Characterization of a novel embryonic stem cell line from an ICSI-derived blastocyst in the African green monkey

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Abstract

Several cell types from the African green monkey (*Cercopithecus aethiops*), such as red blood cells, primary culture cells from kidney, and the Vero cell line, are valuable sources for biomedical research and testing. Embryonic stem (ES) cells that are established from blastocysts have pluripotency to differentiate into these and other types of cells. We examined an *in vitro* culture system of zygotes produced by ICSI in African green monkeys and attempted to establish ES cells. Culturing with and without a mouse embryonic fibroblast (MEF) cell monolayer resulted in the development of ICSI-derived zygotes to the blastocyst stage, while culturing with a buffalo rat liver cell monolayer yielded no development (3/14, 21.4% and 6/31, 19.4% vs 0/23, 0% respectively; $P < 0.05$). One of the nine blastocysts, which had been one of the zygotes co-cultured with MEF cells, formed flat colonies consisting of cells with large nuclei, similar to other primate ES cell lines. The African green monkey ES (AgMES) cells expressed pluripotency markers, formed teratomas consisting of three embryonic germ layer tissues, and had a normal chromosome number. Furthermore, expression of the germ cell markers *CD9* and *DPPA3* (*STELLA*) was detected in the embryoid bodies, suggesting that AgMES cells might have the potential ability to differentiate into germ cells. The results suggested that MEF cells greatly affected the quality of the inner cell mass of the blastocysts. In addition, AgMES cells would be a precious resource for biomedical research such as other primate ES cell lines.

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Introduction

In vitro culturing is an important technique for effectively producing individuals from embryos that were manipulated *in vitro*. However, the *in vitro* culturing for the African green monkey (*Cercopithecus aethiops*) embryos has not been examined. In rhesus monkey embryos, co-culture with a buffalo rat liver (BRL) cell monolayer in CMRL-1066 has been utilized (Zhang *et al.* 1994, Nusser *et al.* 2001). On the other hand, mouse embryonic fibroblast (MEF) cells have been widely utilized for the establishment of embryonic stem (ES) cells, showing that MEF cells affect establishment of the ES cells from the inner cell mass (ICM), where develop to an individual in the future, of blastocyst. We therefore considered that MEF might also enhance the growth of African green monkey embryos cultured *in vitro*.

Primate ES cell lines were established in the rhesus monkey (*Macaca mulatta*) for the first time in 1995 (Thomson *et al.* 1995) and were subsequently

established in the common marmoset (*Callithrix jacchus*) in 1996 (Thomson *et al.* 1996), in humans in 1998 (Thomson *et al.* 1998), and in the cynomolgus monkey (*Macaca fascicularis*) in 2001 (Suemori *et al.* 2001), aiming at utilization in biomedical research. In particular, application to regenerative medicine is expected. For this purpose, methods to induce ES cells to develop into specific differentiated cells have been examined, based on the pluripotency of ES cells, as well as their self-renewal and stable karyotype characteristics, which are important properties for not only regenerative or transplantation medicines but also research or inspection at the cytological level.

The red blood cells and primary culture cells from kidney of the African green monkey have been used for the inspection of measles infection and human polio vaccine production respectively. In addition, the Vero cell line established from the kidney of the monkey has been used to test viral infections and to introduce foreign genes. Furthermore, spontaneous systemic amyloidosis was

Table 1 Attempt to establish embryonic stem (ES) cell lines in African green monkeys.

	Culture of zygotes	Number of blastocysts	Treatment of blastocysts ^a	Number of outgrowths	Passage methods	Colony appearance	Passage methods	Number of ES cell lines established
I	With MEF monolayer	1	Removal of ZP and TE	1	Enzyme and needles	1	Enzyme and needles	1
II		2	Removal of ZP	2	Enzyme and needles	1	Enzyme and pipetting	0
III	Without cell monolayer	3	Removal of ZP and TE	1	Enzyme and pipetting	1	Enzyme and pipetting	0
IV		3	Removal of ZP	0	–	–	–	–

^aZP, zona pellucida; TE, trophectoderm.

recently reported in this monkey (Nakamura *et al.* 2008). The African green monkey and the differentiated cells derived from African green monkey ES (AgMES) cells may help to elucidate the mechanism of amyloidosis in humans and contribute to the treatment. Those unique characteristics show that the cells of the monkey are very valuable for biomedical research and testing.

ES cells may be useful as a source of cells with the same genetic background, even if we do not collect them from living individuals. In short, we could reduce the number of monkey needed to supply cells. Here, we report an *in vitro* culture system of ICSI-derived zygotes

and the establishment of a novel ES cell line in the African green monkey. We believe that AgMES cells would be a precious resource for biomedical research in addition to other primate ES cell lines.

Results

In vitro culture of ICSI-derived embryos

Blastocyst stage embryos were prepared from zygotes produced by ICSI as described in our previous report (Shimozawa *et al.* 2007). A total of 68 zygotes with two pronuclei and a second polar body were cultured with or

Figure 1 Establishment of the AgMES cell line. Parts of the inner cell mass isolated from an ICSI-derived blastocyst (A) were transferred onto a mitomycin C-treated MEF cell monolayer, which then formed an outgrowth (B). At the first passage dividing the outgrowth into small clusters with collagenase and needles, a new colony appeared (C) and was passaged further. The colonies formed multiple new colonies (D). Hereafter, these colonies were regularly passaged with collagenase and pipetting. Karyotyping analysis revealed that cells examined at passage 19 had a normal chromosome number of 60 and sex chromosomes of XX (E). Bar represents 100 µm (A and B) and 500 µm (C and D).

without BRL cell monolayer or MEF cell monolayer, and then nine of those developed to the blastocyst stage. Culturing with and without the MEF cell monolayer yielded development to the blastocyst stage, while culturing with the BRL cell monolayer yielded no development (3/14, 21.4% and 6/31, 19.4% vs 0/23, 0% respectively; $P < 0.05$).

Establishment of ES cells

Nine blastocysts were used to establish ES cells (Table 1). Parts of the ICM isolated by removing the trophectoderm (TE) and the zona pellucida (ZP) with needles (one blastocyst cultured with MEF, hereafter designated I, Fig. 1A; and three without MEF, designated III) or the blastocysts in which the ZP was dissolved with an enzyme (two blastocysts with MEF, designated II; and three without MEF, designated IV) were transferred to ES cell culture medium (ESM) with mitomycin C-treated STO cell or MEF cell monolayer. Of nine blastocysts, four formed outgrowths (one of I blastocysts: Fig. 1B, two of II, and one of III). At the first passage using collagenase and pipetting or dividing into small clusters with needles, three colonies from three outgrowths (one each of I: Fig. 1C, III and IV) appeared and passaged further. The colonies from I passaged with collagenase

and needles newly formed multiple colonies (Fig. 1D), but the other colonies passaged with collagenase and pipetting disappeared. Thereafter, these colonies from I were regularly passaged with collagenase and pipetting, and showed flat-formed colony morphology consisting of cells with large nuclei, similar to other primate ES cell lines.

Characterization of undifferentiated ES cells

We examined karyotype and the expression of undifferentiated markers in the AgMES cell line established. Karyotyping analysis revealed that 88% (88/100) of cells examined at passage 19 had a normal chromosome number of 60 and sex chromosomes of XX (Fig. 1E). Immunofluorescence revealed that this cell line strongly expressed POU5F1 (Oct-3), TRA-1-60, TRA-1-81, and NANOG, but not SSEA1 (Fig. 2A–E), and showed alkaline phosphatase activity (Fig. 2F). The expression of SSEA3 and SSEA4 was vaguely observed. Furthermore, gene expression analysis of the pluripotency markers by RT-PCR demonstrated that *POU5F1* (*OCT3/4*), *NANOG*, *SOX2*, and *REX1* were present in AgMES cells, but *FOXD3* was not, although three primer sets for *FOXD3* were used (Fig. 2G). On the other hand, in CMK6 (AGC Techno Glass Co., Ltd, Chiba, Japan),

Figure 2 Characterization of undifferentiated AgMES cells. AgMES cells were stained with antibodies against pluripotency markers and alkaline phosphatase (A–F). POU5F1 (A), NANOG (B), TRA-1-60 (C), TRA-1-81 (D), SSEA1 (E), and alkaline phosphatase (F). Nuclei were counterstained with Hoechst 33342 (blue) (A–E). A double staining with POU5F1 (A) and NANOG (B) was conducted in the same specimen. Bar represents 50 μ m (A–E) and 200 μ m (F). Gene expression analysis of pluripotency markers by RT-PCR in AgMES cells was conducted (G). The results were compared with those for CMK6, a cynomolgus monkey ES cell line established by Suemori *et al.* (2001), as a control.

Figure 3 Characterization of derivatives from AgMES cells. (A) EBs (a) at 2 weeks after unattached culture spontaneously differentiated to neuron-like cells (b), pigment cells (c), and myocardial-like cells with beating clusters (d). In the derivatives, the expression of β -tubulin III (e, ectoderm), α -smooth muscle actin (f, mesoderm), α -fetoprotein (g, endoderm), and CDX2 (h, trophoblast) was confirmed. Nuclei were counterstained with Hoechst 33342 (blue) (e–h). Bar represents 250 μ m (a–d) and 50 μ m (e–h). (B) Histological analysis of teratomas formed from AgMES cells. Characteristic morphologies; neural (ectoderm, a), osseous (mesoderm, b), and gut tissues (endoderm, c) were confirmed. Each figure includes insets for showing high magnification of typical cells, such as neuroblasts (a), osteoblasts with osseous matrix (b), and goblet cells (c). Bars represent 100 μ m, 40 μ m (inset a), and 20 μ m (inset b and c). (C) Gene expression analysis of germ cell markers by RT-PCR in EBs at days 7, 14, 21, and 28 of culture.

the cynomolgus monkey ES cell line established by Suemori *et al.* (2001) and employed here as a control, the expression of all markers examined was detected (Fig. 2G).

Pluripotency analysis

To examine the pluripotency of AgMES cells, we induced the development of embryoid bodies (EBs) and teratomas *in vitro* and *in vivo* respectively. EBs were developed by the floating culture of ES cell colonies recovered with collagenase treatment (Fig. 3A; a). After 2 weeks of culturing, most EBs formed solid type clusters. EBs were transferred to tissue culture dishes and then attached. Attached EBs showed outgrowths and spontaneously formed the various differentiated cells such as neuron-like cells, pigment cells, and beating myocardial-like cells (Fig. 3A; b–d). These spontaneous differentiated cells from EBs showed the expression of β -tubulin III (ectoderm), α -smooth muscle actin (mesoderm), α -fetoprotein (endoderm), and CDX2 (TE; Fig. 3A; e–h).

Histological analysis revealed that the ES cells transferred into two immunodeficient mice formed teratomas consisting of neural and dermal tissues as ectoderm (Fig. 3B-a; neural tissue), smooth muscle, osseous, cartilage, fatty, fibrous, and vascular tissues as mesoderm (Fig. 3B-c; osseous tissue), and gut tissue as endoderm tissue (Fig. 3B-e). The teratoma formation rate was 100% (2/2). In EBs at days 7, 14, 21, and 28 of culture, we examined the expression of the germ cell markers *CD9*, *DPPA3* (*STELLA*), and *DDX4* (*VASA*) by RT-PCR. Expression of *CD9* and *DPPA3* was detected in all samples, while *DDX4* was not, although two primer sets for *DDX4* were used (Fig. 3C).

Characterization of single-cell subcultured ES cells

To further characterize the AgMES cell line, we examined whether this cell line could be subcultured by dividing it into single cells with trypsin. The average replating efficiency and rate of colonies with undifferentiated morphology until 52 passages were 20.2 ± 5.7

Figure 4 Characterization of single-cell subcultured AgMES cells. (A) The expression of the pluripotency markers, POU5F1 (a), NANOG (b), TRA-1-60 (c), and TRA-1-81 (d), was confirmed. Nuclei were counterstained with Hoechst 33342 (blue) (a–d). A double staining with POU5F1 (a) and NANOG (b) was conducted in the same specimen. Bar represents 50 μ m. (B) Karyotyping of single-cell subcultured AgMES cells. At passage 34, 80% (40/50) of the single-cell subcultured AgMES cells had a normal karyotype (60 chromosomes, XX). (C) Histological analysis of teratomas formed from single-cell subcultured AgMES cells. The teratomas showed neural (ectoderm, a), osseous (mesoderm, b), and gut (endoderm, c) tissues. Each figure includes insets for showing high magnification of typical cells, such as neuroblasts (a), osteoblasts with osseous matrix (b), and goblet cells (c). Bars represent 100 μ m, 40 μ m (inset a), and 20 μ m (inset b and c).

and $15.4 \pm 5.8\%$ respectively and $74.5 \pm 13.8\%$ of the subcultured single cells formed the undifferentiated colonies. Immunofluorescence revealed that this cell line strongly expressed POU5F1, TRA-1-60, TRA-1-81, and NANOG but not SSEA1 (Fig. 4A). Karyotyping analysis revealed that 80% (40/50) of cells examined at passage 34 after single-cell subculture had a normal chromosome number of 60 (Fig. 4B). Histological analysis revealed that teratomas by single-cell subcultured ES cells in two immunodeficient mice consisted of three embryonic germ layer tissues, e.g. neural and dermal tissues as ectoderm (Fig. 4C-a; neural tissue), musculoskeletal, fatty, fibrous, and vascular tissues as mesoderm (Fig. 4C-b; osseous tissue), and gut tissue as endoderm tissue (Fig. 4C-c). The teratoma formation rate was 100% (2/2).

Discussion

We examined *in vitro* culturing of ICSI-derived zygotes and the establishment of an ES cell line, rather than examining developmental potential to term, in the African green monkey. As a result, we succeeded in developing the zygotes to the blastocyst stage and were able to establish a novel primate ES cell line from nine of the blastocysts. The cells derived from African green monkeys are utilized in many types of research and tests.

Therefore, it is expected that the AgMES cell line described in this report could contribute to these applications as a novel biological resource.

Reproductive technologies in the African green monkey have hardly been reported. Development to the blastocyst stage was only reported by *in vitro* culture in a simple medium following production of the fertilized eggs with IVF in 1997 (Sankai *et al.* 1997). We reported follicle growth stimulus methods and the fertilization ability by ICSI in 2007 (Shimozawa *et al.* 2007). Herein, we examined an *in vitro* culture system using zygotes produced by ICSI. *In vitro* culturing with and without a MEF cell monolayer supported development to the blastocyst stage, while culturing with a BRL cell monolayer did not. Although a BRL cell monolayer supported development to the blastocyst stage of rhesus monkey embryos (Zhang *et al.* 1994, Nusser *et al.* 2001), the reason for this negative effect by BRL cells in African green monkey embryos in the present study is uncertain. On the other hand, the ES cell line was established from the blastocysts that originated from the culture system with the MEF cell monolayer. The number of cells constituting the blastocysts or their ICMs could not be examined because we gave priority to the establishment of the ES cell line. Consequently, it was impossible to compare effects between the culture systems in detail. However, one of the reasons for the successful

establishment of an ES cell line may have been that MEF cells greatly affected the quality of the ICM.

The novel established AgMES cell line showed characteristics similar to those of other primate ES cell lines (Thomson *et al.* 1995, 1996, 1998, Suemori *et al.* 2001). The ratio of nuclei to the cytoplasm in an ES cell was very high, and the colony morphology was flat. Expression of the primate ES cell-specific markers was confirmed by immunofluorescence staining and RT-PCR analysis, and we confirmed that ES cells spontaneously differentiated into three embryonic germ cells via the EBs under *in vitro* culturing and formed teratomas that consisted of three embryonic germ cells by being transplanted to SCID mice. In addition, 88% of the examined cells had maintained the normal chromosome number of 60. As described above, the cell line established herein demonstrated that it is an exact match to the ES cell line.

Immunofluorescence revealed that AgMES vaguely expressed SSEA3 and SSEA4. This may be due to antibodies not suitable for African green monkey epitopes. However, it is possible that expressions of SSEA3 and SSEA4 are not indispensable for ES cells (Brimble *et al.* 2007). The cynomolgus monkey ES cells are used for basic experiments aimed at application in regenerative medicine (Sánchez-Pernaute *et al.* 2005, Takagi *et al.* 2005, Shibata *et al.* 2006, Osakada *et al.* 2008). Reports of the differentiation from cynomolgus monkey ES cells to various types of cell suggest that the expression of SSEA3 may not be necessary.

The gene expression analysis also showed that AgMES cells were positive for *POU5F1*, *NANOG*, *SOX2*, and *REX1*. The expression of *FOXD3* was not detected, despite the use of three primer sets for *FOXD3*. However, in EB, the expression of *FOXD3* was detected (data not shown). It has been reported that *FOXD3* has the antagonistic effect on *POU5F1* (Guo *et al.* 2002) and was not expressed in undifferentiated human and common marmoset ES cells (Ginis *et al.* 2004, Mandal *et al.* 2006, Müller *et al.* 2009). We demonstrated that *FOXD3* of African green monkey was not expressed in undifferentiated cells but was detected during differentiation the same as human and common marmoset. In addition, a difference in the gene expression of the pluripotency marker *REX1* in ES cells has been observed among species. *REX1* expression has been detected in human ES cells (Ginis *et al.* 2004, Mandal *et al.* 2006) but not in rhesus monkey ES cells (Mitalipov *et al.* 2006). In the present study, both of African green and cynomolgus monkey ES cells expressed *REX1*, suggesting that the characterization of different ES cell lines within the same species may differ.

Several researchers have examined the differentiation of primate ES cells into germ cells (Clark *et al.* 2004, Chen *et al.* 2007, Sparman *et al.* 2009). When we examined the expression of three germ cell markers, *CD9*, *DPPA3*, and *DDX4*, in EBs derived from AgMES

cells by RT-PCR, the expression of *CD9* and *DPPA3* was detected. This suggested that AgMES cells might have the potential ability to differentiate into germ cells, although further study is necessary. Differentiation into germ cells may be enabled in the primates by doing the comparative study using primate and mouse ES cell lines. Additionally, it was reported that epiblast stem cells (EpiSCs) established in mice did not possess the ability to differentiate into germ cells *in vivo* (Brons *et al.* 2007, Tesar *et al.* 2007). Primate ES cells may also lack the potential to differentiate into germ cells because EpiSCs show characteristics similar to primate ES cells such as culture condition and colony morphology. However, it is unclear whether primate ES cells can differentiate into germ cells. It will thus be very important to study the differentiation of nonhuman primate ES cells into germ cells both *in vivo* and *in vitro*. We consider that the AgMES cells could become a highly valuable research tool as well as rhesus, cynomolgus, and common marmoset monkey ES cell lines.

Next, we examined whether single AgMES cells can continuously be passaged. When the subcultures of single ES cells dissolved with trypsin were repeated 52 times, a replating rate of 20.2% and an undifferentiation colony formation rate of 15.4% were obtained. These ES cells were positive for the primate ES cell-specific markers, formed teratomas, and had normal karyotype, which demonstrated that the AgMES cells maintained by the single-cell subculture method had the same characteristics of undifferentiation and pluripotency. Human ES cell lines were difficult to continue to subculture by single cells, and because the undifferentiation colony formation rate was ~1%. Improvements of culture environment by using some feeder cells or chemical compounds have been examined (Amit *et al.* 2000, Hasegawa *et al.* 2006, Ellerström *et al.* 2007, Watanabe *et al.* 2007). However, single AgMES cells showed 15% of the undifferentiation colony formation rate in the same culture environment as the subculture by small clusters. The AgMES cell line has the potential for easy application in various examinations under *in vitro* culture and to contribute to the research into the proliferation mechanisms underlying colony formation from a single cell in the primate ES cells.

The primary culture cells or the Vero cells that originate from the kidney of the African green monkey have been used for applications such as vaccine developments and virus inspections. In addition, the red blood cells of the African green monkey are used for the measles virus test. We think that studies of the erythropoiesis or hematopoiesis differentiation in cynomolgus ES cells might be able to contribute to differentiation of the red blood cells from AgMES (Hiroyama *et al.* 2006, Umeda *et al.* 2006). The characteristics and chromosomes of kidney-derived cells may have mutated naturally by repeating the subculture for a long time. Inducing differentiation of

pluripotent ES cells, whose chromosomes are relatively steady compared with the various cell lines, may facilitate their becoming a source of cells with uniform properties. This shows the possibility that the red blood cells and the cells of kidney differentiated from the AgMES cells may become a substitute for cells from living monkeys. Producing the various cells from the ES cells would alleviate the need to collect the cells from living animals.

Materials and Methods

Animals

The mature African green monkeys (*C. aethiops*) used in this study were bred and maintained in an air-conditioned room with controlled illumination (12 h light:12 h darkness), temperature ($25 \pm 2^\circ\text{C}$), humidity ($60 \pm 5\%$), and ventilation (10 cycles/h), and were given 70 g of commercial food (Type AS; Oriental Yeast Co., Ltd, Tokyo, Japan) and 100 g of apples daily, and unlimited access to tap water at the Tsukuba Primate Research Center (Tsuchida *et al.* 2008). Every morning, the health condition (e.g. viability, appetite, fur-coat appearance) and menstruation status of each female monkey were monitored. For ovarian stimulation, eight female monkeys were used. Maintenance of animals was conducted according to the guidelines set by the National Institutes of Biomedical Innovation (NIBIO) for the care, use, and biohazard countermeasures of laboratory animals. This study was approved by the Animal Welfare and Animal Care Committee of NIBIO.

Ovarian stimulation and oocyte collection

For ovarian stimulation, the administration of human FSH (hFSH: Fertinorm, Merck Serono), equine chorionic gonadotropin (eCG: Serotropin, ASKA Pharmaceutical, Tokyo, Japan), or human menopausal gonadotropin (hMG) followed by human chorionic gonadotropin (hCG: Gonatropin, ASKA Pharmaceutical) was conducted. Protocols of hFSH and eCG administration, and oocyte collection were reported previously (Shimozawa *et al.* 2007). Briefly, on the first day of menstruation, the female monkeys were administered leuprorelin acetate s.c. as a GnRH agonist (GnRHa; Leuplin, Takeda Pharmaceutical, Osaka, Japan). At 2–3 weeks after that, the follicular growth of these monkeys was stimulated by 25 IU/kg per day hFSH administered for 9 days, 200 IU/head per day eCG administered i.m. for 6 days, or 37.5 IU/head per day hMG administered i.m. daily for 6 days. Thirty-six hours after the final hFSH, eCG, or hMG administration, 1200 IU/head hCG was administered. At 36–38 h after hCG administration, females were anesthetized by 10 mg/kg ketamine hydrochloride (Ketalar, Bayer Yakuhin) and 1 mg/kg xylazine hydrochloride (Seractarl, Bayer Yakuhin) administration. Ovaries were exposed through an abdominal incision, and the contents of the follicles were aspirated through a 25-gauge needle. The collected follicular contents were immediately placed in TYH medium modified by adding Hepes (Hepes-TYH) containing 2.5 IU/ml heparin (Novo Nordisk Pharma, Tokyo, Japan). The oocytes were freed from cumulus cells by

pipetting after treatment with 0.1% hyaluronidase (Sigma) in Hepes-TYH medium. Collected oocytes were then transferred to CMRL-1066 (Invitrogen) medium containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), Gluta-MAX ($\times 100$, Invitrogen), and penicillin–streptomycin solution ($\times 100$, Sigma), hereafter simply called CMRL.

ICSI and embryo culture

Sperm collection and ICSI were performed as described previously (Shimozawa *et al.* 2007). Briefly, fresh semen was collected in TYH medium by rectal probe electrostimulation. Semen suspension was layered onto 90% Percoll (GE Healthcare UK Ltd, Buckinghamshire, England) diluted with 9% NaCl solution and then centrifuged at 800 *g* for 10 min. The precipitate containing sperm was suspended in CZB modified by adding Hepes (Hepes-CZB). Sperm suspended in Hepes-CZB with 10% polyvinylpyrrolidone (Sigma) was used for ICSI. Mature oocytes with a polar body were subjected to ICSI using a micromanipulation system equipped with a piezo drive unit (Primetech, Ibaraki, Japan) under an inverted microscope (Nikon, Tokyo, Japan). An immobilized spermatozoon drawn into the injection pipette was injected into the mature (MII) oocyte by using a few piezo pulses, and then injected oocytes were transferred into CMRL microdroplets. The fertilized embryos were judged to be normal based on the formation of two pronuclei and the release of a second polar body 15–16 h after ICSI. Embryos were cultured with or without a MEF cell monolayer or a BRL cell monolayer in 500 μl CMRL using a four-well multidish (Nunc, Rochester, Denmark). Half of the CMRL was changed every other day. Culturing was performed under CMRL covered with mineral oil at 37.5°C in a humidified atmosphere of 5% CO_2 , 5% O_2 , and 90% air.

Establishment of ES cells

The TE and ZP of blastocysts were removed mechanically with 27-G needles or dissolved in the ZP with 0.5% actinase. Treated blastocysts were cultured with mitomycin C-treated STO cell or MEF cell monolayer on gelatin-coated dishes in ESM consisting of DMEM/F12 (1:1) supplemented with 20% knockout serum replacement (KSR, Invitrogen), 1% GlutaMax (Invitrogen), 0.1 mM β -mercaptoethanol (Sigma), 1% nonessential amino acids (Invitrogen), and 10 ng/ml human recombinant leukemia inhibitory factor (hLIF, Millipore, Billerica, MA, USA). In one case, ESM in which FBS replaced KSR and that was supplemented with 4 ng/ml human recombinant basic fibroblast growth factor (hbFGF, Wako Pure Chemical Industries, Ltd, Osaka, Japan) was used. The extended colony was treated with 0.1% collagenase (Wako) in DMEM (Sigma) and divided mechanically into small clusters with pipetting or with 27-G needles, then passaged onto a new feeder layer. The resulting stem cell colony was expanded using 0.1% collagenase and passaged with pipetting or 27-G needles into a 6-cm dish (BD Falcon, Franklin Lakes, NJ, USA). ES cell colonies were divided into small clusters using 0.1% collagenase and pipetting.

Table 2 Primer sequences used for RT-PCR analyses.

Gene	Forward	Reverse	bp
<i>POU5F1</i>	TTGGAGACCTCTCAGCCTGA	ACACATGTTCTTGAAGCTAA	326
<i>SOX2</i>	CCCCCGGCGGCAAC GCA	TCGGCGCCGGGGAGATACAT	448
<i>NANOG</i>	CAGAAGGCCTCAGCACCTAC	GACTGTTCCAGGCCTGATTGT	217
<i>REX1</i>	CAGATCGAAACAGCTCGCAGAAT	CGTACGCAAATTGAAGTCCAGG	305
<i>FOXD3</i>	TACATCGCGCTCATCACCATG	GTTGTGCAACATGTCCTCGGA	246
<i>CD9</i>	AAATAGCTGCGGCCATCTGGGGATA	G CCCCCAGCCAAACCACAGCA	167
<i>DPPA3</i>	GTTACTCGGCAGAGTTCGTA	TGAAGTGGCTTGGTGTCGTA	167
<i>DDX4</i>	AGGATGAGGACTCCATCTTGCACATTAT	CAGACCTGTTTGAGCACAAGCCA	252
<i>GAPDH</i>	TGGACCTGACCTGCCGTCT	GGAAGAGTGGGTGTCGTGT	152

For the investigation of undifferentiated colony formation from a single ES cell, ES cell colonies recovered after collagenase treatment were dispersed to single cells with 0.25% trypsin/0.1 m EDTA (Sigma), and a thousand single ES cells were cultured with MEF cell monolayer in a 3.5-cm dish (BD Falcon).

Characterization

Alkaline phosphatase was detected using Alkaline Phosphatase Chromogen Kit (Biomeda, Plovdiv, Bulgaria). For immunofluorescence, cells were fixed with 4% paraformaldehyde in PBS for 20 min. Following permeabilization with 0.2% Triton X-100 in PBS for 10 min and blocking with 5% skim milk in PBS for 30 min, cells were incubated with primary antibodies overnight at 4 °C and visualized by IgG or IgM conjugated with Alexa 488 (A11001, A21042, and A21212) or 555 (A21428) (1:1000, Invitrogen). Primary antibodies used were as follows: POU5F1 (1:50, 611202; BD Biosciences, San Jose, CA, USA), NANOG (1:50, RCAB0003P, ReproCELL Inc., Tokyo, Japan), SSEA1 (MAB4301), SSEA3 (MAB4303), SSEA4 (MAB4304), TRA-1-60 (MAB4360), TRA-1-81 (MAB4381) (1:80, Millipore), Nestin (1:50, MAB1259), α -smooth muscle actin (1:100, MAB1420, R&D Systems, Minneapolis, MN, USA), β -tubulin III (1:50, T 8660), α -fetoprotein (1:500, A 8452, Sigma), and CDX2 (1:100, GTX15258, GeneTex, San Antonio, TX, USA). The nuclei were stained with 10 μ g/ml Hoechst 33342 (Calbiochem, Darmstadt, Germany) in PBS.

For pluripotency analysis, EBs and teratomas were prepared from ES cells. EBs were grown by culturing unattached ES cell colonies in ESM without hLIF and hbFGF. After 2 weeks, EBs were transferred to 6-cm dishes in DMEM containing 10% FBS for attachment culture. Spontaneous differentiated cells from EB were observed and analyzed by immunofluorescence. Teratoma formation was as follows: six 6-cm dishes of small cluster subcultured and single-cell subcultured ES cells suspended in ESM without hLIF and hbFGF were injected into the hind leg muscle of two immunodeficient mice (NOD/SCID, Charles River Japan, Kanagawa, Japan) respectively. Tumors were fixed with 4% paraformaldehyde in PBS, then embedded in paraffin, and sectioned for histological analysis by hematoxylin and eosin staining. No tumors formed in two NOD/SCID mice that were injected with ESM without hLIF and hbFGF as a control.

Karyotype analyses were performed at the International Council for Laboratory Animal Science Monitoring Center (Kanagawa, Japan) or the Chromosome Science Labo Inc (Hokkaido, Japan).

Gene expression analysis

Undifferentiated AgMES cells and EBs at days 7, 14, 21, and 28 of culture were treated with RNA^{later} (Ambion, Austin, TX, USA). RNA was isolated using an RNAqueous Kit (Ambion) according to the manufacture's protocol. First-strand cDNA was primed via random hexamers, and RT-PCR was performed with the primer sets showing in Table 2. The expected sizes of the PCR products were inferred from rhesus monkey and human sequences.

Statistical analysis

Data were analyzed using Fisher's exact probability test. $P < 0.05$ was considered significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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