (FACS) and subcloning.

The nuclear donors were introduced into the nuclear transfer procedure without treatment for cell cycle regulation. About 1 h before nuclear transfer, the cells from the Second to eighth passage were dispersed by treatment with 0.5% trypsin–5.3 mM EDTA solution and then kept in M2 medium at 4°C until use. The chromosome number and karyotype of the donor cells were analyzed as reported previously (Nesbitt and Francke, 1973; Sugawara et al., 2006).

Removal of chromosomes from recipient oocytes

The zona pellucida of the oocytes was slit with a glass needle along 10–20% of its circumference, close to the position of the first polar body. The MII chromosomes and spindle were located in the cortex of the oocyte near the first polar body and identified with differential interference microscopy without any staining. The oocytes were placed in PBI medium containing 5 g/mL CB and a small amount of cytoplasm containing the MII chromosomes was aspirated with an enucleation pipette. When confirmation of the removal operation was necessary, aspirated cytoplasm was stained with 10 μg/mL Hoechst 33342 and chromosomes were observed using a fluorescent microscope.

Nuclear transfer

After chromosome removal, the cytoplasms of the *in vitro* matured oocytes were used as recipients for nuclear transfer. Transplantation of donor nuclei into the recipient cytoplasms was performed through cell membrane fusion by electrical stimulation or sensitization of inactivated Sendai virus (HVJ, hemagglutinating virus of Japan). Using electrical stimulation, two electrical pulses of 150 V/mm, lasting 50 μsec each, were applied to Zimmerman's cell fusion medium after injection of donor cells into the perivitelline space of the recipient egg. In the case of inactivated Sendai virus, a donor cell was introduced into the perivitelline space with three to five times the volume of the virus, which was prepared at 2700 hemagglutinating units (HAU)/mL.

To construct diploid and tetraploid SCNT (NT-dip(o) and NT-tetra(dd)) embryos, single and two donor cell(s) were transferred to the oocyte cytoplasm after chromosome removal, respectively. Tetraploid SCNT [NT-tetra(od)] embryos with both oocyte- and donor-cell-derived nuclei were constructed by transferring a single donor cell to the oocyte

TABLE 2. PRODUCTIVE EFFICIENCY AND *IN VITRO* DEVELOPMENT OF CLONED EMBRYOS PRODUCED BY DIFFERENT PROCDURES

		Number of eggs (%)			*Number of cloned embryos developed to (%)****			
Timing of NT	*Fusion method**	*Used*	*Fused*	*Activated***	*Two-Cell*	*Eight-Cell*	*Morula*	*Blastocyst*
After activation	EP	43	27 (62.8)[a]	27 (100)	20 (74.1)[a]	4 (14.8)[a]	0 (0)	0 (0)
Immediately after activation	EP	91	54 (59.3)[a]	54 (100)	52 (96.3)[b]	13 (20.1)[a]	0 (0)	0 (0)
Before activation	HVJ	45	45 (100)[b]	43 (95.6)	34 (79.1)[a]	21 (48.8)[b]	0 (0)	0 (0)
Parthenogenetic control	—	—	—	86 (—)	78 (90.7)[b]	16 (18.6)[a]	0 (0)	0 (0)

In this experient, clone embryos were produced using embryonic fibroblasts as nuclear donor.
[a,b]Different letters in same column indicate the significant differences ($p < 0.05$).
*EP = two electrical pulses of 150 V/mm, lasting 50 μsec each. HVJ = inactivated Sendai virus applied at 2700 HAU/mL.
**All activated eggs were introduced to *in vitro* culture.
***Percentages were calculated from the number of activated eggs.

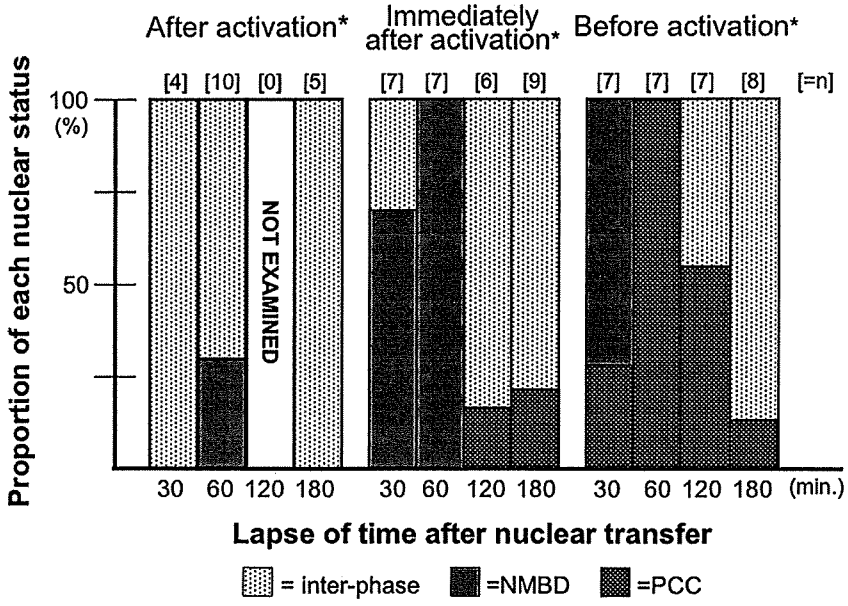


FIG. 1. Status of donor nuclei after transfer to recipient cytoplasm. Light-colored boxes show nuclei at interphase; gray boxes show nuclei that have undergone nuclear membrane breakdown; dark-colored boxes show nuclei that have undergone premature chromosome condensation. *Timing of nuclear transfer (into the recipient cytoplasm).

cytoplasm without chromosome removal. After parthenogenetic activation, because the emission of a second polar body was suppressed by CB treatment, it follows that oocyte-derived haploid genomes within the constructed embryos formed diploidy. Therefore, NT-tetra(od) embryos formed tetraploidy, by combining the donor cell-derived diploid genomes with the oocyte-derived diploid genomes.

In vitro *culture of embryos*

The *in vitro* culture of constructed embryos was performed using ISM1 and ISM2 culture medium (Nosan, Tokyo, Japan, 10500010 and 10510010). The embryos were cultured in ISM1 medium for the first 48 h (from days 1 to 3) and then co-cultured with inactivated marmoset embryonic fibroblasts in ISM2 medium supplemented with 10% FBS for 7 days. During culture in ISM2, 50% of the culture medium was exchanged every 48 h. The vapor phase conditions for culture were 38°C, 5% CO_2, and humidity saturation.

Analysis of the transplanted nuclei

Some constructed embryos were applied to whole-mount specimens 30, 60, 120, and 180 min after nuclear transfer. The status of the nuclei was classified into three groups: interphase (with a nuclear membrane), nuclear membrane breakdown (NMBD, with/without a slight nuclear membrane and without condensed chromosomes), and premature chromosome condensation (PCC, with condensed chromosomes).

Statistical assessment

Differences were analyzed using the chi-square test. Statistical significance was set at the $p < 0.05$ level.

Results

In vitro *maturation of oocytes*

For the present experiments, 1092 GV oocytes were collected from 104 marmosets. After 22–24 h incubation, 578 (52.9%) oocytes with a polar body were confirmed to be matured oocytes at metaphase II. Then, the matured oocytes were used in *in vitro* fertilization (IVF) to assess their viability. Of the IVF embryos, 5.3% (5/95) developed to the blastocyst stage, suggesting that the conditions of *in vitro* maturation may not have been optimal.

Oocyte activation

We conducted parthenogenetic stimulation using electrical pulses or strontium chloride to examine the activation procedure of *in vitro* matured oocytes in marmosets (Table 1). A large percentage of the oocytes could be effectively activated by electrical stimulation, with no need for supplementary treatment with DMAP. In contrast, oocyte activation did not occur with strontium treatment, even though this procedure has been successful in mice and rats. This finding suggests that the sensitivity of marmoset oocytes to chlorination strontium differs from that of mice and rats.

Development of cloned embryos

To construct viable SCNT embryos, we examined the transfer of donor nuclei into recipient cytoplasm and the timing of activation treatment (Table 2). In this experiment, embryonic fibroblasts were used as the nuclear donors. The induction of cells was performed 1 h before, immediately after, or 1 h after activation using electrical pulses or inactivated Sendai virus. The results showed that 95 to 100% of

TABLE 3. *IN VITRO* DEVELOPMENT OF DIPLOID AND TETRAPLOID CLONED EMBRYOS AND PARTHENOGENETIC EMBRYOS

		Genome			Number of embryos developed to (%)			
Types of embryos	*Ploidy*	*Oocyte*	*Donor cell*	*No. of embryos cultured*	*Two-Cell*	*Eight-Cell*	*Morula*	*Blastocyst*
NT-dip(o)	2n	–	+	29	29 (100)	21 (72.4)[a]	0 (0.0)	0 (0.0)
NT-tetra(od)	4n	+	+	21	21 (100)	19 (90.5)[b]	14 (66.7)	14 (66.7)
NT-tetra(dd)	4n	–	++	21	20 (90.5)	12 (57.1)[a]	0 (0.0)	0 (0.0)
PG-dip	2n	+	–	18	17 (94.4)	9 (50.0)[c]	0 (0.0)	0 (0.0)
PG-tetra	4n	++	–	9	9 (100)	4 (44.4)[c]	0 (0.0)	0 (0.0)

Nuclear transfer (fusion of donor cells) was performed using inactivated HVJ.
[a,b,c]Different letters in same column indicate the significant differences compared with NT-dip(o) embryos ($p < 0.05$).

the eggs in each group were successfully classified as pronuclear-stage eggs, while the induction rate was significantly high using HVJ. After *in vitro* culture of the constructed embryos, the highest developmental rate was observed when nuclear transfer was performed 1 h before activation. However, SCNT embryos in all groups and parthenogenetically activated oocytes did not develop beyond the eight-cell stage. These findings suggest that the most viable SCNT embryos were efficiently obtained by transferring donor cells into a nonactivated oocyte cytoplasm using HVJ.

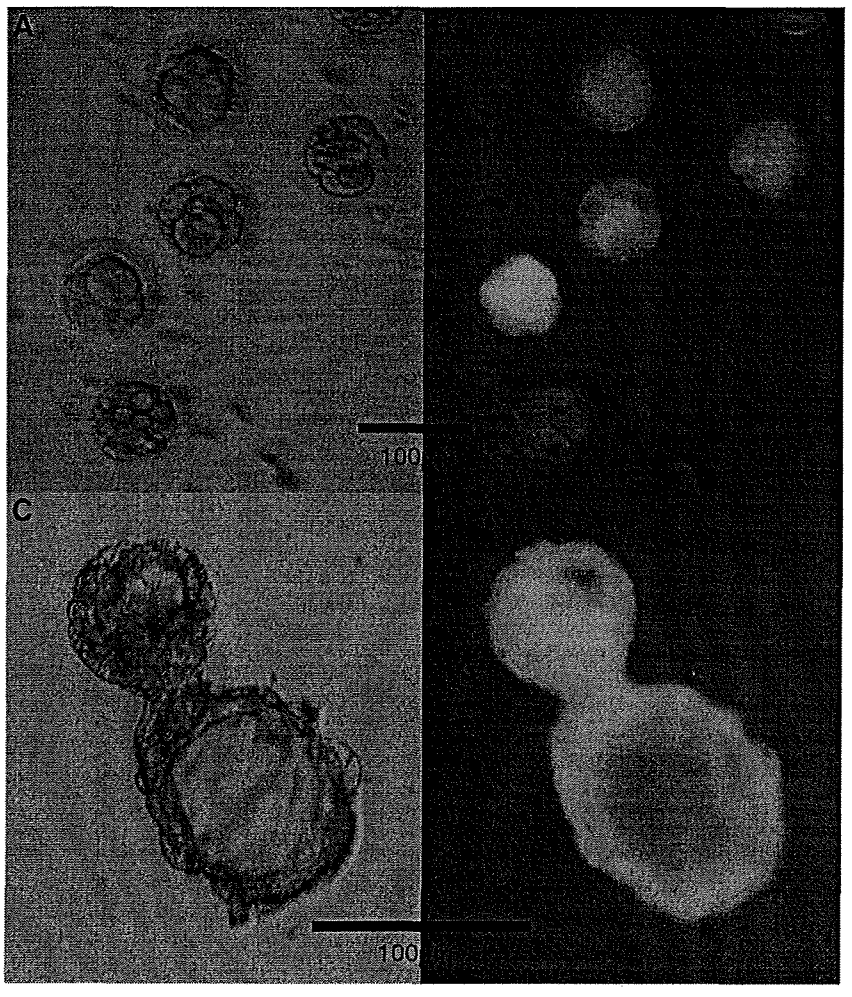


FIG. 2. Tetraploid SCNT [NT-tetra(od)] embryos at the four- to six-cell stage on day 3 (**A, B**) and at the blastocyst stage on day 8 (**C, D**), under bright-field and fluorescence microscopy, respectively. NT-tetra(od) embryos were produced by transferring an EGFP-gene-transfected MBMMNCs cell into a recipient oocyte cytoplasm with the MII chromosome. Living embryos expressed gene derived from donor cells.

Chromosome number and karyotype of donor cells

To assess cytogenetic quality, we examined the chromosome number and karyotype of the donor cells used for nuclear transfer (after the second to eighth passage). In embryonic fibroblast and MBMMNC lines, at least 74.0% (37/50) and 72.0% (36/50) of the analyzed cells, respectively, were confirmed to have a normal diploid chromosome number of 46 with estimated sex chromosomes of XX (female) and XY (male). Because chromosome analysis is commonly accompanied by an artificial error, it is inferred that almost all of the cells might be cytogenetically normal.

Status of the transplanted nuclei

When the donor nuclei were transplanted to an activated recipient cytoplasm, NMBD was partially observed but not PCC (Fig. 1). NMBD and PCC were observed when the nuclei were transferred to a nonactivated recipient. All nuclei transferred to the recipient 1 h before activation underwent PCC 60 min after nuclear transfer. These observations suggest that MPF activity in the marmoset oocyte is reduced 2 to 3 h after activation stimulation.

Effects of removal of oocyte genome and nuclear ploidy

To investigate the causes of low developmental potential of SCNT embryos, we determined whether the presence of the oocyte genome in recipient ooplasm and ploidy of constructed embryos affected the developmental potential, by creating diploid and tetraploid SCNT embryos or parthenogenetic embryos (Table 3). In this experiment, MBMMNCs and nonactivated oocytes were used as the nuclear donor and recipient cytoplasm, respectively, to construct SCNT embryos. As a result, only tetraploid NT-tetra(od) embryos produced by transferring a donor cell into a recipient bearing the MII chromosome (oocyte genome) developed into blastocysts (66.7%). In contrast, neither PG-dip/PG-tetra parthenogenetic embryos nor NT-dip(o)/NT-tetra(dd) embryos (whether diploid or tetraploid) produced using enucleated oocytes developed past the eight-cell stage.

Moreover, to confirm the contribution of donor nuclei to embryo development, NT-tetra(od) embryos were constructed using EGFP gene-transfected MBMMNCs, and were examined under fluorescence microscopy. The fluorescence signal of EGFP was observed in the cytoplasm of the embryos at six- to eight-cell and blastocyst stages (Fig. 2), confirming that donor cells certainly contributed to the development of NT-tetra(od) embryos until blastocyst stage.

Discussion

Embryo cloning in mammals can roughly be divided into two types of procedure depending on the timing of donor nucleus transplant and activation of the recipient egg cytoplasm. The first type involves reprogramming of the donor nucleus while using an activated or immediately after-activated oocyte cytoplasm as the recipient, as observed in sheep and cows (Campbell et al., 1996; Kato et al., 1998; Wilmut et al., 1997). The marmosets employed in the present study belong to the second type, as employed for mice, rats, and rhesus monkeys (Ogura et al., 2000; Ono et al., 2001a; Wakayama et al., 1998; Zhou et al., 2003; 2006), whereby a SCNT embryo with the highest developmental ability to the eight-cell stage is constructed when the donor cells have been transferred into the recipient prior to activation. However, the developmental ability of marmoset SCNT embryos was found to be extremely limited. On the other hand, tetraploid SCNT embryos produced by transferring a donor cell into a recipient oocyte cytoplasm with the MII chromosome [NT-tetra(od)] developed to the blastocyst stage, but neither tetraploid parthenogenetic embryos nor tetraploid SCNT embryos produced by transferring two donor cells into an enucleated oocyte cytoplasm [NT-dip(o) and NT-tetra(dd)] did. These results suggest that the presence of MII chromosome or cytoplasm proximal to the MII chromosome, but not the genome constitution of tetraploidy, plays an important role in the development of SCNT embryos to the blastocyst stage. However, parthenogenetic embryos did not develop to blastocyst, showing that the intactness of *in vitro* mature oocytes cannot support preimplantation development alone. Based on these observations, although the obvious mechanism is still unclear, it is concluded that the synergistic effects of an intact oocyte cytoplasm/genome, and some factors from donor cells passed through a fertilization process are necessary to gain developmental viability.

Previous studies on embryo cloning have shown that MPF activity in the cytoplasm of a meiotic oocyte at metaphase II plays an important role in reprogramming donor nuclei (McGrath and Solter, 1984; Roble et al., 1986; Wakayama et al., 2000). With exposure to high MPF activity surroundings, chromosome condensation is induced in nuclei at interphase through NMBD, and the status of early-stage embryos is initiated (Barns et al., 1993; Campbell et al., 1993; Colls and Robl, 1991; Szollosi et al., 1986). In the present study, whole-mount analysis confirmed that chromosome condensation of donor nuclei occurred after the transfer of nonactivated oocytes into the cytoplasm, resulting in the production of cloned embryos with the highest developmental ability. A similar observation was also reported in the rhesus monkey (Zhou et al., 2006). On the other hand, condensation did not occur in the case of activated oocytes, and the embryos showed poor development. These findings reconfirmed that sufficient exposure of the nuclear genome to high MPF activity surroundings is a principal event for cloned embryos to gain the benefit of reprogramming factor in the recipient cytoplasm. Moreover, a recent topical report in a clone study, which shows that zygotic cytoplasm at metaphase is also actively involved in the reprogramming of differentiated nuclei at metaphase (Egli et al., 2007), could support this logic.

Cloned animals have been obtained using nuclei from various kinds of mammalian cells, including ES cells, although the selection of donor cells remains controversial with regard to cloning procedures. The chromosome number/karyotype is one of the most essential factors related to the construction of viable healthy cloned embryos; however, concerning gene manipulation with donor cells, proliferation activity (ideally with motility) during *in vitro* culture is also an important factor. Here, when the cell lines used as the nuclear donor were subsequently cultured, the proliferation of a skin-derived fibroblast cell line decreased after the 10th passage, but that of the MBMMNC line did not (data not shown). From this point of view, fibroblasts may not be suitable as donors, although they are easy to collect.

The MBMMNCs used in this study have not been characterized by cell selective markers such as CD45 or differentiation potency to adipocytes, chondrocytes, and osteocytes. It is, however, likely that part of these MBMMNCs are mesenchymal stem cells because mesenchymal stem cell lines are established from MBMMNCs (Fuchs and Segre, 2000; Prockop, 1997). These findings suggest that MBMMNCs are convenient for advanced cloning procedures.

In the SCNT procedure in mammals, transfer of donor nuclei into an egg cytoplasm is performed with membrane fusion of the whole cell/karyoplast (nuclei with surrounding cytoplasm) and egg, or direct injection of nuclei through the egg membrane (Kimura and Yanagimachi, 1995). In this study, nuclear transfer was attempted through membrane fusion using physical stimulation in the form of electrical pulses (Vienken and Zimmermann, 1982; Willadsen, 1986; Wolfe and Kraemer, 1992), or the hemagglutinating activity of HVJ (Sendai virus) (McGrath and Solter, 1983). With the electrical pulses, nuclear transfer was successfully achieved, but the efficiency was evidently improved with HVJ. HVJ has a wide host range, with mice, rats, including cotton rats, hamsters, guinea pigs, rabbits, ferrets, pikas, pigs, and marmosets, all showing sensitivity. Especially in mice, HVJ is often used for nuclear transfer; however, the fusion activity of cell membranes in other species remains unclear. Here, HVJ was also shown to be an effective tool for nuclear transfer in marmosets.

In the present study, parthenogenetic development of marmoset embryos was not observed beyond the eight-cell stage. As reported previously, in the monkey *Macaca mulatta* (Mitalipov et al., 2001; 2002), in mammals such as mice (Graham, 1970; Kaufman, 1973), rats (Jiang, et al., 2002; Krivokharchenko et al., 2003), cows (Campbell et al., 2000; Liu et al., 1998), pigs (Grupen et al., 1999; Wang et al., 1999), and so on, in which *in vitro* culture of preimplantation fertilized eggs has been established, it has been confirmed that parthenogenetically activated oocytes can develop to the blastocyst stage *in vitro*. Also, in marmosets (Marshall et al., 1998), parthenogenetic embryos can undergo implantation after transfer to a recipient female, in which the parthenogenetic embryos were derived from *in vivo* mature oocytes and transferred at the four-cell stage. Thus, as our study showed poor development of the parthenogenetic embryos, there might be room for the improvement in *in vitro* maturation/culture conditions of marmoset oocytes/embryos.

In the marmoset, the optimal procedure for viable SCNT embryo has not yet been established. Meanwhile, considering the necessary characteristics of laboratory primates, marmosets are considered as a model animal for preclinical experiments, as already shown for spinal cord injuries (Iwanami et al., 2005a, 2005b). This study also indicated the possibility of advanced applications using the marmoset, similar to those that have been established in mice, and perhaps the production of cloned individuals aimed at gene manipulation (Rideout et al., 2000) and the establishment of somatic cell nuclear transfer-derived ES cells aimed at regenerative medicine (Kishigami et al., 2006; Rideout et al., 2002). However, although there is a gradual progress in the gene expression analysis of cloned embryos or fetuses (Blelloch et al., 2006; Hiiragi and Solter, 2005; Humpherys et al., 2001; Inoue et al., 2002; Kang et al., 2001; Ogawa et al., 2003; Suemizu et al., 2003), the cause of developmental abnormality, and thus, the low success rate (Eggan et al., 2001; Hill et al., 1999; Ono et al., 2001a, 2001b; Renard et al., 1999; Shimozawa et al., 2002, 2003, 2006; Wakayama and Yanagimachi, 1999; Tamashiro et al., 2000, 2002) and the precise mechanism of nuclear reprogramming remain unclear. Prior to the introduction of cloning techniques through SCNT for therapeutic application to human patients, these problems need to be addressed, and accordingly, simultaneous progress in clone studies regarding basic biological mechanisms and preclinical application is expected.

Acknowledgments

This study was supported by a grant-in-aid for Scientific Research (18500336) to E.S. from the Japan Society for the Promotion of Science, a grant from Japan Science and Technology Agency (SORST) to H.O., and a Grant-in-aid for 21st Century and Global COE program to Keio University from The Ministry of Education, Culture, Sports, Science, and Technology (MEXT). A part of this study is the result of "Highly creative animal model development for brain sciences" carried out under the Strategic Research Program for Brain Sciences by the Ministry of Education, Culture, Sports, Science and Technology of Japan. We thank Dr. T. Kono (Tokyo University of Agriculture, JP) and Dr. N. Maeda (Hiroshima University, JP) for helpful discussion. We also thank Ms. M. Kamioka, Ms. F. Toyota and Ms. S. Oba (JAC Inc., JP) for their professional animal cares. Lenti viral vector was kindly provided by Dr. H. Miyoshi at RIKEN BRC.

Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

References

Barnes, F.L., Collas, P., Powell, R. et al. (1993). Influence of recipient oocyte cell cycle stage on DNA synthesis, nuclear envelope breakdown, chromosome constitution, and development in nuclear transplant bovine embryos. Mol. Reprod. Dev. 36, 33–41.

Blelloch, R., Wang, Z., Meissner, A., et al. (2006). Reprogramming efficiency following somatic cell nuclear transfer is influenced by the differentiation and methylation state of the donor nucleus. Stem Cells 24, 2007–2013.

Bradley, A., Evans, M., Kaufman, M.H., et al. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature 309, 255–256.

Byrne, J.A., Pedersen, D.A., Clepper, L.L., et al. (2007). Producing primate embryonic stem cells by somatic cell nuclear transfer. Nature 450, 497–502.

Campbell, K.D., Reed, W.A., and White, K.L. (2000). Ability of integrins to mediate fertilization, intracellular calcium release, and parthenogenetic development in bovine oocytes. Biol. Reprod. 62, 1702–1709.

Campbell, K.H., Ritchie, W.A., and Wilmut, I. (1993). Nuclear-cytoplasmic interactions during the first cell cycle of nuclear transfer reconstructed bovine embryos: implications for deoxyribonucleic acid replication and development. Biol. Reprod. 49, 933–942.

Campbell, K.H., McWhir, J., Ritchie, W.A., et al. (1996). Sheep cloned by nuclear transfer from a cultured cell line. Nature 380, 64–66.

Cibelli, J.B., Stice, S.L., Golueke, P.J., et al. (1998). Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science 280, 1256–1258.

Collas, P., and Robl, J.M. (1991). Relationship between nuclear remodeling and development in nuclear transplant rabbit embryos. Biol. Reprod. 45, 455–465.

Delimitreva, S., Zhivkova, R., Isachenko, E., et al. (2006). Meiotic abnormalities in in vitro-matured marmoset monkey (*Callithrix jacchus*) oocytes: development of a non-human primate model to investigate causal factors. Hum. Reprod. 21, 240–247.

Eggan, K., Akutsu, H., Loring, J., et al. (2001). Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. Proc. Natl. Acad. Sci. USA 98, 6209–6214.

Egli, D., Rosains, J., Birkhoff, G., et al. (2007). Developmental reprogramming after chromosome transfer into mitotic mouse zygotes. Nature 447, 679–685.

Fuchs, E., and Segre, J.A. (2000). Stem cells: a new lease on life. Cell 100, 143–155.

Gilchrist, R.B., Nayudu, P.L., and Hodges, J.K. (1997). Maturation, fertilization, and development of marmoset monkey oocytes in vitro. Biol. Reprod. 56, 238–246.

Graham, C.F. (1970). Parthenogenetic mouse blastocysts. Nature 226, 165–167.

Grupen, C.G., Verma, P.J., Du, Z.T., et al. (1999). Activation of in vivo- and in vitro-derived porcine oocytes by using multiple electrical pulses. Reprod. Fertil. Dev. 11, 457–462.

Hibino, H., Tani, K., Ikebuchi, K., et al. (1999). The common marmoset as a target preclinical primate model for cytokine and gene therapy studies. Blood 93, 2839–2848.

Hiiragi, T., and Solter, D. (2005). Reprogramming is essential in nuclear transfer. Mol. Reprod. Dev. 70, 417–421.

Hill, J.R., Roussel, A.J., Cibelli, J.B., et al. (1999). Clinical and pathologic features of cloned transgenic calves and fetuses (13 case studies). Theriogenology 51, 1451–1465.

Hirabayashi, M., Kato, M., Ishikawa, A., et al. (2003). Factors influencing chromosome condensation and development of cloned rat embryos. Cloning Stem Cells 5, 35–42.

Humpherys, D., Eggan, K., Akatsu, H., et al. (2001). Epigenetic instability in ES cells and cloned mice. Science 293, 95–97.

Inoue, K., Kohda, T., Lee, J., et al. (2002). Faithful expression of imprinted genes in cloned mice. Science 295, 297.

Ito, J., Hirabayashi, M., Kato, M., et al. (2005). Contribution of high p34cdc2 kinase activity to premature chromosome condensation of injected somatic cell nuclei in rat oocytes. Reproduction 129, 171–180.

Iwanami, A., Yamane, J., Katoh, H., et al. (2005a). Establishment of graded spinal cord injury model in a non-human primate: the common marmoset. J. Neurosci. Res. 80, 172–181.

Iwanami, A., Kakneko, S., Nakamura, M., et al. (2005b). Transplantation of human neural stem/progenitor cells promotes functional recovery after spinal cord injury in common marmoset. J. Neurosci. Res. 80, 182–190.

Jiang, J.Y., Mizuno, S., Mizutani, E., et al. (2002). Parthenogenetic activation and subsequent development of rat oocytes in vitro. Mol. Reprod. Dev. 61, 120–125.

Kang, Y.K., Koo, D.B., Park, J.S., et al. (2001). Aberrant methylation of donor genome in cloned bovine embryos. Nat. Genet. 28, 173–177.

Kato, Y., Tani, T., Sotomaru, Y., et al. (1998). Eight calves cloned from somatic cells of a single adult. Science 282, 2095–2098.

Kaufman, M.H. (1973). Parthenogenesis in the mouse. Nature 242, 475–476.

Kimura, Y., and Yanagimachi, R. (1995). Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. Development 121, 2397–2405.

Kishigami, S., Wakayama, S., van Thuan, N., et al. (2006). Cloned mice and embryonic stem cell establishment from adult somatic cells. Hum. Cell 19, 2–10.

Krivokharchenko, A., Popova, E., Zaitseva, I., et al. (2003). Development of parthenogenetic rat embryos. Biol. Reprod. 68, 829–836.

Kurita, R., Sasaki, E., Yokoo, T., et al. (2006). Tal1/Scl gene transduction using a lentiviral vector stimulates highly efficient hematopoietic cell differentiation from common marmoset (*Callithrix jacchus*) embryonic stem cells. Stem Cells 24, 2014–2022.

Li, P., Tong, C., Mehrian-Shai, R., et al. (2008). Germline competent embryonic stem cells derived from rat blastocysts. Cell 135, 1299–1310.

Liu, L., Ju, J.C., and Yan, X. (1998). Parthenogenetic development and protein patterns of newly matured bovine oocytes after chemical activation. Mol. Reprod. Dev. 49, 298–307.

Lopata, A., Summers, P.M., and Hearn, J.P. (1988). Births following the transfer of cultured embryos obtained by in vitro and in vivo fertilization in the marmoset monkey (*Callithrix jacchus*). Fertil. Steril. 50, 503–509.

Mansfield, K. (2003). Marmoset models commonly used in biomedical research. Comp. Med. 53, 383–392.

Marshall, V.S., Wilton, L.J., and Moore, H.D. (1998). Parthenogenetic activation of marmoset (*Callithrix jacchus*) oocytes and the development of marmoset parthenogenones in vitro and in vivo. Biol. Reprod. 59, 1491–1497.

Marshall, V.S., Browne, M.A., Knowles, L., et al. (2003). Ovarian stimulation of marmoset monkeys (*Callithrix jacchus*) using recombinant human follicle stimulating hormone. J. Med. Primatol. 32, 57–66.

McGrath, J., and Solter, D. (1983). Nuclear transplantation in the mouse embryo by microsurgery and cell fusion. Science 220, 1300–1302.

McGrath, J., and Solter, D. (1984). Inability of mouse blastomere nuclei transferred to enucleated zygotes to support development in vitro. Science 226, 1317–1319.

Mitalipov, S.M., Nusser, K.D., and Wolf, D.P. (2001). Parthenogenetic activation of rhesus monkey oocytes and reconstructed embryos. Biol. Reprod. 65, 253–259.

Mitalipov, S.M., Yeoman, R.R., Nusser, K.D., et al. (2002). Rhesus monkey embryos produced by nuclear transfer from embryonic blastomeres or somatic cells. Biol. Reprod. 66, 1367–1373.

Miyoshi, H., Blömer, U., Takahashi, M., et al. (1998). Development of a self-inactivating lentivirus vector. J. Virol. 72, 8150–8157.

Nayudu, P.L., Wu, J., and Michelmann, H.W. (2003). In vitro development of marmoset monkey oocytes by pre-antral follicle culture. Reprod. Domest. Anim. 38, 90–96.

Nesbitt, M.N., and Francke, U. (1973). A system of nomenclature for band patterns of mouse chromosomes. Chromosoma 41, 1451–1458.

Ogawa, H., Ono, Y., Shimozawa, N., et al. (2003). Disruption of imprinting in mouse cloned fetuses from ES cells. Reproduction 126, 549–557.

Ogura, A., Inoue, K., Ogonuki, N., et al. (2000). Production of male cloned mice from fresh, cultured, and cryopreserved immature Sertoli cells. Biol. Reprod. 62, 1579–1584.

Ono, Y., Shimozawa, N., Ito, M., et al. (2001a). Cloned mice from fetal fibroblast cells arrested at metaphase by a serial nuclear transfer. Biol. Reprod. 64, 44–50.

Ono, Y., Shimozawa, N., Muguruma, K., et al. (2001b). Production of cloned mice from embryonic stem cells arrested at metaphase. Reproduction 122, 731–736.

Prockop, D.J. (1997). Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276, 71–74.

Renard, J.P., Chastant, S., Chesne, P., et al. (1999). Lymphoid hypoplasia and somatic cloning. Lancet 353, 1489–1491.

Rideout, W.M., 3rd, Wakayama, T., Wutz, A., et al. (2000). Generation of mice from wild-type and targeted ES cells by nuclear cloning. Nat. Genet. 24, 109–110.

Rideout, W.M., 3rd, Hochedlinger, K., Kyba, M., et al. (2002). Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. Cell 109, 17–27.

Robertson, E., Bradley, A., Kuehn, M., et al. (1986). Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. Nature 323, 445–448.

Robl, J.M., Gilligan, B., Critser, E.S., et al. (1986). Nuclear transplantation in mouse embryos: assessment of recipient cell stage. Biol. Reprod. 34, 733–739.

Sasaki, E., Hanazawa, K., Kurita, R., et al. (2005). Establishment of novel embryonic stem cell lines derived from the common marmoset *(Callithrix jacchus)*. Stem Cells 23, 1304–1313.

Schnieke, A.E., Kind, A.J., Ritchie, W.A., et al. (1997). Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science 278, 2130–2133.

Shimozawa, N., Ono, Y., Kimoto, S., et al. (2002). Abnormalities in cloned mice are not transmitted to the progeny. Genesis 34, 203–207.

Shimozawa, M., Tajima, S., Azuma, N., et al. (2003). Histological study of the hypertrophic placentas and open eyelids observed in cloned fetuses. J. Reprod. Dev. 49, 221–226.

Shimozawa, N., Sotomaru, Y., Eguchi, N., et al. (2006). Phenotypic abnormalities observed in aged cloned mice from embryonic stem cells after long-term maintenance. Reproduction 132, 435–441.

Suemizu, H., Aiba, K., Yoshikawa, T., et al. (2003). Expression profiling of placentomegaly associated with nuclear transplantation of mouse ES cells. Dev. Biol. 253, 36–53.

Sugawara, A., Goto, K., Sotomaru, Y., et al. (2006). Current status of chromosomal abnormalities in mouse embryonic stem cell lines used in Japan. Comp. Med. 56, 31–34.

Szollosi, D., Czolowska, R., Soltynska, M.S., et al. (1986). Remodelling of thymocyte nuclei in activated mouse oocytes: an ultrastructural study. Eur. J. Cell Biol. 42, 140–151.

Tamashiro, K.L., Wakayama, T., Blanchard, R.J., et al. (2000). Postnatal growth and behavioral development of mice cloned from adult cumulus cells. Biol. Reprod. 63, 328–334.

Tamashiro, K.L., Wakayama, T., Akutsu, H., et al. (2002). Cloned mice have an obese phenotype not transmitted to their offspring. Nat. Med. 8, 262–267.

Thomas, K.R., and Capecchi, M.R. (1987). Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell 51, 503–512.

Thomson, J.A., Kalishman, J., Golos, T.G., et al. (1996). Pluripotent cell lines derived from common marmoset *(Callithrix jacchus)* blastocysts. Biol. Reprod. 55, 254–259.

Vienken, J., and Zimmermann, U. (1982). Electric field-induced fusion: electro-hydraulic procedure for production of heterokaryon cells in high yield. FEBS Lett. 137, 11–13.

Wakayama, T., Perry, A.C., Zuccotti, M., et al. (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394, 369–374.

Wakayama, T., and Yanagimachi, R. (1999). Cloning of male mice from adult tail-tip cells. Nat. Genet. 22, 127–128.

Wakayama, T., Tateno, H., Mombaerts, P., et al. (2000). Nuclear transfer into mouse zygotes. Nat. Genet. 24, 108–109.

Wang, W.H., Machaty, Z., Ruddock, N., et al. (1999). Activation of porcine oocytes with calcium ionophore: effects of extracellular calcium. Mol. Reprod. Dev. 53, 99–107.

Willadsen, S. (1986). Nuclear transplantation in sheep embryos. Nature 320, 63–65.

Wilmut, I., Schnieke, A.E., McWhir, J., et al. (1997). Viable offspring derived from fetal and adult mammalian cells. Nature 385, 810–813. Erratum (1997) in Nature 386, 200.

Wilton, L.J., Marshall, V.S., Piercy, E.C., et al. (1993). In vitro fertilization and embryo development in the marmoset monkey (*Callithrix jacchus*). J. Reprod. Fertil. 97, 481–486.

Wolfe, B.A., and Kraemer, D.C. (1992). Methods in bovine nuclear transfer. Theriogenology 37, 5–15.

Yang, S.H., Cheng, P.H., Banta, H., et al. (2008). Towards a transgenic model of Huntington's disease in a non-human primate. Nature 453, 921–924.

Zhou, Q., Renard, J.P., Le Friec, G., et al. (2003). Generation of fertile cloned rats by regulating oocyte activation. Science 302, 1179.

Zhou, Q., Yang, S.H., Ding, C.H., et al. (2006). A comparative approach to somatic cell nuclear transfer in the rhesus monkey. Hum. Reprod. 21, 2564–2571.

Address correspondence to:
Dr. Yusuke Sotomaru
Natural Science Center for Basic Research and Development
Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima
Hiroshima, Japan 734-8551

E-mail: sotomaru@hiroshima-u.ac.jp

Vol 459|28 May 2009|doi:10.1038/nature08090 nature

ARTICLES

Generation of transgenic non-human primates with germline transmission

Erika Sasaki[1], Hiroshi Suemizu[1], Akiko Shimada[1], Kisaburo Hanazawa[2], Ryo Oiwa[1], Michiko Kamioka[1], Ikuo Tomioka[1,3], Yusuke Sotomaru[5], Reiko Hirakawa[1,3], Tomoo Eto[1], Seiji Shiozawa[1,4], Takuji Maeda[1,4], Mamoru Ito[1], Ryoji Ito[1], Chika Kito[1], Chie Yagihashi[1], Kenji Kawai[1], Hiroyuki Miyoshi[6], Yoshikuni Tanioka[1], Norikazu Tamaoki[1], Sonoko Habu[7], Hideyuki Okano[4] & Tatsuji Nomura[1]

The common marmoset (*Callithrix jacchus*) is increasingly attractive for use as a non-human primate animal model in biomedical research. It has a relatively high reproduction rate for a primate, making it potentially suitable for transgenic modification. Although several attempts have been made to produce non-human transgenic primates, transgene expression in the somatic tissues of live infants has not been demonstrated by objective analyses such as polymerase chain reaction with reverse transcription or western blots. Here we show that the injection of a self-inactivating lentiviral vector in sucrose solution into marmoset embryos results in transgenic common marmosets that expressed the transgene in several organs. Notably, we achieved germline transmission of the transgene, and the transgenic offspring developed normally. The successful creation of transgenic marmosets provides a new animal model for human disease that has the great advantage of a close genetic relationship with humans. This model will be valuable to many fields of biomedical research.

The use of transgenic mice has contributed immensely to biomedical science. However, the genetic and physiological differences between primates and mice—including their neurophysiological functions, metabolic pathways, and drug sensitivities—hamper the extrapolation of results from mouse disease models to direct clinical applications in humans. Thus, the development of non-human primate models that mimic various human systems would accelerate the advance of biomedical research. In particular, genetically modified primates would be a powerful human disease model for preclinical assessment of the safety and efficacy of stem-cell or gene therapy.

The common marmoset (*Callithrix jacchus*) is a small New World primate that, because of its size, availability, and unique biological characteristics[1], has attracted considerable attention as a potentially useful biomedical research animal in fields such as neuroscience, stem cell research, drug toxicology, immunity and autoimmune diseases, and reproductive biology. Marmosets have a relatively short gestation period (about 144 days), reach sexual maturity at 12–18 months, and females have 40–80 offspring during their life. Therefore, the application of transgenic techniques to marmosets may be feasible, and would greatly facilitate the study of human disease. In contrast, the more commonly used Old World primates, such as the rhesus monkey (*Macaca mulatta*) and cynomolgus monkey (*Macaca fascicularis*), show slow sexual maturation (about 3 years) and have fewer offspring (around 10) over the female lifespan. Thus, even though marmosets are less closely related to humans than either apes or Old World primates, their potential as transgenic primate models of human disease means they may be uniquely valuable.

Obtaining large numbers of oocytes from primates for transgenic experiments is limited by ethical and economic constraints. However, because retroviral vectors allow the efficient integration of a provirus into the host genome[2–4], their use requires fewer oocytes than some other techniques. Furthermore, the injection of a lentiviral vector into the perivitelline space of a pre-implantation embryo, which is less invasive than injection into the pronucleus, is an advantageous method for generating transgenic animals. In fact, transgenic modification of rhesus monkeys using retroviral vectors and a lentiviral vector[5–7] has been attempted. In these studies, genomic integration and expression of the transgene was observed in the placenta, but not in the infants' somatic tissues, by objective analyses such as PCR with reverse transcription (RT–PCR) or western blotting.

The recombinant adeno-associated virus has been used for the targeted knockout of the cystic fibrosis transmembrane conductance receptor gene in swine fetal fibroblasts, and targeted gene knockout pigs have been generated by somatic cell nuclear transfer (SCNT) of the fibroblast nuclei into oocytes[8,9]. Although conceptually this method could be used to make targeted gene-knockout primates, marmoset SCNT techniques are not available at present.

Here we successfully produced transgenic marmosets, by injecting a lentiviral vector containing an enhanced green fluorescent protein (EGFP) transgene[10] into marmoset embryos. Four out of five transgenic marmosets expressed the EGFP transgene in neonatal tissues; the fifth expressed it in the placenta. Two showed transgene expression in the germ cells, and one fathered a healthy transgenic neonate. Our method for producing transgenic primates promises to be a powerful tool for studying the mechanisms of human diseases and developing new therapies.

Production of transgenic marmosets

In a pilot study, we showed that pre-implantation marmoset embryos obtained through natural intercourse had much better developmental potential than embryos obtained by *in vitro* fertilization (IVF). Therefore, both natural and IVF embryos were used in this study.

[1]Central Institute for Experimental Animals, 1430 Nogawa, Miyamae-ku, Kawasaki, Kanagawa 216-0001, Japan. [2]Department of Urology, Juntendo University Nerima Hospital 3-1-10 Takanodai, Nerima-ku, Tokyo 177-8521, Japan. [3]Center for Integrated Medical Research, [4]Department of Physiology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. [5]Natural Science Centre for Basic Research and Development, Hiroshima University 1-2-3, Kasumi, Minami-ku, Hiroshima 734-8551, Japan. [6]Subteam for Manipulation of Cell Fate, RIKEN BioResource Centre, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan. [7]Department of Immunology, Tokai University School of Medicine, Bohseidai, Isehara, Kanagawa 259-1193, Japan.

To introduce the EGFP gene into the marmoset embryo, three kinds of self-inactivating lentiviral vectors were constructed on the basis of human immunodeficiency virus type 1 (HIV-1), and each carried a different promoter, CAG, CMV or EF1-α. The self-inactivating lentiviral vectors were named CAG-EGFP, CMV-EGFP and EF1-α-EGFP, respectively.

All lentiviral vector injections were performed at the earliest embryonic stage possible using an Eppendorf FemtoJet express and a Narishige micromanipulator. Twenty-seven IVF embryos and 64 natural embryos were injected with a high titre of the lentiviral vector, from 5.6×10^9 to 5.6×10^{11} transducing units per ml (Table 1). Because the perivitelline space of the marmoset early embryo is rather small, 16 of the 27 IVF embryos, and 49 of the 64 natural embryos, at the pronuclear-to-morula stage, were first placed in 0.25 M sucrose in PB1 medium (0.25 M sucrose medium), which made the perivitelline space expand 1.2–7.5-fold (data not shown). The lentiviral vector was then injected into the perivitelline space (Supplementary Data 1). Virus was injected into the blastocoel of the remaining 11 IVF and 15 natural embryos at the blastocyst stage, without the 0.25 M sucrose treatment (Supplementary Data 1).

Immediately after injection, 4 of the IVF and 12 of the natural embryos were transferred to recipient females. The rest were examined for the expression of EGFP, starting 48 h after injection. Among the sucrose-treated IVF and natural early embryos at 48 h after injection, 68.8% and 97.7% expressed EGFP, respectively; of the non-sucrose-treated IVF and natural embryos injected with lentivirus as blastocysts, 85.7% and 87.5% expressed EGFP, respectively (Supplementary Data 1). Therefore, 61 of the natural embryos and 19 of the IVF embryos were transferred to surrogate mothers (Table 1). For the transfers, the recipients were synchronized with the donor oocyte cycle; each recipient received 1–3 embryos per cycle, and 50 surrogate mother animals were used.

Of the surrogate mothers, seven that received natural or IVF embryos became pregnant. Three recipients miscarried on days 43, 62 and 82, and the other four delivered five healthy offspring (three singletons, one pair of twins), one male (number 666) and four females, on days 144–147 after ovulation (Fig. 1). For the infants, the lentiviral vector injection had been performed at the four-cell stage (584), the pronuclear stage (587), and the morula stage (588, 594 and 666). The EGFP transgene was driven by the CAG promoter in three newborns (584, 587 and 588) and by the CMV promoter in the other two (594 and 666; Supplementary Data 1).

Table 1 | Production rates of transgenic marmosets

	Artificial reproductive technique	Natural
Number of GV oocytes	460	No data
Number of matured oocytes (only MII)	201	No data
Number of IVFs performed (including MI)	272	No data
Number of fertilized oocytes	121	No data
Fertilization rate (fertilization per GV)	26.3%	No data
Fertilization rate (fertilization per IVF)	44.5%	No data
Lentiviral injections	27	64
EGFP expression confirmed after 48 h or later	23	52
EGFP expression	17	50
EGFP expression rate	73.9%	96.2%*
ETs	19	61
Number of surrogates	13	37
Number of pregnancies	1	6
Number of deliveries	1	3
Births	1	4
Birth rate (birth per ET)	5.20%	6.55%
Number of Tgs	1	4
Production rate (Tg per injection)	3.70%	6.25%
Production rate (Tg per ET)	5.26%	6.25%
Production rate (Tg per birth)	100	100

ET, embryo transfer; GV, germinal vesicle; MI, metaphase I; MII, metaphase II; Tg, transgenes.
*$P < 0.01$, chi-squared analysis.

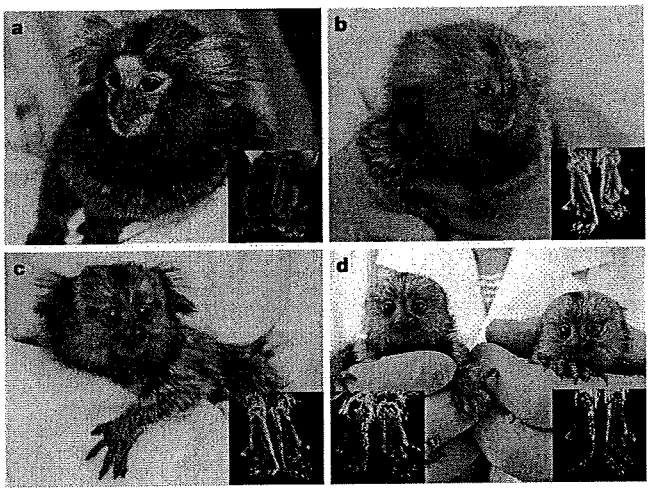


Figure 1 | Self-inactivating lentiviral vector-derived EGFP transgenic marmosets. a–d, The transgenic marmoset infants are shown. Shown are 584 (Hisui) (**a**), 587 (Wakaba) (**b**), 588 (Banko) (**c**), and twin infants 594 (Kei)/666 (Kou) (**d**). 584, 587 and 588 contained CAG-EGFP and 594/666 carried CMV-EGFP. Inset boxes in each panel show epifluorescent images of the paw of a transgenic animal (right), compared to a wild-type animal's foot pad (left). All animals except 588 expressed EGFP in their paw. 666 expressed EGFP at a slightly lower level.

EGFP transgene integration in the genome

The integration, transcription and expression of the transgene in the infant marmosets were examined using tissues that could be acquired noninvasively (placenta, hair roots, skin and peripheral blood cells). Because marmosets usually eat the placenta after delivery, only three placentae (584, 588 and that shared by twins 594/666) were collected and available for analysis[11].

The placental DNA from infants 584 and 588 showed high levels of the transgene content by real-time PCR, whereas that from 594/666 showed a relatively low level (Supplementary Data 2). The transgene was detected in the hair roots, skin and peripheral blood from infants 584, 587, 594 and 666.

Copy numbers of the integrated transgene were determined by Southern blotting analysis. At least four copies of the transgene were integrated into the genome of animal 584, and two copies were present in the genome of animal 587 (Fig. 2). Several integration sites in the genomic DNA of skin fibroblast cells, peripheral blood, the placenta of 594 and 666, and the placenta of 588 were found. Infant 588 showed transgene integration only in the placenta (Fig. 2).

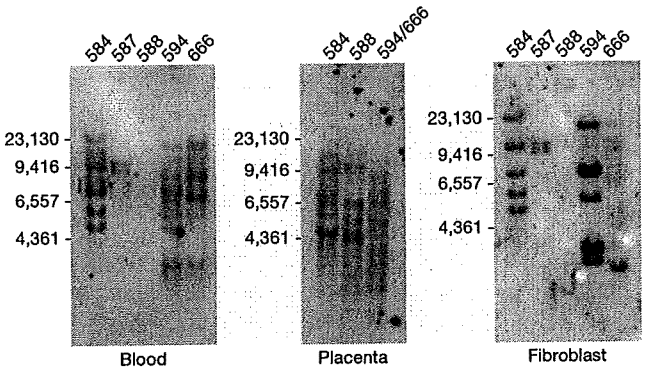


Figure 2 | Transgene insertions in several infant tissues. Southern blot analysis. All infants except 588 showed transgene integration in the skin fibroblast cells and blood, whereas 588 showed transgene integration in the placenta. The lane markers on the left of each gel represent base pairs.

To identify the chromosomal transgene integration sites, fluorescence *in situ* hybridization (FISH) was performed. Consistent with the Southern blotting analysis, the FISH results showed several integration sites in the chromosomes of peripheral blood mononuclear cells (MNCs), and further showed that each infant had different transgene integration patterns with patterns the sometimes varied among different MNCs (Supplementary Fig. 1 and Supplementary Data 3). In 584, four transgene integration sites were seen, on chromosomes 2, 7 and 13; in 587, two distinct signals were recognized in the peripheral blood lymphocyte DNA, on chromosomes 3 and 12. No signal was detected in the peripheral blood lymphocyte samples from 588, and several transgene integration patterns were seen in 594 and 666. Infant 594 had at least three different transgene integration patterns, and more than six patterns may have occurred. Infant 666 showed the largest number of integration patterns, up to 13. Moreover, although this animal was male, of the 13 investigated karyograms, eight samples were of the female karyotype, owing to haematopoietic chimaerism caused by blood exchange with his twin, 594.

Expression of the EGFP transgene

EGFP messenger RNA was detected in the hair roots of all the infants except 588 and in the peripheral blood cells of 584 and 587, by RT–PCR. Transcription of the EGFP gene was indicated in all of the placental samples, 584, 588 and 594/666 (Fig. 3a–c).

To assess EGFP expression in tissues, EGFP fluorescence was examined directly by fluorescence microscopy, and immunohistochemical analysis of the hair roots, frozen sections of a small piece of ear tissue, and placenta samples was performed (Fig. 3d–g). EGFP was strongly expressed in the epidermal cells of the ear tissue and stromal cells of the placenta. In all of the animals except 588, EGFP expression was observed in the hair roots and skin. Placental samples from 584 and 588 also showed high levels of EGFP, but it was undetectable in 594/666 (Supplementary Figs 2–4).

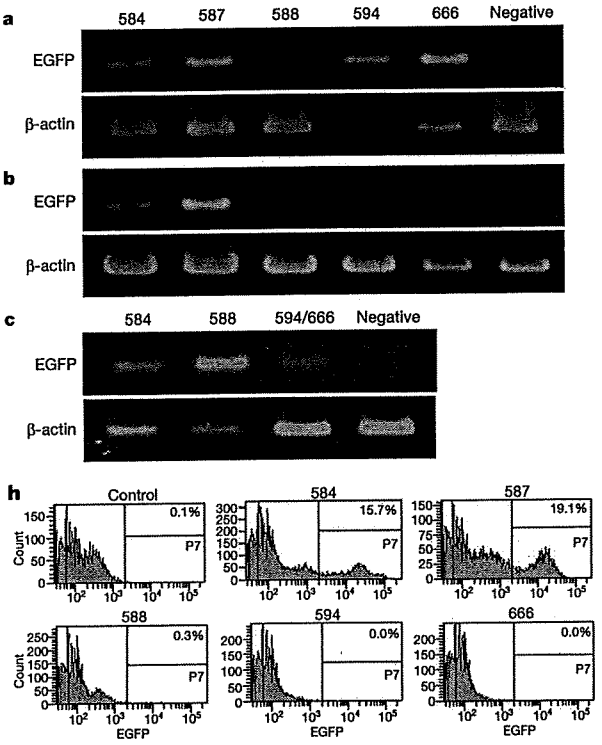
Peripheral blood samples were subjected to flow cytometric analysis using a FACScant. FACS analysis showed EGFP-positive peripheral blood MNCs in 584 and 587. The proportion of EGFP-positive cells was 15.7 and 19.1%, respectively (Fig. 3h). The flow cytometry results corresponded well with those from RT–PCR. Among the peripheral blood cells, the EGFP-positive percentage of granulocytes, lymphocytes and monocytes was 34.5, 3.3 and 18.0% in 584, and 47.7, 4.6 and 20.0% in 587, respectively (Supplementary Fig. 5).

Germline transmission of the transgene

At the moment when two of the animals (666 and 584) became sexually mature, the transgene expression in their gametes was analysed. Semen samples were collected from 666, and live spermatozoa were obtained by the swim-up method in TYH medium. RT–PCR analysis demonstrated the presence and expression of the transgene in the germ cells of 666 (Fig. 4a). IVFs were then performed using semen collected from 666 and wild-type oocytes to analyse the fertility of the germ cells carrying the transgene. Fluorescence microscopy showed that 20–25% of the IVF embryos strongly expressed EGFP, as shown in Fig. 4b. Furthermore, three pre-implantation live natural embryos were collected from female animal 584, and one of these embryos strongly expressed EGFP. The IVF embryos from 666 and two of the natural blastocyst embryos from 584 were shown to express the EGFP transgene by RT–PCR (Fig. 4a). Three EGFP-positive IVF embryos from the male animal (666) were then transferred into a surrogate mother. One neonate (687) was delivered at full term by caesarean section, and this neonate carried the EGFP gene and expressed the transgene in skin (Fig. 4c–e), but not in the placenta and hair.

Discussion

To our knowledge, this is the first report of transgenic non-human primates showing not only the transgene expression in somatic tissues, but also germline transmission of the transgene with the full, normal



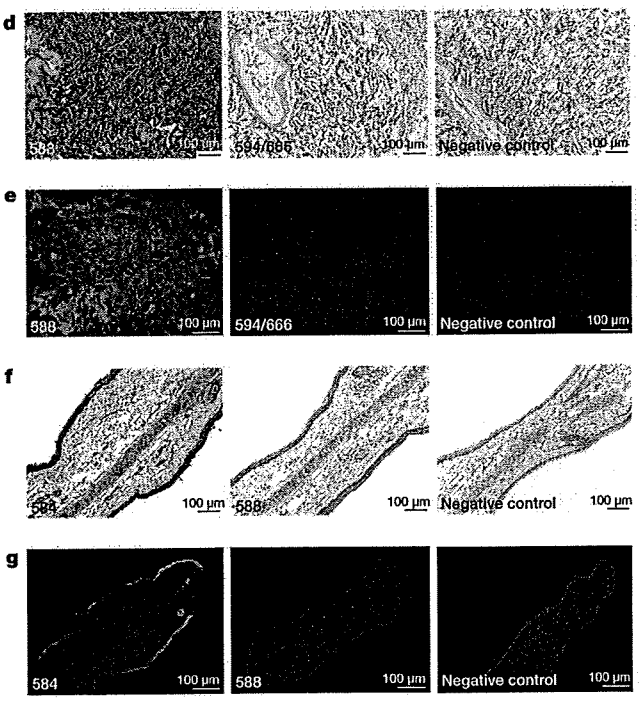


Figure 3 | Transgene transcription and expression in several infant tissues. **a–c**, RT–PCR results from hair roots (**a**), peripheral blood (**b**) and placenta (**c**). Each lane indicates the animal number. **d–g**, Immunohistochemical (**d**, **f**) and epifluorescence (**e**, **g**) analyses using an anti-EGFP antibody, of frozen ear tissues (**f**, **g**) and placentae (**d**, **e**). Scale bars, 100 μm. **h**, Results of FACS analysis using whole peripheral blood cells. The percentage of EGFP-positive cells is shown in the top right of each panel.

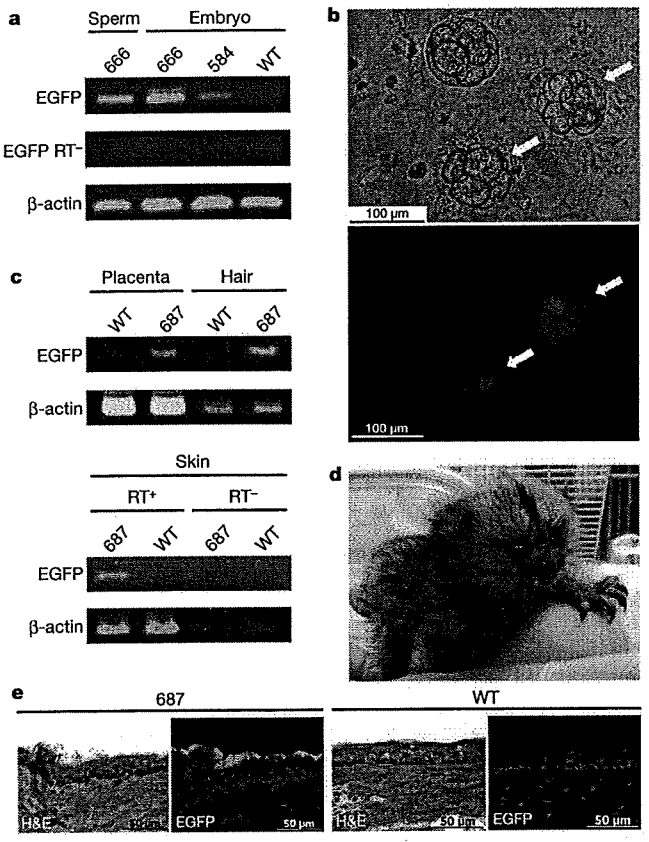


Figure 4 | Germline transmission of the transgene. **a**, RT–PCR analysis of spermatozoa and IVF embryos from 666, and natural embryos from 584. RT− denotes the absence of reverse transcriptase as a control. **b**, Bright-field and dark-field of epifluorescence images of IVF embryos. EGFP-positive IVF embryos produced with 666 spermatozoa are indicated by white arrows. **c**, PCR (top panel) and RT–PCR (bottom panel) analysis of the tissues from the F_1 neonate. **d**, Photograph of the F_1 offspring (687) from 666. **e**, Haematoxylin and eosin (H&E) staining and epifluorescence imaging of frozen skin tissue from the neonate. WT, wild-type control.

development of the embryo. We obtained five transgenic marmosets, four of which expressed the transgene in several somatic cell lineages, such as hair root, skin fibroblast and peripheral blood cells. The remaining animal expressed the transgene only in the placenta. Two of these animals reached sexual maturity and showed the transgene insertion and expression in germ cells. Epifluorescence microscopic observation and RT–PCR analysis of embryos generated by transgene-bearing gametes strongly indicated that the transgenic germ cells from animals 666 and 584 were fertile, and this was proved for the male (666) who fathered one healthy, transgenic infant (687) with the transgene expression in the somatic cells. These findings suggest that it should be possible to establish transgenic non-human primate colonies, opening the door to their use in biomedical research.

Because the manipulation of embryos for viral injection and their subsequent culture may affect embryonic development, the birth rate after embryo transfer (6.25%) was lower than that for normal embryos (30.7%, data not shown). The miscarriage rates were not significantly different between embryonic transfers performed using normal embryos (28.6%) and transgenic embryos (42.6%). Despite considerable effort, transgenic marmosets have not been produced by DNA pronuclear microinjection. The production rate that we obtained using lentivirus (5.26–6.25%) suggests that our technique is sufficiently effective for the production and use of genetically modified marmosets as human disease models.

The 100% birth rate of transgenic marmosets achieved in the present study could be due to several technical advantages. First, we used EGFP as the transgene, enabling us to monitor the presence and expression of the transgene at each experimental step in live embryos and the transgenic animals. Accordingly, we were able to select unambiguously EGFP-expressing embryos for transfer into surrogate mothers. This selection was effective, not only for increasing the birth rate of transgenic animals, but also for reducing the number of surrogate mother animals needed.

Second, we used pre-implantation embryos obtained by natural intercourse, high-titre lentiviral vectors, and 0.25 M sucrose solution as a medium for injection. Even though the birth rates (birth per embryonic transfer) were no different between IVF and natural embryos, the fertilization rate of the germinal vesicle-stage oocytes was quite low. Because it is difficult to collect large quantities of oocytes, it was advantageous to use marmoset natural embryos. To inject as much lentiviral vector as possible into the perivitelline space, the embryos were placed in 0.25 M sucrose medium at the time of lentiviral vector injection, which expanded the volume of the perivitelline space 1.2–7.5-fold. For example, the estimated volume of the perivitelline space of one marmoset pronuclear stage embryo was approximately 31.5 pl, but when placed in 0.25 M sucrose medium, it expanded to about 231 pl. A high titre of the lentiviral vector solution was used so that many lentiviral vector particles were injected into the expanded perivitelline space; approximately 1.3×10^3–1.3×10^5 transducing units of lentiviral vector were injected in this study. Each of these steps probably contributed to the successful production of transgenic marmosets.

The high number of injected lentiviral vector particles resulted in several transgene integrations, as observed by Southern blot analysis and FISH. The embryos injected with the transgene before the four-cell stage (584 and 587) showed fewer than four copies of the transgene per genome by Southern blotting and FISH. The three other embryos (588, 594 and 666), which received the injection at the morula stage, exhibited several integrations of the transgene by Southern blotting and FISH. As the FISH analysis was performed using only peripheral blood MNCs, other patterns of transgene integration cells may have existed in other tissues. The FISH results for 666 were consistent with this hypothesis, as the integration sites in the chimaeric blood MNCs from his twin, 594, were different from those in the blood MNCs of 594.

The lentiviral vector used in the present study can be used to transmit only relatively small transgenes, 8.5 kilobases of DNA or less. Therefore, further study will be necessary to enable the introduction of larger transgenes into marmoset embryos. Furthermore, to study human diseases involving the malfunctions of specific genes, targeted gene-knockdown marmosets could be developed using RNA interference (RNAi) lentiviruses.

The results of the present study indicate that transgenic marmosets may be used as experimental animals for biomedical research. Recently, somatic cell nuclear-transferred embryonic stem cells from the rhesus macaque and induced pluripotent stem cells from adult human fibroblasts were reportedly established[12–15]. Those studies indicated that the obstacle caused by immunogenetic incompatibility has at least theoretically been resolved, and that a new era of regenerative medicine using somatic cell nuclear-transferred embryonic stem cells in primates[14] or human induced pluripotent stem cells[12,13,15] has become possible. However, before such stem cells can be used in clinical applications, preclinical assessments of their safety and efficacy are essential. We previously reported that marmosets with injured spinal cords can recover motor function after the transplantation of human neural stem/progenitor cells[16], highlighting the usefulness of the marmoset for assessing the safety and efficacy of, not only these cells, but also of other stem cells, such as human embryonic stem cells[17] or induced pluripotent stem cells. Human disease models in non-human primates have so far been limited to mechanical injury models (for example, spinal cord injury[18]) and drug administration models (for example, MPTP-induced Parkinson's[19,20]). The only transgene-induced primate disease model is of Huntington's disease[7], in rhesus

monkeys expressing a mutant human huntingtin gene. In that report, although the transgene was inserted into the genome of founder infants and its expression was detected in post-mortem animals, the germline transmission of the transgene has not yet been confirmed[7]. Thus, at this point, it is not certain how reproducible the effects of various therapeutic interventions would be using a large number of animals.

The technique by which we achieved transgene expression in several tissues, along with germline transmission, may provide the means to obtain genetically modified non-human primate models for translational research, investigations of regenerative medicine and gene therapy, and clarification of the scientific gaps among transgenic mice, human disease models, and real human diseases.

METHODS SUMMARY

All animal experiments were approved by the institutional animal care and use committee, and were performed in accordance with Central Institution for Experimental Animal (CIEA) guidelines.

To obtain oocytes, recombinant human follicle stimulating hormone (r-hFSH; 50 international units (IU); Fertinome, Serono) was administered daily by intramuscular injection for 11 days. Human chorionic gonadotropin (hCG; 75 IU; Gonatropin, Teikoku-zouki) was administered by intramuscular injection at 17:30 on day 12. On day 13, the animals were anaesthetized and follicular aspiration was performed surgically. Oocytes were incubated for 24 h at 38 °C, 5% CO_2 in air, for *in vitro* maturation. After incubation, only matured oocytes (metaphase II) were collected and used for IVF.

Ejaculated semen was collected in TYH medium (Mitsubishi Kagaku Iatron), using a Ferti Care personal vibrator. Hyaluronidase-treated oocytes were placed in 70-μl drops of TYH, and an aliquot of sperm (4×10^5) was added to each oocyte incubation drop. After 26–30 h of insemination, the fertilized oocytes were placed into ISM1 (Medicult) medium, and lentiviral vector injection was performed in 0.25 M sucrose.

Natural embryo collection was performed as previously described[21]. Embryos at the pronuclear-to-morula stage were placed in 0.25 M sucrose supplemented PB1 medium (Mitsubishi Chemical Medience Corporation) and injected with lentiviral vector. Blastocysts were not treated with sucrose. Lentiviral vector injection was performed using an Eppendorf FemtoJet express and a Narishige micromanipulator. The embryos were cultured until GFP expression was confirmed.

The ovulation cycles of donor and recipient animals were synchronized, and EGFP-expressing embryos were transferred as previously described[22,23]. After embryo transfer, the recipients were tested for pregnancy by plasma progesterone once a week. The resulting infants were analysed for transgene integration, transcription and expression, by real-time PCR, Southern blot analysis, RT–PCR, immunohistochemical analysis, FACS and FISH.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 27 September 2008; accepted 30 April 2009.

1. Mansfield, K. Marmoset models commonly used in biomedical research. *Comp. Med.* 53, 383–392 (2003).
2. Chan, A. W., Homan, E. J., Ballou, L. U., Burns, J. C. & Bremel, R. D. Transgenic cattle produced by reverse-transcribed gene transfer in oocytes. *Proc. Natl Acad. Sci. USA* 95, 14028–14033 (1998).
3. Hofmann, A. *et al.* Efficient transgenesis in farm animals by lentiviral vectors. *EMBO Rep.* 4, 1054–1060 (2003).
4. Hofmann, A. *et al.* Generation of transgenic cattle by lentiviral gene transfer into oocytes. *Biol. Reprod.* 71, 405–409 (2004).
5. Chan, A. W., Chong, K. Y., Martinovich, C., Simerly, C. & Schatten, G. Transgenic monkeys produced by retroviral gene transfer into mature oocytes. *Science* 291, 309–312 (2001).
6. Wolfgang, M. J. *et al.* Rhesus monkey placental transgene expression after lentiviral gene transfer into preimplantation embryos. *Proc. Natl Acad. Sci. USA* 98, 10728–10732 (2001).
7. Yang, S. H. *et al.* Towards a transgenic model of Huntington's disease in a non-human primate. *Nature* 453, 921–924 (2008).
8. Rogers, C. S. *et al.* Disruption of the *CFTR* gene produces a model of cystic fibrosis in newborn pigs. *Science* 321, 1837–1841 (2008).
9. Rogers, C. S. *et al.* Production of *CFTR*-null and *CFTR-ΔF508* heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. *J. Clin. Invest.* 118, 1571–1577 (2008).
10. Miyoshi, H., Blomer, U., Takahashi, M., Gage, F. H. & Verma, I. M. Development of a self-inactivating lentivirus vector. *J. Virol.* 72, 8150–8157 (1998).
11. Ross, C. N., French, J. A. & Orti, G. Germ-line chimerism and paternal care in marmosets (*Callithrix kuhlii*). *Proc. Natl Acad. Sci. USA* 104, 6278–6282 (2007).
12. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861–872 (2007).
13. Yu, J. *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318, 1917–1920 (2007).
14. Byrne, J. A. *et al.* Producing primate embryonic stem cells by somatic cell nuclear transfer. *Nature* 450, 497–502 (2007).
15. Nakagawa, M. *et al.* Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nature Biotechnol.* 26, 101–106 (2008).
16. Iwanami, A. *et al.* Transplantation of human neural stem cells for spinal cord injury in primates. *J. Neurosci. Res.* 80, 182–190 (2005).
17. Thomson, J. A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145–1147 (1998).
18. Iwanami, A. *et al.* Establishment of graded spinal cord injury model in a nonhuman primate: the common marmoset. *J. Neurosci. Res.* 80, 172–181 (2005).
19. Eslamboli, A. Marmoset monkey models of Parkinson's disease: which model, when and why? *Brain Res. Bull.* 68, 140–149 (2005).
20. Ando, K. *et al.* Neurobehavioral protection by single dose l-deprenyl against MPTP-induced parkinsonism in common marmosets. *Psychopharmacology (Berl.)* 195, 509–516 (2008).
21. Sasaki, E. *et al.* Establishment of novel embryonic stem cell lines derived from the common marmoset (*Callithrix jacchus*). *Stem Cells* 23, 1304–1313 (2005).
22. Lopata, A., Summers, P. M. & Hearn, J. P. Births following the transfer of cultured embryos obtained by *in vitro* and *in vivo* fertilization in the marmoset monkey (*Callithrix jacchus*). *Fertil. Steril.* 50, 503–509 (1988).
23. Summers, P. M., Shephard, A. M., Taylor, C. T. & Hearn, J. P. The effects of cryopreservation and transfer on embryonic development in the common marmoset monkey, *Callithrix jacchus*. *J. Reprod. Fertil.* 79, 241–250 (1987).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank F. Toyoda, S. Ohba, T. Inoue, Y. Sawada and M. Yokoyama for technical assistance with the animal experiments and care. E.S. is an associate professor of the Global COE program for human metabolomic systems biology assigned to Keio University. This study was also supported by the Global COE program for Education and Research Centre for Stem Cell Medicine from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), the Japanese Government to Keio University. This study was also supported by funds from Solution-Oriented Research for Science and Technology (SORST) of the Japan Science and Technology Agency and grants from MEXT to H.O. and from Special Coordination Funds for Promoting Science and Technology of MEXT to S.H.

Author Contributions E.S. designed the experiments, conducted the project, and wrote the paper. A.S., Y.S., T.E., I.T. and R.H. assisted in embryological technique development. K.H., R.O. and M.K. developed surgical techniques for embryo collection and transfer. H.S., C.K. and C.Y. performed or assisted with the real-time PCR and parentage evaluation test. S.S. and T.M. assisted with the Southern blot analysis and tissue collection. M.I. raised the anti-marmoset CD45 antibody. R.I. performed the FACS analysis, and K.K. performed the immunohistochemical analysis. H.M. provided the lentiviral vectors. Y.T., H.O., S.H., N.T. and T.N. designed the project, and H.O., S.H. and N.T. also participated in writing the paper. The whole project was supervised by E.S. and H.O.

Author Information Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to E.S. (esasaki@ciea.or.jp) or H.O. (hidokano@sc.itc.keio.ac.jp).

METHODS

Animals. Adult common marmosets more than 2 years old were obtained from a marmoset breeding colony for experimental animals. Female marmosets with normal ovarian cycles were paired with intact males for natural embryo collection. Recipient females were paired with vasectomised males or intact females. This study was approved by the Institutional Animal Care and Use Committee of CIEA, and was performed in accordance with CIEA guidelines.

***In vitro* fertilization.** Semen was collected as previously described for common marmosets[24]. Ejaculated semen was collected in TYH medium (Mitsubishi Kagaku Iatron) and washed twice with TYH. The semen was placed in a CO_2 incubator for 10 min in a test tube inclined at a 30° angle to allow the sperm to swim up. Hyaluronidase-treated metaphase-II-arrested oocytes were inseminated with a final concentration of 5×10^6 sperm ml^{-1} for 26–30 h. Fertilized embryos were cultured in ISM medium (Medicult, Nosan Corp.).

Embryo collection and transfer. Embryo collection and transfer were performed as previously described[25]. After embryo transfer, the recipients were monitored for pregnancy by measuring their plasma progesterone once a week until the pregnancies could be monitored by transabdominal palpation of the uterus.

Lentiviral vector preparation and transduction. The lentiviral vectors were produced as previously described[26]. The medium containing viral particles was spun at 4 °C, 50,000*g* for 4 h, and the viral pellet was then resuspended in ISM2 medium, in 1/1,000 of the volume of the original lentiviral vector supernatant. To measure the lentivirus titre, serially diluted (10^{-2} to 10^{-8}) lentiviral vector was used to infect 10^5 293T cells. The number of EGFP-positive cells was counted by FACS to quantify the titre.

Pronuclear-to-morula stage embryos were placed in 0.25 M sucrose supplemented PB1 medium (Mitsubishi Chemical Medience Corporation), and the virus was injected into the perivitelline space. For blastocyst embryos, the viral vector was injected into the blastocoel. All viral injections were performed using an Eppendorf FemtoJet Express and a Narishige micromanipulator.

Southern blot analysis. Five micrograms of genomic DNA was digested with BamHI for animals that had been injected with CAG-EGFP, and with EcoRI for those that had been injected with CMV-EGFP. The digested genomic DNA was separated on a 0.8% agarose gel and transferred to a Hybond-N+ nylon membrane (GE Healthcare Biosciences). Southern blot analysis was performed using the DIG system (Roche Diagnostics K.K.), according to the manufacturer's protocol. CMV-EGFP was digested with EcoRI and then labelled with DIG using the PCR DIG probe synthesis kit, according to the manufacturer's instructions (Roche Diagnostics K.K.).

RT–PCR. To detect EGFP gene expression, EGFP5-5 (5′-GCACAAGCTGGAGT ACAACTACAACAGC-3′) and EGFP3-1 (5′-TCACGAACTCCAGCAGGACC AT-3′) primers were used. To detect β-actin expression, β-actin 001 (5′-TCCTG ACCCTGAAGTACCCC-3′) and β-actin 002 (5′-GTGGTGGTGAAGCTGTA GCC-3′) primers were used. PCR was performed for 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 s at 58 °C for EGFP primers or 62 °C for β-actin primers, and elongation at 72 °C for 30 s.

To detect EGFP gene expression in germ cells and neonatal tissues, PCR was performed using the EGFP5-4 (5′-CAAGGACGACGGCAACTACAAGACC-3′) and EGFP3-3es (5′- GCTCGTCCATGCCGAGAGTGA-3′) primers. Then, 1 μl of the PCR products was re-amplified with the EGFP5-6 (5′-TCGAGCTGA AGGGCATCGAC-3′) and EGFP3-1 (5′-TCACGAACTCCAGCAGGACCAT-3′) primers. To detect β-actin expression, the PCR primers β-actin 003 (5′-TGGACTTCGAGCAGGAGAT-3′) and β-actin 006R (5′-CCTGCTTGCTG ATCCACATG-3′) were used. Then, 0.5 μl of the PCR products was re-amplified with the 004 (5′-TCCCTGGAGAAGAGCTATG-3′) and 005R (5′-GAGC CACCAATCCACACTGA-3′) primers. PCR was performed for 30 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 10 s, and elongation at 72 °C for 30 s.

Immunohistochemical analysis. Tissues were embedded in OCT compound, frozen in liquid nitrogen, and sliced into 5-μm sections, which were fixed in 4% paraformaldehyde for 30 min at 4 °C. Endogenous peroxidase activity was quenched using 0.03% hydrogen peroxidase for 30 min at room temperature. The slides were blocked with 10% goat serum (Nichirei) for 10 min at room temperature and then reacted with the rabbit anti-GFP polyclonal antibody (A.v. peptide antibody, BD Bioscience) overnight at 4 °C. The slides were incubated with the biotinylated secondary antibody Simple Stain Mouse MAX PO (Nichirei) for 30 min at room temperature. The bound antibodies were detected with DAB (3,3-diaminobenzidine tetrahydrochloride) horseradish peroxidase complex. The samples were then stained with H&E and examined by microscopy.

FACS analysis. Whole blood cells were washed with PBS and suspended in 0.13 M NH_4Cl. The pellet was incubated with the mouse IgG1 anti-marmoset CD45, 6C9 antibody for 30 min on ice[27], then mixed with an allophycocyanin (APC)-labelled anti-mouse IgG antibody, and incubated for 30 min on ice. The sample was washed with PBS and resuspended in 200 μl of propidium iodide solution. FACScant analysis was then performed.

Fluorescent *in situ* hybridization. Peripheral blood samples were cultured in RPMI 1640 containing phytohaemagglutinin, concanavalin A, lipopolysaccharide, and 2-mercaptoethanol for 2–3 days. After 2–3 h of incubation with BrdU (final concentration 30 μg ml^{-1}), colcemid (final concentration 0.02 μg ml^{-1}) was added to the medium, and the samples were incubated for another 2 h. After lymphocyte fixation, the cells were spread on slides and air-dried overnight, then stained with Hoechst 33258 and treated with ultraviolet light. CAG-EGFP was labelled with digoxigenin-11-dUTP as a probe, and hybridized at 37 °C overnight. After stringent washes, the bound label was detected using anti-Dig-Cy3. For karyotyping, Leica CW4000 FISH and Leica CW4000 Karyo were used.

24. Kuederling, I., Schneiders, A., Sonksen, J., Nayudu, P. L. & Hodges, J. K. Non-invasive collection of ejaculates from the common marmoset (*Callithrix jacchus*) using penile vibrostimulation. *Am. J. Primatol.* 52, 149–154 (2000).
25. Summers, P. M., Shephard, A. M., Taylor, C. T. & Hearn, J. P. The effects of cryopreservation and transfer on embryonic development in the common marmoset monkey, *Callithrix jacchus*. *J. Reprod. Fertil.* 79, 241–250 (1987).
26. Bai, Y. *et al.* Effective transduction and stable transgene expression in human blood cells by a third-generation lentiviral vector. *Gene Ther.* 10, 1446–1457 (2003).
27. Ito, R. *et al.* Novel monoclonal antibodies recognizing different subsets of lymphocytes from the common marmoset (*Callithrix jacchus*). *Immunol. Lett.* 121, 116–122 (2008).

CD73, a Novel Cell Surface Antigen That Characterizes Retinal Photoreceptor Precursor Cells

Hideto Koso,[1] *Chiharu Minami,*[1,2] *Yoko Tabata,*[1] *Mariko Inoue,*[1] *Erika Sasaki,*[3] *Shinya Satoh,*[1] *and Sumiko Watanabe*[1]

Purpose. The authors sought to identify cell surface markers of photoreceptor and its precursor cells.

Methods. The expression of surface CD antigens that label both temporally and spatially distinct populations of mouse retinal cells were examined. Of the antibodies that showed positive signals in retinal cells, CD73 was focused on for more detailed analyses.

Results. Mouse retinal subpopulations that expressed CD73 first appeared around birth and subsequently increased dramatically in number, eventually representing more than 90% of the retinal cells in the adult. $CD73^+$ cells were postmitotic and mostly rhodopsin-negative at postnatal day 1. However, in the adult retina, most of these cells expressed rhodopsin but not s-opsin. In reaggregation cultures, $CD73^+$ cells differentiated into rhodopsin-positive cells more rapidly than $CD73^-$ cells, which supports the idea that CD73 is an early photoreceptor lineage marker. The effects of ectopic expression in retinal cells of Nrl and Crx, both of which are transcription factors known to be expressed in photoreceptor lineage, suggest that CD73 is genetically downstream of Crx in the rod cell differentiation lineage. Adult retina of the common marmoset monkey also showed correlation of the expression pattern of rhodopsin and CD73.

Conclusions. CD73 is a cell surface marker of cone/rod common precursors and mature rod cells in mice and is genetically localized between Nrl and Crx. The expression of CD73 was conserved in primate rod cells, and CD73 provides an useful tool to purify photoreceptor cells for transplantation aimed at the regeneration of photoreceptors. (*Invest Ophthalmol Vis Sci.* 2009;50:5411–5418) DOI:10.1167/iovs.08-3246

The vertebrate neural retina consists of six types of neurons and one type of glial cells, which are organized into a laminar structure. The outer nuclear layer (ONL) consists of photoreceptors, and specific loss of these cells causes several severe retinal diseases, among them retinitis pigmentosa.[1,2] Regeneration of photoreceptor cells is an important step in the regeneration of vision, and considerable effort is being invested in understanding these processes. The isolation of retinal progenitor cells or precursors of the photoreceptors lineage is one of the strategies used to achieve neural retina regeneration by transplantation.[3] However, these cell populations have not yet been adequately characterized, in part because of a lack of markers that can be used to identify the distinct stages and lineages of retinal cells. Although the patterns of expression of transcriptional factors reported to be involved in retinal development may reflect the developmental stage, these molecules are intracellular, which limits their usefulness for cell enrichment. Therefore, it is important to define surface markers that can be used to label specific retinal cell subpopulations. Surface antigens permit the isolation of a specific subset of cells from a cell mixture without damaging the cells, which facilitates the characterization of cell lineages and the identification of factors that regulate cell proliferation and differentiation. We evaluated candidate markers using flow cytometry and cell sorting in combination with retinal in vitro cultures. We screened the mouse retina at various developmental stages for reactivities with a panel of antibodies directed against cell-surface antigens and obtained unique expression patterns for more than 30 antigens. Among these, SSEA-1 (CD15) and c-kit (CD117) have been shown previously to represent the early and late immature stages of retinal progenitor cells, respectively.[4,5]

In the present study, we focused on the CD73 antigen, the expression of which was seen to increase concomitantly with retinal development. CD73, which is also known as ecto-5′-nucleotidase, is a 70-kDa glycosylphosphatidylinositol (GPI)-anchored cell surface molecule that catalyzes the extracellular conversion of 5′-adenosine monophosphate to adenosine.[6,7] We identified CD73 as a marker of the early stages of the photoreceptor lineage. CD73 is assumed to be localized genetically downstream of Crx. This is the first report describing a cell surface marker of immature photoreceptor cells.

Materials and Methods

Mice, Common Marmoset, and Cultures

EGFP transgenic mice, which were kindly provided by Masaru Okabe (Osaka University, Japan),[8,9] were maintained in a C57BL/6J background. ICR mice were obtained from Japan SLC. Common marmoset was maintained in the Central Institute for Experimental Animals (CIEA) in accordance with CIEA guidelines. Retinal explant cultures and reaggregation cultures were prepared as described previously[4,10] and were infected with retroviruses as described previously.[10,11] All animal experiments were approved by the Animal Care Committee of the Institute of Medical Science, University of Tokyo and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Fluorescence-Activated Cell Sorting

Neural retinas were isolated and dispersed to single cells using trypsin and were stained with antibodies, as described previously.[4] The following antibodies were used: anti-CD73 (BD Bioscience, Franklin Lakes, NJ), anti-Ki67 (BD Biosciences), anti-Rho4D2 (kind gift from

From the [1]Department of Molecular and Developmental Biology, Institute of Medical Science, University of Tokyo, Tokyo, Japan; the [2]Tokyo College of Biotechnology, Tokyo, Japan; and the [3]Central Institute for Experimental Animals, Kawasaki, Kanagawa, Japan.

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

Submitted for publication December 2, 2008; revised April 12, 2009; accepted August 7, 2009.

Disclosure: **H. Koso**, None; **C. Minami**, None; **Y. Tabata**, None; **M. Inoue**, None; **E. Sasaki**, None; **S. Satoh**, None; **S. Watanabe**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Sumiko Watanabe, Department of Molecular and Developmental Biology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan; sumiko@ims.u-tokyo.ac.jp.

Robert Molday, University of British Columbia, Canada), anti-Nestin (BD Biosciences), anti-PNR (Perseus Proteomics, Tokyo, Japan), and anti-protein kinase C (anti-PKC; Oncogene Research Products, San Diego, CA). Nonlabeled antibodies were visualized with the appropriate secondary antibody conjugated with Alexa488 (Molecular Probes, Eugene, OR). At least 10,000 events for healthy cells were analyzed (FACSCalibur; BD Biosciences). Cell sorting was carried out (FACSVantage or FACSAria; BD Biosciences), as described previously,[4] and results (FACSAria; BD Biosciences) were replotted (FlowJo software; Tree Star, Ashland, OR)

Construction and Immunostaining

The full-length cDNAs of the mouse *Crx*, *Nrl*, and *CD73* genes were cloned by polymerase chain reaction (PCR) according to the sequences in the database using mouse retinal cDNA. The PCR products were cloned into a vector (pGEM-T-Easy; Promega, Madison, WI), and the inserts were subcloned into the pMX-IRES-EGFP retrovirus vector.[10] Immunostaining for sectioned or dissociated retinas was performed as described previously.[4,10] The primary antibodies used were anti-CD73, anti-rhodopsin (Rho4D2), anti-s-opsin (Chemicon, Temecula, CA), anti-glutamine synthetase (Chemicon), and anti-m-opsin (Chemicon). The primary antibodies were visualized using the appropriate secondary antibodies conjugated to Alexa-488 or Alexa-546 (Molecular Probes). DAPI was used for nuclear staining. Samples were mounted (Vecta-Shield; Vector Laboratories, Burlingame, CA) and analyzed under a microscope (Axioplan; Zeiss, Thornwood, NY).

RT-PCR

Total RNA was purified from $CD73^+$ and $CD73^-$ retinal cells using the reagent (TRIZOL; Gibco BRL, Grand Island, NY), and cDNA was synthesized by reverse transcriptase (Superscript II; Gibco BRL). All the primer sets were tested for different numbers of cycles (25–35 cycles) using rTaq (Takara, Shiga, Japan), and the semiquantitative cycle number was determined for each primer set. DNA bands were visualized with ethidium bromide.

Results

Characterization of $CD73^+$ Cells in the Developing Retina

By screening the expression patterns of various CD antigens in the mouse retina at various developmental stages, we noted that the expression levels of some antigens changed as retinal development progressed. The expression of the CD73 antigen increased dramatically with retinal development. Initially, we examined CD73 expression in the mouse neural retina at various developmental stages using flow cytometry (Fig. 1A). CD73 expression was not detected in the retina at embryonic day (E) 14 and was first observed in approximately 10% of the cells derived from an E16 mouse (Fig. 1A). The population of CD73-expressing cells increased along with mouse development until postnatal day (P) 6, and 90% of the cells in the adult mouse retina expressed CD73.

Double immunostaining of CD73 and Ki67, which is a nuclear cell proliferation-associated antigen,[12] showed that CD73 expression in postmitotic cells (Fig. 1B). However, between 10% and 20% of $CD73^+$ cells are Ki67/CD73 double positive at P1 and E17 (data not shown), suggesting that retinal progenitor cells begin to express CD73 at the end of their proliferative stage. We also examined whether $CD73^+$ cells had the characteristics of differentiated lineages of the retinal subpopulation by costaining. Nestin is expressed in neural progenitor cells.[13] None of $CD73^+$ cells coexpressed nestin at P9, which confirms that the $CD73^+$ cell fraction is enriched for differentiated cells. PKC, which is a marker of bipolar cells, was expressed only in a negligible fraction (3%) of the $CD73^{low}$ cells at P9 (Fig. 1C). Similar results were obtained by staining adult retinal cells with antibodies against CD73 and PKC or Islet-1, which are markers of bipolar or amacrine cells, respectively (Fig. 1D). In addition to these results, our immunostaining data do not support the idea that CD73 is expressed in retinal cells other than rod photoreceptors. However, our observation that 85% to 90% of the adult retinal cells were $CD73^+$ by FACS analysis is higher than the generally accepted value for rod photoreceptors (70%). One possible explanation for this discrepancy is the selective loss of retinal cells other than rod photoreceptors during the preparation of single cells by dissociation for FACS analysis.

Rod photoreceptor cells express rhodopsin and represent the largest of the retinal cell subpopulations; these cells differentiate during the last stage of retinal development.[14] More than 80% of the retinal cells were rhodopsin positive at P9, and the most of these cells were double positive for rhodopsin and CD73 (Fig. 1F), which suggests that CD73 is a marker of rhodopsin-positive cells. Previous reports demonstrated the expression of 5′-nucleotidase enzyme activities in photoreceptor cells of the rat retina,[15] which is consistent with our present results. Therefore, we examined in more detail the coexpression of CD73 and rhodopsin during retinal development. Retinal cells from mice at P1, P6, and P9 were dissociated and immunostained with the anti-CD73 and anti-rhodopsin antibodies. Some of the cells were replated and used for immunohistochemistry with the same set of antibodies (Fig. 1E). Less than 30% of the $CD73^+$ cells expressed rhodopsin in P1 mouse retinas, and more than 50% of the $CD73^+$ cells expressed rhodopsin in P6 mouse retinas. Interestingly, although a significant number of $CD73^+$ cells were observed at P6, not all cells were rhodopsin-positive cells at this stage (Fig. 1F). At the more advanced stage of P9, most of the cells were double-positive for CD73 and rhodopsin (Figs. 1E, 1F), and less than 10% of the cells in the P9 and adult samples were rhodopsin-positive/CD73-negative. We attempted to characterize these cells; however, we have no explanation for our observation. Taken together, these results suggest that retinal cells that commit to the rod photoreceptor cell lineage initially express CD73 and subsequently become double-positive for CD73 and rhodopsin. To examine this hypothesis at the transcriptional level, we examined the temporal transitions of mRNA expression of CD73 and rhodopsin by semiquantitative RT-PCR (see Fig. 4C). Weak expression of CD73 mRNA was observed in the retina at E16, and the strength was increased until P5, which is consistent with the transition of CD73 protein expression revealed by FACS analysis. This suggests that the expression of CD73 is regulated mainly at the transcriptional level. The onset of CD73 expression occurred earlier than that of rhodopsin, which supports the idea that CD73 is an earlier marker than rhodopsin of the photoreceptor lineage.

We also examined the spatial localization of $CD73^+$ cells in the developing retina by immunohistochemistry and compared this expression pattern with that of rhodopsin (Fig. 1G). In P1 retinas, CD73 was observed in the outer half of the neuroblastic layer, which corresponds to the area in which the photoreceptors start to differentiate. At P5, the ONL became visible, and CD73 expression was evident throughout the ONL. When we examined adult mouse-derived retinas, CD73 expression was strong and widespread in the ONL nucleus and in the outer region of the nucleus, and rhodopsin expression was strong in the outer region of the ONL, which corresponds to the outer segment of the photoreceptor. Although a previous study reported the expression of CD73 in Müller glial cells,[16] we did not observe the expression of CD73 in the inner nuclear layer (INL), in which the Müller glial cell body is

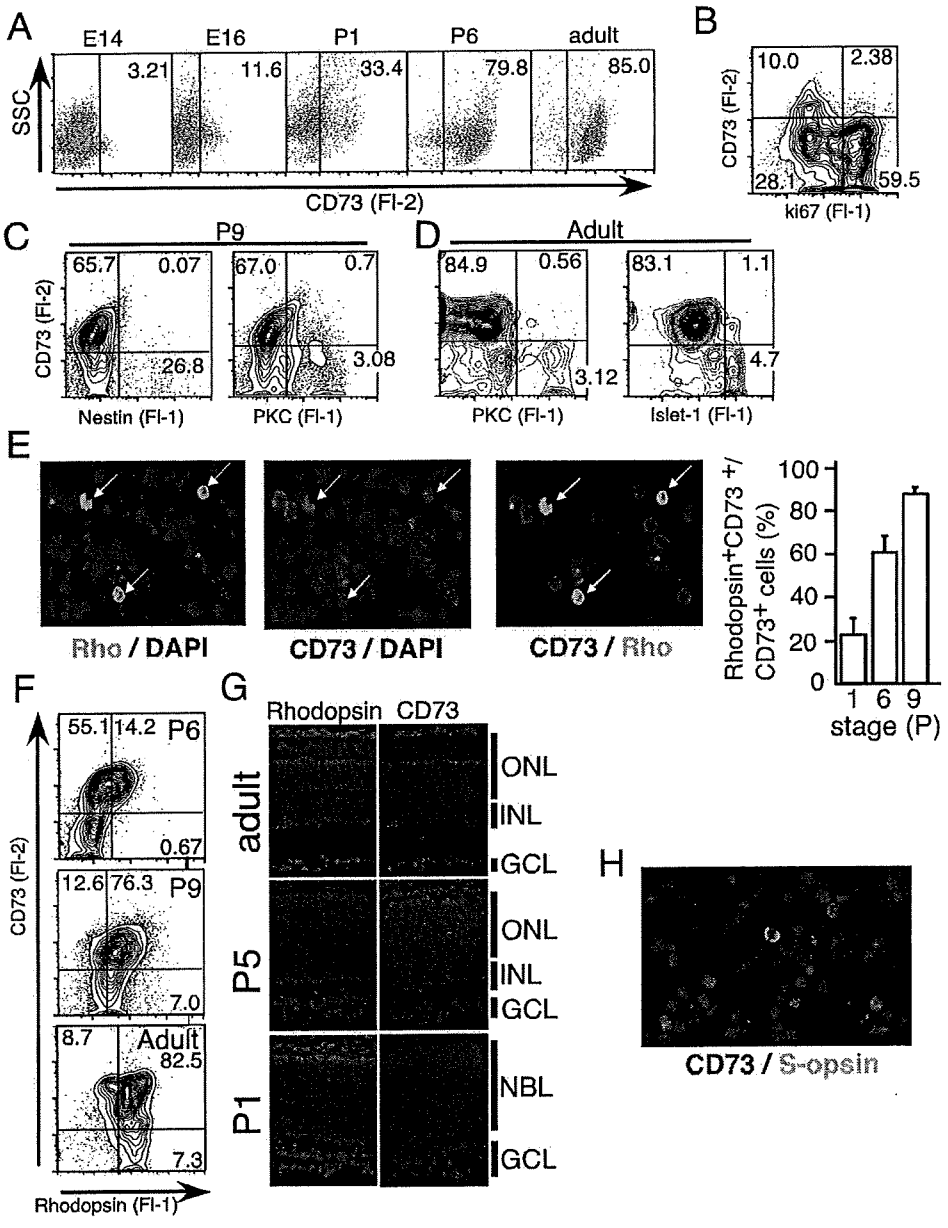


FIGURE 1. Characterization of CD73 expression. (A) Flow cytometric analysis of the expression of CD73 at various developmental stages of the mouse retina. The dot plot patterns of side scatter (SSC) versus CD73 are shown. (B–D) Contour plot patterns of double staining of the mouse retina at P1 with anti- CD73 versus anti-Ki67 (B), and of the mouse retina at the P9 stage with antibodies against nestin, protein kinase C (PKC) (C), and at the adult stage with antibodies against PKC and islet-1 (D). (E) Co-expression of CD73 (*red*) and rhodopsin (*green*) in dissociated retinal cells was examined. Retinal cells at the indicated stages were dissociated on the plate and immunostained with anti-CD73 and anti-rhodopsin antibodies. DAPI was used to stain the nuclei. *Left*: views under the microscope of samples derived from P9 mice. *Right*: the percentages of rhodopsin/CD73 double-positive subpopulation of $CD73^+$ cells are shown. (F) Contour plot pattern of double-staining for CD73 and rhodopsin of a retina at P6, P9, and adult. (G) Immunostaining for CD73 and rhodopsin of frozen-sectioned retina derived from mice at P1, P5, and adulthood. GCL, ganglion cell layer; NBL, neuroblastic layer; INL, inner nuclear layer; ONL, outer nuclear layer. (H) Double-staining of mouse retinal cells at the P2 stage with anti-CD73 (*red*) and anti-s-opsin (*green*) antibodies.

localized, at any of the stages examined (Fig. 1G and data not shown). This notion was confirmed by the lack of expression of mRNA of glutamine synthetase (GS), which is a marker of Müller glial cells, in the purified $CD73^+$ cell population (see Fig. 4A). Because GS antibody cannot be used for FACS analysis (data not shown), we examined its expression in dissociated $CD73^+$ retinal cells derived from P15 mice and found that only a negligible number (1/150 $CD73^+$ cells) of GS/CD73 double-positive cells was observed, further supporting this notion. In the ONL, in addition to the rod cells, cone cells were detected. We examined whether CD73 was expressed in cone cells by double immunostaining the adult mouse retina with the cone marker s-opsin and CD73. We found that $CD73^+$ cells never expressed s-opsin (Fig. 1H). However, when we used semi-quantitative RT-PCR to examine the expression of s-opsin mRNA in $CD73^+$ and $CD73^-$ cells from the retinas of developing mice, s-opsin expression was observed in both cell fractions (see Fig. 4B).

In Vitro Differentiation and Proliferation of $CD73^+$ Cells

We next examined the differentiation and proliferation activities of isolated $CD73^+$ cells using an in vitro culture system. We used reaggregation cultures, which have been shown to be an excellent model system for examining the intrinsic proliferation and differentiation of retinal progenitors in vitro.[4,5] In this system, the cells proliferate and differentiate into rod photoreceptor cells in a manner similar to that seen in vivo.[17] By culturing labeled donor cells with an excess number of unlabeled host retinal cells, we could evaluate the intrinsic proliferation and differentiation of the donor cells in a defined environment.[18] To distinguish transplanted cells from host cells, we used neural retinas derived from EGFP transgenic (Tg) mice[8,9] as donor cells.[4,5] $CD73^+$ and $CD73^-$ cells of EGFP Tg mice at P1 were purified in a cell sorter (Fig. 2A) and mixed with an excess number of dissociated unfractionated host retinal cells from normal mice at P1 to prepare reaggregation

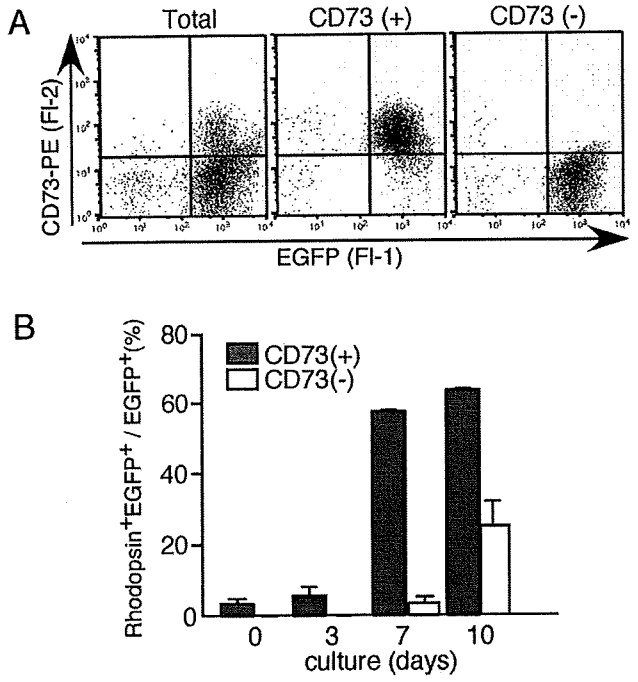


FIGURE 2. Differentiation of $CD73^+$ and $CD73^-$ cells in reaggregation cultures. (**A**) Dot plot pattern of EGFP (Fl-1) versus anti-CD73 antibody staining (PE [Fl-2]) of E17 neural retina derived from EGFP Tg mice. Whole cells displaying the pattern shown on the *left* were fractionated according to CD73 expression in the cell sorter, as shown in the *middle* ($CD73^+$) and *right* ($CD73^-$) panels. The purities of the fraction were approximately 90% and >95%, respectively. (**B**) Reaggregation cultures that consisted of a mixture of donor cells and host cells were prepared to analyze the differentiation of $CD73^+$ and $CD73^-$ cells. $CD73^+$ or $CD73^-$ retinal cells derived from the EGFP Tg mice at P1 were mixed with a large excess of retinal cells from normal mice of the same age. The rhodopsin-expressing cells (%) in the EGFP-positive cell population are shown. Reaggregation cultures were harvested at the indicated days of culture, and the cells were replated on a chamber glass slide and immunostained with antibodies against GFP and rhodopsin. The experiments were performed at least twice, with essentially the same results.

cultures. When we examined the expression of rhodopsin before starting the culture, less than 5% of $CD73^+$ cells were rhodopsin positive, but no expression of rhodopsin was observed in $CD73^-$ cells (Fig. 2B). After 3 days of culture, the number of rhodopsin-positive cells increased, but these cells were still only observed in the $CD73^+$ cell reaggregates. On day 7 of culture, nearly 60% of the $CD73^+$ cells were rhodopsin positive, whereas only a low percentage of the $CD73^-$ cells were rhodopsin positive. In this culture system, the efficiency of cell differentiation was lower than in the in vivo situation (using unfractionated retinal cells, the maximum percentage we observed as rhodopsin-positive cells was 60%; data not shown). On day 10 of culture, although the proportion of rhodopsin-positive cells in the $CD73^-$ cell population had increased significantly, it was far lower than the proportion of rhodopsin-positive cells in the $CD73^+$ population. This indicates that $CD73^+$ cells are in a more advanced stage of the rod photoreceptor cell lineage than $CD73^-$ cells.

Role of CD73 in Retinal Rod Photoreceptor Cell Differentiation

CD73 is an enzyme with ecto-5′-nucleotidase activity, and the enzymatic product of CD73 is adenosine.[6,7] Of the four types of adenosine receptor (A1, A2a, A2b, A3),[19] A2a and A2b have previously been reported to be expressed in the neural retina of the rat.[20,21] We examined the temporal expression of the mRNAs for these receptors by semiquantitative RT-PCR (Fig. 3A). We found that A1 was expressed in the neural retina from an early stage (E16) of retinal development and that the A1 expression level increased as retinal development progressed (Fig. 3A). Very weak bands were observed for A2a and A2b in the embryonic retina; these receptors started to be expressed in the postnatal retina, and the expression level increased concomitantly with development. We also used semiquantitative RT-PCR of RNA samples isolated from purified $CD73^+$ and

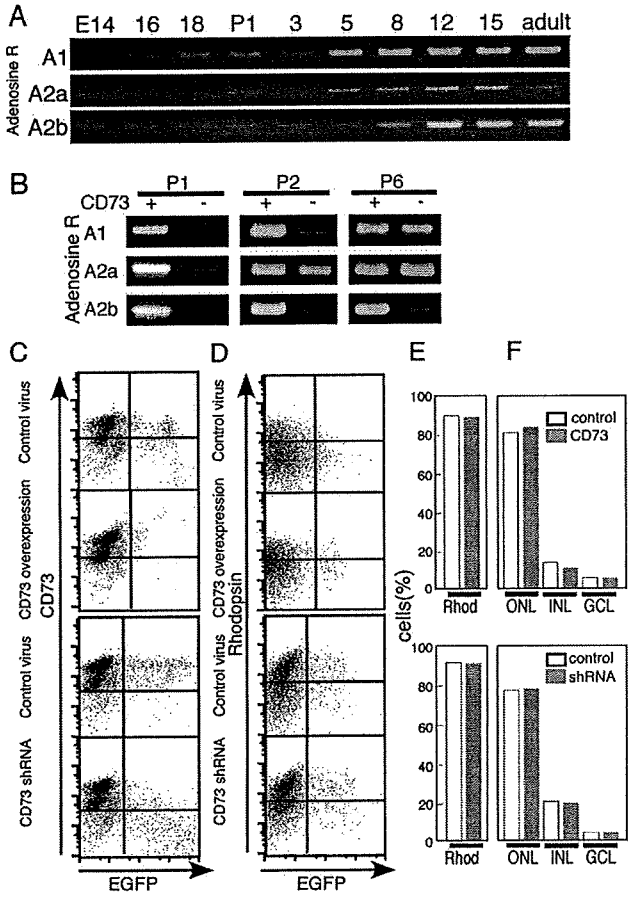


FIGURE 3. The roles of CD73 in retinal development. Temporal expression of mRNA for adenosine receptors in the developing retina. (**A**) Semiquantitative RT-PCR for adenosine receptors A1, A2a, and A2b was carried out using total RNA samples purified from mouse retinas at various developmental stages. (**B**) The expression of adenosine receptors A1, A2a, and A2b in $CD73^+$ and $CD73^-$ cells at the indicated stages was examined by semiquantitative RT-PCR. (**C–F**) Gain- and loss-of-function analyses of CD73. Retroviruses that encode IRES-EGFP or CD73-IRES-EGFP (for overexpression experiments) and CMV EGFP or shRNA against CD73-CMV EGFP (for downregulation experiments) were transduced into retinal explants at E16, followed by FACS analysis of the expression of CD73 (**C**) and rhodopsin (**D**) after 14 days of culture. (**E**) Quantitative results for rhodopsin-positive cells calculated from (**D**). The rhodopsin-positive cells in the EGFP-positive cell population (shown in **D**) are expressed as the relative percentage of those in the EGFP-negative cells population in each experiment. (**F**) Distributions of EGFP-positive cells in the ONL, INL, and GCL. Retroviruses that encode IRES-EGFP, CD73-IRES-EGFP, CMV-EGFP, or shRNA against CD73-CMV EGFP were transduced into retinal explants at E16. After 14 days of culture, frozen sections were produced, and the distribution of EGFP-positive cells in each layer was examined.

$CD73^-$ cells at various stages to examine whether these receptors were expressed in $CD73^+$ cells (Fig. 3B). Interestingly, all three adenosine receptor subtypes were predominantly expressed in $CD73^+$ cells at P1 (Fig. 3B). At P2, weak expression of all three receptors was observed in the $CD73^-$ cells; at P6, significant expression of A1 and A2a was observed in both the $CD73^+$ and $CD73^-$ cells, and A2b was detected primarily in the $CD73^+$ fraction.

To examine the role of CD73 in retinal development, we performed gain- and loss-of-function analyses of CD73 using retinal explant culture. For the gain-of-function analysis, CD73 was expressed by retrovirus-mediated gene expression in the retinal explant. A retrovirus that encoded the wild-type CD73-IRES-EGFP was transduced into retinal explants prepared from an E16 mouse. After 14 days of culture, CD73 expression was examined by FACS analysis (Fig. 3C). The control EGFP virus-expressing population contained both $CD73^+$ and $CD73^-$ cells. In contrast, the CD73-IRES-EGFP virus-transduced cell population contained only $CD73^+$ cells (Fig. 3C). In addition, the expression level of CD73 in this cell population was higher than that of the control samples. After 14 days of retinal explant culture, the expression of rhodopsin was examined by FACS analysis (Figs. 3D, 3E). In the CD73-overexpressed fraction, the proportion of rhodopsin-positive cells was similar to that observed in the control EGFP virus-infected population (Fig. 3E). The downregulation of CD73 was achieved using shRNA, which was introduced into the retinal explant cultures by retroviruses. The suppression of CD73 expression was confirmed by FACS analysis (Fig. 3C). The levels of rhodopsin expression were similar in the EGFP-positive and EGFP-negative cells after 14 days of culture (Fig. 3D). Once again, the levels of rhodopsin expression (Fig. 3E) and distribution of cells into subretinal layers (Fig. 3F) were similar in the EGFP-positive, CD73-downregulated cells and the control EGFP virus-infected cells. Taken together, these results suggest that CD73 does not mediate autonomous cellular signals to promote rhodopsin lineage differentiation. We also examined whether the overexpression or downregulation of CD73 affects other cell lineages, such as Müller glia, ganglion, and amacrine cells by immunostaining with specific markers such as glutamine synthetase and HuC/D. We did not detect significant differences between the two fractions and the controls (data not shown).

CD73 Expressed Downstream of Crx

To analyze the detailed molecular mechanisms, we examined the gene expression patterns of $CD73^+$ and $CD73^-$ cells by semiquantitative RT-PCR. As expected, rhodopsin was expressed exclusively in $CD73^+$ fractions (Fig. 4B). Crx and Nrl are transcription factors that play important roles in photoreceptor cell development.[22,23] Nrl was observed exclusively in $CD73^+$ cells, whereas Crx was observed in both $CD73^+$ and negative cells, which suggests that Crx is expressed earlier than CD73. We also examined the expression of early retinal progenitor markers, such as Chx10[24] and Hes1, and found that they were expressed exclusively in $CD73^-$ cells, as expected (Fig. 4A).

We examined in more detail the time course of gene expression (Fig. 4C). We first examined the temporal transitions of mRNA expression of CD73 and rhodopsin by semiquantitative RT-PCR (Fig. 4C). Weak expression of CD73 mRNA was observed in the retina at E16, and the strength was increased until P5, which is consistent with the transition of CD73 protein expression revealed by FACS analysis. This suggests that the expression of CD73 is regulated mainly at the transcriptional level. The onset of CD73 expression occurred earlier than that of rhodopsin, which supports the idea that CD73

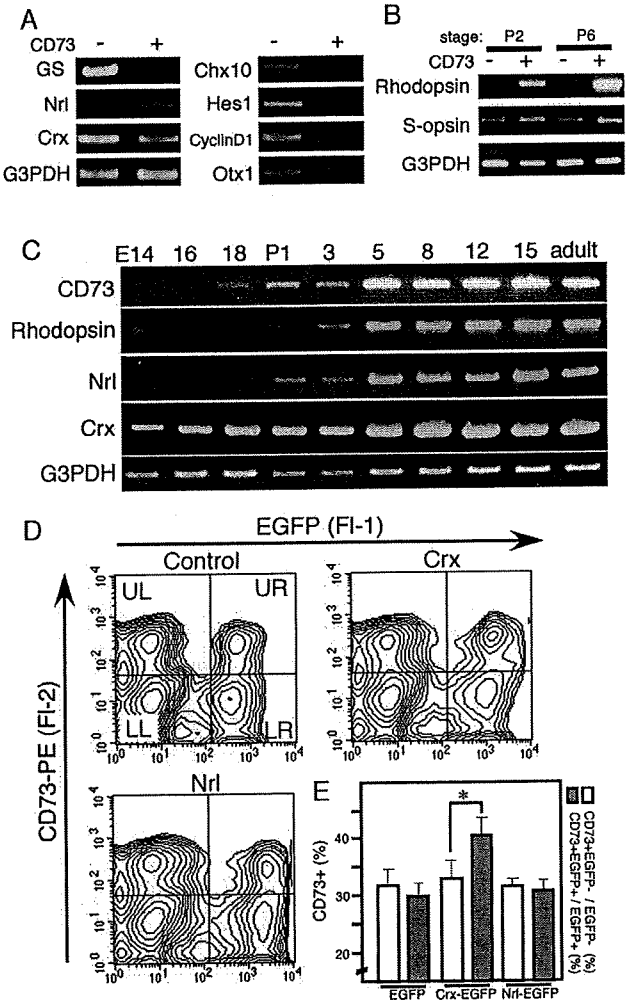


FIGURE 4. Mapping of CD73 in the hierarchy of genes involved in photoreceptor cell differentiation. (A, B) Semiquantitative RT-PCR of $CD73^+$ and $CD73^-$ populations from the P1 (A) and P2 and P6 (B) mouse retina. The RT-PCR products were separated in a 1% agarose gel and visualized with ethidium bromide. G3PDH was used as a control. The experiments were performed at least twice, for all the primers, using independently prepared samples, with essentially the same results. (C) Kinetics of gene expression in the mouse retina. Semiquantitative RT-PCR was performed using RNA from mouse retinas at various developmental stages. G3PDH was used as a control. (D, E) Effects of retrovirus-mediated expression of Crx and Nrl transcription factors on CD73 expression. Retinal explant cultures prepared from E16 mouse retinas were infected with retroviruses that contained EGFP, Crx-IRES-EGFP, or Nrl-IRES-EGFP, and the cells were cultured for 4 days and then dissociated. The expression of CD73 in the EGFP-positive and EGFP-negative cells was examined by flow cytometry. The flow cytometric patterns (D) and the calculated values for the $CD73^+$ cells in the EGFP-positive or EGFP-negative populations are shown (E). The experiments were performed at least three times with essentially the same results.

is an earlier marker than rhodopsin of the photoreceptor lineage. Crx was found to be expressed even at E14, and CD73 expression started around E16 to E18. A transition in Nrl expression in the developing mouse retina was previously reported. Bibb et al.[25] reported that Nrl was expressed after birth, whereas Akimoto et al.[26] reported that Nrl was first expressed during early embryogenesis around E12. In our study, Nrl and rhodopsin expression began around P1. The difference in Nrl expression between these previous studies

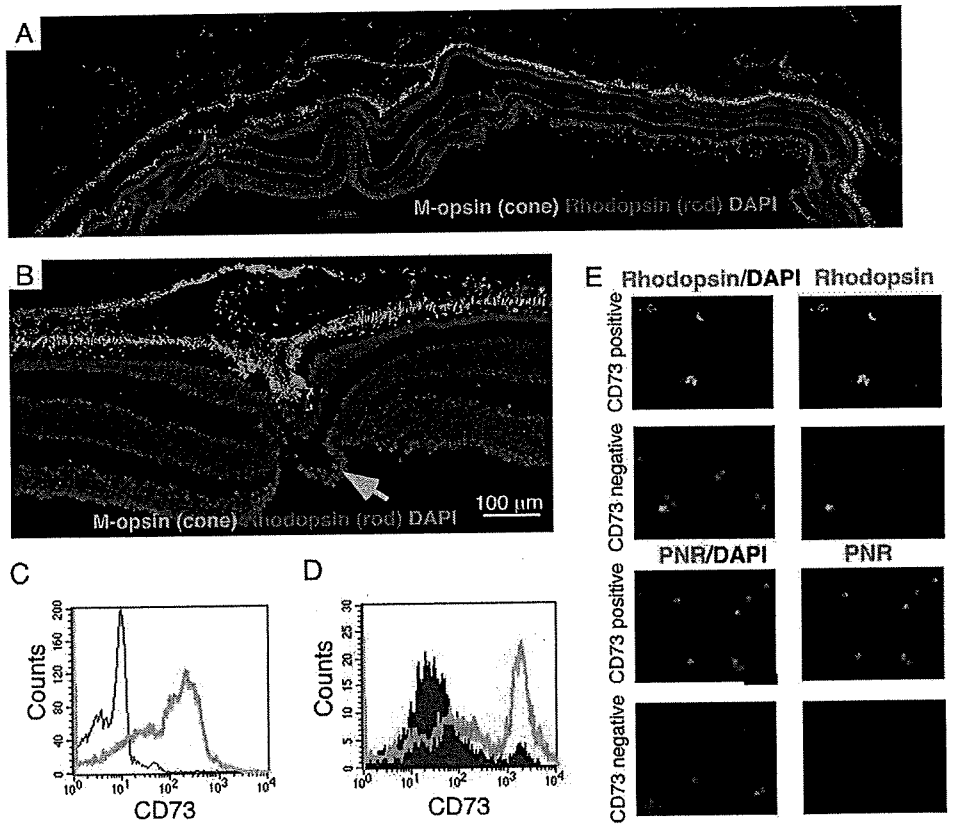


FIGURE 5. Expression of CD73 in marmoset retina. (A, B) Expression of M-opsin and rhodopsin in adult common marmoset retina. Immunostaining was performed using frozen-sectioned retina, and nuclei were visualized by DAPI. (C) FACS pattern of CD73 staining of whole retina of adult common marmoset. *Green line*: CD73 staining. *Black line*: control IgG staining. (D) Adult common marmoset retina was mechanically divided into fovea and other regions, and FACS analysis of CD73 expression was performed for both fractions. *Violet region, green line*: staining patterns of fovea and other regions, respectively. (E) Whole retina of adult common marmoset was dissociated and sorted to $CD73^+$ and $CD73^-$ populations by cell sorter, and the cells were replated on a chamber glass slide and immunostained with antibodies against rhodopsin (*upper four panels*) and PNR (*lower four panels*). Nuclei were stained with DAPI.

and our own may be attributed to a difference in primer sensitivity. Our results confirm that the onset of CD73 expression occurs between Crx and Nrl expression. Therefore, we examined the role of Crx in CD73 expression. To test the effects of Crx, we used Nrl for comparison purposes and incorrectly expressed these genes in retinal explants derived from E16 mice by retroviral-mediated gene transfer.[11] Retinal explants were infected with retrovirus that encoded Crx- or Nrl-IRES-EGFP; they were cultured for 4 days, harvested, and dissociated. CD73 expression was analyzed by flow cytometry (Figs. 4D, 4E). In the control EGFP virus-transfected cells, approximately 30% of the cells expressed CD73 in both the EGFP-positive and EGFP-negative fractions. However, in the Crx retrovirus-infected explant samples, more than 40% of the EGFP-expressing cells also expressed CD73, which was significantly higher than that of the control cells (Fig. 4E). In the Nrl-expressing cell population, the proportion of $CD73^+$ cells in the EGFP-positive fraction was almost the same as in the uninfected parental cells or control EGFP virus-infected cells (Figs. 4D, 4E). Taken together, these results suggest that the activation of Crx positively regulates CD73-expressing cells and that Crx (but not Nrl) is upstream of CD73 in the photoreceptor development hierarchy.

CD73, a Marker for Rod Photoreceptor Cells in Retina of Common Marmoset (*Callithrix jacchus*)

Finally, we asked whether our finding that CD73 is a marker for rod photoreceptor is also applicable for primates. Retinas from adult common marmoset were isolated, and we first examined the expression of rhodopsin and M-opsin in the frozen sectioned retina, including the fovea region. The fovea pit is depicted at the central region of Figure 5A, identified by its unique bended structure of nuclear layers (Fig. 5B). As previously reported,[27] M-opsin was predominantly expressed in the fovea region, and, in contrast, rhodopsin was distributed in regions other than the fovea (Figs. 5A, 5B). Although anti-human CD73 antibody did not give reliable signals of frozen sectioned retina by immunohistochemistry, FACS analysis using whole retina showed strong expression of CD73 (Fig. 5C). Therefore, under a microscope, we mechanically divided the fovea and other regions with the use of a scalpel and used FACS to examine the CD73 expression of these two fractions (Fig. 5D). Strong CD73 expression was observed with retina except for fovea, and only a small population of cells at the foveal region expressed CD73. We purified $CD73^+$ and $CD73^-$ cells of the adult marmoset retina by cell sorter, and the cells were seeded on the slide glass and immunostained of the purified cells with anti-rhodopsin and -PNR, which is rod photoreceptor specific marker, antibodies (Fig. 5E). Almost all cells in $CD73^+$ fractions were rhodopsin or PNR positive; in contrast, only a few positive cells were observed in $CD73^-$ cells. Taken together, we concluded that CD73 is a marker of rod photoreceptor cells in the mature retina of the common marmoset.

DISCUSSION

In the present study, we show that CD73 labels precursor and mature populations of photoreceptor cells. The present report is the first to show specific expression of a cell surface molecule in a photoreceptor cell lineage and the application of this molecule for the enrichment of photoreceptor precursor cells. This is important in terms of applications related to transplantation for the treatment of retinal diseases. Using reaggregation cultures of retinal cells, we show that the purification of CD73 is an efficient way to achieve photoreceptor cell generation by transplantation. This is an in vitro model system, and we are in the process of developing an in vivo transplantation system.

Recently, in vivo retinal repair by transplantation of photoreceptor precursors has been reported.[3] Successful regeneration of functional rod photoreceptors in the mouse is encouraging for researchers working on regenerative medicine of the neural retina. However, in the previous report, the isolated retinas were from transgenic mice that expressed EGFP under the control of the Nrl promoter, which limits the use of this protocol for human applications. CD73 is a cell surface molecule and an anti-CD73 antibody is commercially available, allowing for transplantation and regeneration using CD73-expressing cells without risk for gene transfer. Furthermore, it is important that we found that CD73 is also a marker for the common marmoset photoreceptor. Thus, we can apply this knowledge easily and immediately to human systems using an anti-human CD73 antigen antibody.

Our observation of exclusive expression of CD73 in a photoreceptor lineage is supported by recent DNA microarray analyses of knockout mice. In Nrl-knockout and rhodopsin-knockout mice, both of which lack rod photoreceptors, DNA microarray analyses comparing the retinas of wild-type mice and the knockout mice have revealed that the expression of CD73 is downregulated in these knockout mice.[28,29] However, previous reports have suggested that the 5′-nucleotidase activity is localized in photoreceptor cells and in Müller cells.[30,31] More recently, immunostaining of the 5′-nucleotidase with polyclonal antibody in the developing retina of the mouse has concluded that this enzyme is distributed in Müller cells.[16] In contrast, we did not observe the expression of CD73 in the INL, where the nuclei of Müller glial cells are localized, and CD73 expression never overlapped with glutamine synthetase expression, which is a marker of Müller glial cells, thereby suggesting that CD73 is not expressed in Müller glial cells. Because the previous report did not describe double immunostaining for the 5′-nucleotidase and markers of retinal cells,[16] it is not possible to compare directly the previous results with our present results. However, given that the polyclonal antibody used in the previous paper was raised against the soluble form of bovine 5′-nucleotidase,[16] it is possible that the antibody recognizes a molecule different from that recognized by the anti-CD73 monoclonal antibody used in our present study. This notion is supported by their observation that the expression of the antigen during embryonic development was also associated with proliferating cellular elements,[16] which contrasts with our finding that CD73 is expressed exclusively in nonproliferating cells. Nevertheless, we cannot exclude the possibility that the tertiary structure of CD73 is different in different cell types and that the antibodies only recognize a certain structure of CD73.

Initially, we expected that cone-specific genes would not be expressed in $CD73^+$ cells. However, cone s-opsin mRNA was detected in CD73 cells derived from neonatal mice. In contrast, in mature retinas, we did not observe CD73 expression in s-opsin-expressing cone cells. We speculate that the transition of CD73 expression during photoreceptor cell differentiation occurs as shown schematically in Figure 6. Thus, CD73 is expressed in the common progenitors of cone and rod cells. After terminal differentiation, CD73 continues to be expressed in rod cells until adulthood, whereas it is downregulated in the cone cell lineage.

Nrl and Crx are key transcriptional factors that control photoreceptor differentiation.[23] The homeodomain protein Crx is required for both rod and cone differentiation and regulates the transcription of many photoreceptor-specific genes.[32] The Maf-family bZIP transcription factor NRL is essential for rod differentiation and controls the expression of most of the rod-specific genes.[23] Based on our observations of differential expression of Crx and Nrl in $CD73^+$ and $CD73^-$ cells, we hypothesize that CD73 is downstream of Crx and upstream or parallel to Nrl in rod cell differentiation. Furthermore, detailed examination of the transition of expression of the mRNAs for Crx, CD73, and Nrl also support the idea of ordered expression of these genes in the developing retina. Previous studies of the promoter for CD73 in humans have revealed the involvement of various immune-related transcription factors such as NFAT, hypoxia-related factor 1, and the Wnt signaling pathway.[33,34] We looked for the Crx consensus-binding sequence (C/T TAATCC[35]) in approximately 1 kb of the 5′ upstream region of the mouse CD73 gene and found one matching sequence at −870 nt from the initiation codon. However, this sequence was not found within the region covering 1 kb of human CD73 5′ upstream. We do not know whether the enhancement of CD73-expressing cells by Crx is due to the direct effects of Crx on CD73 transcription or simply results from an expanded population of rod photoreceptors. The most important outcome of the present study is that CD73 is a marker for cells at defined stages of the photoreceptor lineage and can be used in the isolation and transplantation of these cell populations.

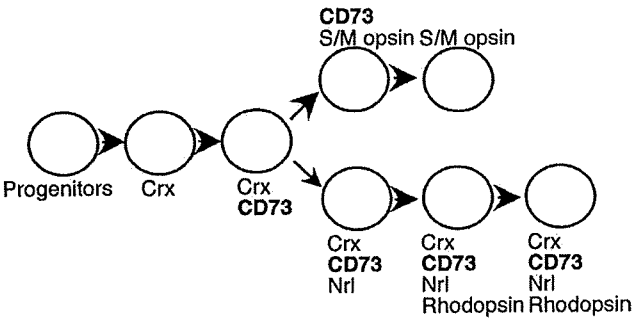


FIGURE 6. Schematic representation of photoreceptor cell differentiation and CD73. CD 73 is first expressed after Crx in the common precursors of the rod and cone cells and continues to be expressed in the rod cell lineage. However, CD73 appears to be downregulated once the cells commit to differentiation into the cone cell lineage.

Acknowledgments

The authors thank Yasuo Ouchi for helpful discussions, and Takashi Shibata and Nobukazu Watanabe of the FACS Core Laboratory (Institute of Medical Science, University of Tokyo, Tokyo, Japan) for technical support with the sorting.

References

1. Rattner A, Nathans J. Macular degeneration: recent advances and therapeutic opportunities. *Nat Rev Neurosci.* 2006;7:860-872.
2. Hartong DT, Berson E, Dryja TP. Retinitis pigmentosa. *Lancet.* 2006;368:1795-1809.
3. MacLaren RE, Pearson RA, MacNeil A, et al. Retinal repair by transplantation of photoreceptor precursors. *Nature.* 2006;444.
4. Koso H, Ouchi Y, Tabata Y, et al. SSEA-1 marks regionally restricted immature subpopulations of embryonic retinal progenitor cells. *Dev Biol.* 2006;292:265-276.
5. Koso H, Satoh S, Watanabe S. c-Kit marks late retinal progenitor cells and regulates their differentiation in developing mouse retina. *Dev Biol.* 2007;301.
6. Zimmermann H. 5′-Nucleotidase: molecular structure and functional aspects. *Biochem J.* 1992;285:345-365.
7. Deussen A, Bading B, Kelm M, Schrader J. Formation and salvage of adenosine by macrovascular endothelial cells. *Am J Physiol.* 1993; 264:692-700.
8. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. 'Green mice' as a source of ubiquitous green cells. *FEBS Lett.* 1997;407:313-319.