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Growth Characteristics of the Nonhuman Primate Embryonic Stem Cell Line Cjes001 Depending on Feeder Cell Treatment

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Abstract

Embryonic stem cells (ESC) hold tremendous potential for therapeutic applications, including regenerative medicine, as well as for understanding basic mechanisms in stem cell biology. Since numerous experiments cannot be conducted in human ESC because of ethical or practical limitations, nonhuman primate ESC serve as invaluable clinically relevant models. The novel marmoset (*Callithrix jacchus*) ESC line cjes001 was characterized using different stem cell markers. The cells were stained positively with Oct4, SSEA-3, SSEA-4, Tra-1-60, Tra-1-81, and Sox-2 underscoring their status as undifferentiated ESC. ESC are typically grown on mouse embryonic fibroblasts (MEF) as feeder cells whose proliferation is arrested either by treatment with Mitomycin C or by γ-irradiation. To assess the impact of these treatments on the ability of MEF to support the growth of undifferentiated ESC, we used an MTT assay to evaluate the cellular metabolic activity of growth arrested feeder cells. There was a significant ($p < 0.02$) difference in γ-irradiated cells displaying a higher metabolic activity compared to Mitomycin C inactivation. Also we quantified 69 soluble factors in the supernatant of both Mitomycin-treated and γ-irradiated MEF by bead-based multiplex analysis, and thus established a profile of MEF-secreted factors. The time course of secretion was analyzed by monitoring the supernatant at 0, 6, 12, and 24 h after changing the medium. Comparing γ-irradiated and Mitomycin-treated MEF suggested higher amounts of some cytokines including FGF or SCF by the former. We also assessed whether the method of inactivation had an effect on growth kinetics and differentiation of primate ESC. There appeared to be a trend to a lower number of differentiated ESC colonies on the γ-irradiated feeder cells, suggesting that this may be the preferable method of growth arrest.

Introduction

Embryonic stem cells (ESC) hold tremendous potential for therapeutic applications because of their ability to differentiate into multiple, clinically applicable cell types (Fleischmann, 2007; Mountford, 2008; Wobus, 2001). Mouse ESC are very commonly used, and methods for culture and differentiation of these stem cell lines are well established. However, mouse ESC significantly differ from human ESC in their characteristics concerning culture, morphology, and gene expressions. It is at best unclear to what extend results established in murine models can be transferred to the human setting. Also, many experiments cannot be conducted in human ESC because of ethical problems, thus elevating nonhuman primate ESC as invaluable clinically relevant models (Fischbach and Fischbach, 2004; Horn et al., 2006; Mountford, 2008; Nakatsuji and Suemori, 2002; Nikol'skii et al., 2007; Suemori, 2006). Especially the common marmoset *Callithrix jacchus* serves as a very useful nonhuman primate model because of its small size, the unproblematic breeding, and long life span.

For clinical applications and to prevent uncontrolled differentiation ESC need to be cultured under defined standardized growth conditions abolishing all undefined compositions of media and feeder cells.

The approaches to cultivate ESC under feeder-free conditions are indeed encouraging but are currently not fully developed to be transferred to all different ESC lines. The specific method of cultivation also depends on the species the ESC are derived from. For human and rhesus ESC it has

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been described that feeder-free cultivation is possible with special media and cultivation terms (Hong-mei and Gui-an, 2006; Zhang et al., 2006). Here, both the additives and the dish-surfaces used play an important role. There are more published approaches where human ESC are cultivated under completely feeder-free conditions (Amit and Itskovitz-Eldor, 2006a; 2006b; Beattie et al., 2005; Bigdeli et al., 2008; Xu, 2001). In these cases additives such as leukemia inhibitory factor (LIF) were substituted to keep the ESC in an undifferentiated and pluripotent status (Humphrey, 2004; Rose-John, 2002). In addition, the O_2 tension is thought to play an important role to keep the cells in an undifferentiated status, which may be due to the fact that early stage embryos develop in low O_2 concentration (hypoxia). However, human ESC are typically cultured in 21% O_2 (normoxia) conditions under which they tend to differentiate spontaneously (Ezashi et al., 2005; Kurosawa et al., 2006).

At present, the majority of laboratories typically grow ESC on mouse embryonic fibroblasts (MEF), whose proliferation is arrested either by treatment with Mitomycin C or by γ-irradiation. Mitomycin C is an antibiotic covalently intercalating the cells' DNA, preventing dissociation essential for replication and transcription, finally causing apoptosis. The damage for the DNA by Ionizing radiation is not fully understood, although it is commonly accepted that the inhibition of transcription and apoptosis is mediated here by upregulation of tumor suppressor protein p53. It appears that ESC receive signals from the feeder layer via cytokines and extracellular matrix–cell surface molecule interaction, which may result in intracellular signal transduction. There is no unique reliable protocol established for feeder free cultivation of most human and nonhuman primate ESC, and unfortunately, the interaction of MEF and ESC is not fully understood. Identifying the best MEF inactivation method in terms of ESC growths and understanding the interaction between MEF from different mouse strains and ESC in more detail could be an important step to develop such a feeder-free protocol. In this study, MEF from two different mouse strains (CF1 and NMRI) were inactivated by Mitomycin C or by γ-irradiation and used to assess the influence of all four conditions on ESC.

We evaluated the impact of these different treatments on the capacity of the MEF to support undifferentiated growth of primate ESC. Also, the secreted factors by MEF and the metabolic activities were measured to point out the differences between the inactivation methods.

Material and Methods

Cultivation of the embryonic stem cell line cjes001

ESC colonies were cultured in medium consisting of Knockout-DMEM (Dulbecco's Modified Eagle Medium) (Gibco Invitrogen GmbH, Karlsruhe, Germany) with 20% Knockout-Serum-Replacement (Gibco Invitrogen GmbH), 1% Pen-Strep (c·c·pro, Oberdorla), 1% MEM nonessential amino acids (Gibco, Invitrogen GmbH), 1 mM L-Glutamine (c·c·pro), 0,2 μM β-Mercaptoethanol (Gibco Invitrogen GmbH), and 10 ng/mL bFGF (basic fibroblast growth factor) (peprotech, Hamburg, Germany). Cultivation was performed on MEF, which were inactivated either by Mitomycin C (10 μg/mL) (Sigma, Steinheim, Germany) treatment or γ-irradiation. MEF were seeded on six-well plates (greiner bio-one, Cellstar, Frickenhausen, Germany) coated with 0,1% gelatine (Stem Cell Technologies Inc, Palo Alto, CA) for 30 min at 37°C. For passaging ESC were treated with trypsin solution ([0.25% trypsin, 1 mM $CaCl_2$, 20% KSR in phosphate-buffered saline (PBS)]) for 4 min at 37°C. After incubation, colonies detach from the feeder layer. To separate ESC, solution were pipetted up and down to dissolve colonies into small cell clumps of 10–50 ESC. ESC were then replated on new inactivated feeder cells in ESC medium described above. ESC were split once a week 1:2 to 1:4, depending on density, and plated on MEF that had been seeded out 24 h earlier. The cjes001 line was maintained up to the present under these culture conditions for more than 2 years and for more than 90 passages.

Characterization of cjes001

The characterization of cjes001 was performed by immunofluorescent staining with stem cell markers Oct4 (Octamer-binding protein 4), SSEA-3 (Stage-Specific Embryonic Antigen-3), SSEA-4 (Stage-Specific Embryonic Antigen-4), the keratane sulfate antigens Tra-1-60 and Tra-1-81 and Sox-2. Detection of alkaline phosphatase (AP) was performed by immunohistochemical staining (Chemicon International, Temecula, CA).

Immunofluorescent staining was performed after fixation with 4% PFA (Paraformaldehyde) for 2–3 min at room temperature. Cells were washed twice with 2 mL PBS and incubated with primary antibody for 30 min at 4°C. Primary antibodies were diluted 1:50 in PBS. After another washing step with PBS cells were incubated with the secondary antibodies Alexa488 (Invitrogen GmbH, Germany) which were diluted 1:50 in PBS. Analysis was performed using the fluorescent microscope BZ-8000 from Keyence (Osaka, Japan).

Preparation of MEF

MEF were prepared by extraction from 13.5-day-old embryos of CF1 and NMRI mice, respectively. Limbs, head, tail, and the fetal liver were removed and the tissue was minced by grinding between two frosted glass slides with 10 mL PBS. After centrifugation at 4°C for 5 min at 200×g, the pellet was resuspended in 20 mL Trypsin-EDTA (0.25%) (c·c·pro) and incubated for 15 min at 37°C. FCS (5 mL) was added, the mixture was given through a 70-μm cell-strainer (BD Biosciences, Heidelberg) and flushed with MEF-media (DMEM, 10% FCS, 1% sodium pyruvate, 1% MEM-nonessential-amino-acids, 1% Antibiotic/Antimycotic). After final centrifugation (400×g, 5 min) the cells were resuspended from the pellet and seeded in a density of 20×10^6 cells on a 500-cm^2 cell culture plate (Corning, New York). The cells could be expanded for two to three passages and were then inactivated as described below or frozen and stored in liquid nitrogen.

Inactivation of MEF

For inactivation cells were seeded on 10-cm dishes (TPP, Trasadingen, Switzerland). The inactivation of MEF by Mitomycin C (10 μg/mL) was performed for 4 h at 37°C, 5% CO_2. To ensure sufficient removal of Mitomycin C cells were trypsinized and washed at least three times before reseeding on gelatine-coated cell culture dishes at a density of 0.5×10^6/six-well.

Alternatively, cells were inactivated by (γ-irradiation with 30 Gy at 2 Gy/min using a Cs-137 source (IBL437C, CIS GmbH,

Dreieich, Germany) and subsequently plated on gelatin-coated cell culture dishes at a density of 0.5×10^6/six-well.

MTT Assay

We utilized a colorimetric microtiter (MTT) assay to quantify the metabolic activity of cells. In general, the cells were incubated with the eponymous substance 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (ICN Biomedicals Inc., Aurora, OH) and the metabolic activity can be quantified by the reduction of MTT into blue-violet hydrophob formazan crystals. In our experiments the feeder cells were seeded (0.17×10^5 cells/well) in a 96-well plate and cultivated overnight at 37°C, 5% CO_2. After 12 h the medium was changed to 100 μL medium and 25 μL MTT-reagent (5 mg MTT in 1 mL PBS) followed by an incubation for 2 h at 37°C. Eventually, 50 μL lysis-buffer (20% SDS in 1:1 DMF (Dimethylformamide): H_2O) was added and incubated for further 12 h. 100 μL medium + 25 μL MTT-reagent + 50 μL lysis-buffer were used as control and reference in an ELISA plate reader at 562 nm (anthos bt3, Anthos Labtec Instruments, Wals, Austria). Further analysis was performed with WinRead software, version 2.36. The test was repeated for three times. Each part (negative control, Mitomycin C treated cells, irradiated cells, frozen/thawed cells) was performed eight times.

Cytokine quantitation using a multiplexed immunoassay

Different cytokines and other soluble factors were quantified using a bead-based multiplex quantitative analysis (Rules-Based Medicine, Austin, TX). Sixty-nine factors were measured to compare different concentrations of these factors in media of Mitomycin C-treated and γ-irradiated MEF, and also in MEF of different mouse strains. To compare the different mouse strains and inactivation types a time course of secreted factors was analyzed by monitoring the supernatant at 0, 6, 12, and 24 h.

Supernatant was taken of Feeder Cells seeded out in six-well-plates (Greiner Bio-One) in a density of 0.5×10^6 cells per six-well after inactivation. In a first approach NMRI and CF1 as different mouse strains as well as γ-irradiation and Mitomycin C-treatment as inactivation methods were compared. Samples were taken 24 h after seeding out the cells. As blank value, MEF-media was taken. Each approach was made independently three times with three replicas each.

In a second experiment cells were prepared for monitoring secreted factors over time. On the one hand, it should be clarified how the concentration of factors is changing over time. On the other hand, the inactivation method should be compared. To collect medium on each time point six six-wells were prepared and an additional one-well with feeder cells for blank value sample was prepared. On each time point medium was taken of one well. The samples were not collected from the same well to avoid the problem that media volume is decrasing in this well, and thus the concentration of the soluble factors will increase.

Counting colonies and Morphology of cjes001

ESC were cultured for this experiment in six-well-plates like described above (Greiner Bio-One, Cellstar, Frickenhausen, Germany). For comparison, ESC were seeded out on irradiated and chemical-treated MEF. Both approaches were dealed the same way. In the beginning we started with a cell number of 500 cells. Cells were passaged weekly 1:4. Passaging was performed like described above by treating with trypsin. The cell number per well was quantified by counting under white field microscopy (Olympus, IMT-2, Japan). Proliferation of ESC was observed for 5 weeks. The undifferentiated cells grow as compact, multicellular colonies with

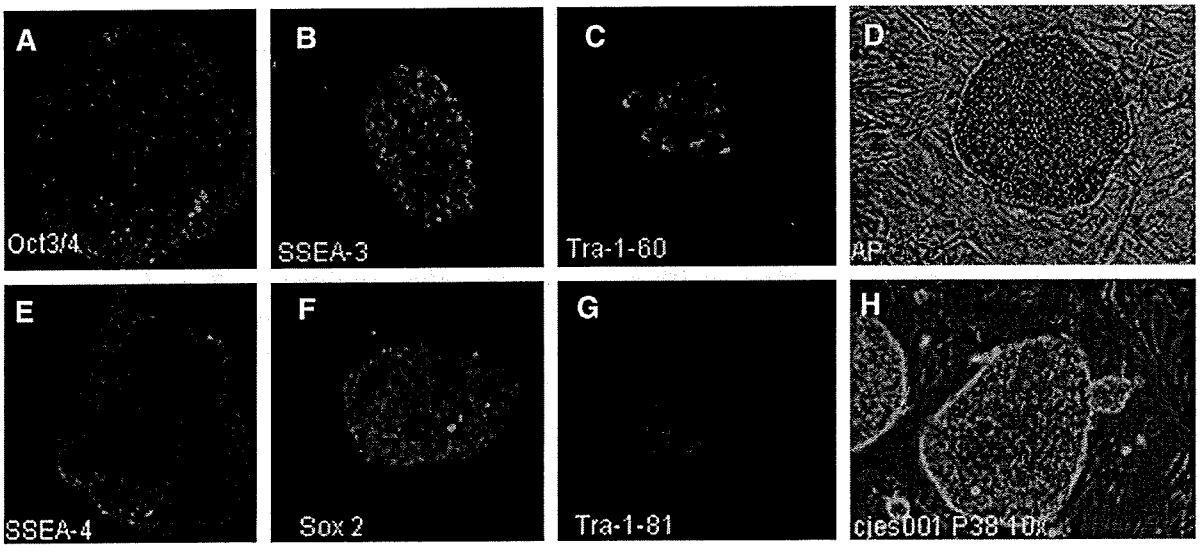

FIG. 1. Immunofluorescent staining with stem cell markers Oct4, SSEA-3, SSEA-4, Tra-1-60, Tra-1-81, and Sox-2 (**A–F**). Immunohistochemical staining of alkaline phosphatase (AP) (**G**). The positively staining underscores their undifferentiated status. Light microscopy picture of a cjes001 colony on inactivated MEF, 4 days after splitting (**H**).

a well-defined border and appear to "gleam" compared to the feeder layer. We anticipate that this is a helpful light microscopy effect originating in the tight packing of the cells in an intact colony. In contrast, differentiated colonies' borders are a blur and lack of the above-mentioned light effect.

Growth rate and differentiation status

Considering the above-mentioned differences in secretion of soluble factors in each feeder cell setup, we assumed a direct effect of feeder cell treatment on ESC growth characteristics, namely, in growth rate and also rate of spontaneous differentiation. To calculate the rate of cell division of ESC on different MEF types we visually counted the number of colonies every sixth day after splitting for 8 weeks. As a second criterion, the "lack shininess/blurred colony borders" versus "shiny and clear borders" of the counted ESC colonies was recorded.

Statistical analysis

The statistical package for social sciences (SPSS version 15 for windows; SPSS Inc., Chicago, IL) software was used for the statistical analysis. Initially, descriptive statistics were employed, and important parameters such as mean, standard deviation, and standard error were determined. To compare the means of different groups, an analysis of variance was performed with subsequent pairwise post hoc tests. In addition, a Kruskal-Wallis H-test was used to define differences between groups. Values of $p < 0.05$ were considered significant for testing the hypothesis.

Results

Characterization of cjes001

The expression of different pluripotency markers in cjes001 was determined by immunofluorescence. Positive immunofluorescent staining of the embryonic stem cell markers was detected for Oct 4, SSEA-3, Tra-1-60, SSEA-4, Sox-2, Tra-1-81 (Fig. 1A–C, E–G). High alkaline phosphatase expression could be detected by immunohistochemistry (Fig. 1D). In comparison, Figure 1H shows an unstained embryonic stem cell colony.

MTT assay

MTT assay was used to assess the metabolic activity of MEF after Mitomycin C treatment or γ-irradiation, respectively. The assay was performed three independent times with eight replicates every time. In all cases data were similar. The assay displayed a significant ($p < 0.02$) difference between the two methods of inactivation with γ-irradiated cells (30 Gy), namely, a higher metabolic activity than Mitomycin C-treated cells. One cycle of freezing and thawing of cells, as typically would be done in most laboratories, further decreased metabolic activity of the feeder cells by roughly 16% (Fig. 2).

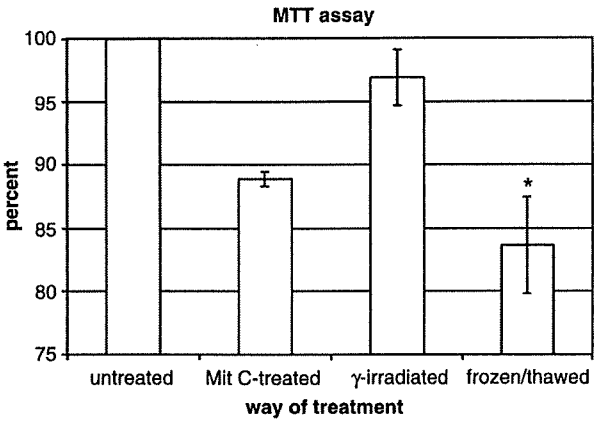

FIG. 2. Metabolic activity (%) of inactivated MEF, analyzed by MTT assay. Student's *t*-test unreveals a significant ($p < 0.02$) difference of metabolic activity between γ-irradiated versus chemically treated cells by Mitomycin C. Furthermore, freezing as well decreases the metabolic activity significantly (indicated by asterisk).

Soluble factors secreted by MEF

A bead-based multiplex quantitative analysis (Rules-Based Medicine, Inc.,) was performed to assess differences in concentrations of cytokines and other soluble factors secreted by MEF. Both the method of inactivation (either by Mitomycin C treatment or γ-irradiation), and MEF of two different mouse strains (NMRI vs. CF1) were compared. In addition, the kinetics of the release of 69 soluble factors by MEF over time by quantitation after 0, 6, 12, and 24 h was measured.

The analysis displayed significant differences in concentrations of both cytokines and soluble factors for Mitomycin C/γ-irradiation as well as between NMRI and CF1.

Mitomycin C treatment versus γ-irradiation

Comparing Mitomycin C treatment and γ-irradiation LIF, basic Fibroblast Growth Factor (FGF-basic) and Vascular Endothelial Cell Growth Factor (VEGF) have a markedly higher concentration in γ-irradiated cells than in Mitomycin C-treated cells (Fig. 3 A–C). In contrast, the concentration of stem cell factor (SCF) is higher in Mitomycin C-treated cells than in γ-irradiated (Fig. 3D). There is no difference in concentration of TNF-alpha between the different inactivated MEF (Fig. 3E).

NMRI versus CF1

The comparison between the different mouse strains NMRI and CF1 showed that in CF1-derived MEF secreted significantly higher amounts FGF-basic than MEF prepared from NMRI mice (Fig. 3C; $p < 0.05$). Also, a trend toward higher secretion of LIF SCF, and VEGF from CF1 MEF compared to NMRI MEF was detected (Fig. 3A, B, and D). TNF-alpha does not show a difference between the secreted amounts from the different mouse strains (Fig. 3E).

Time response

The alteration in concentration over time of 69 soluble factors was measured. Four samples were taken over a time-frame of 24 h. As expected, the levels of most soluble factors secreted by MEF increases over time. But there are also a lot

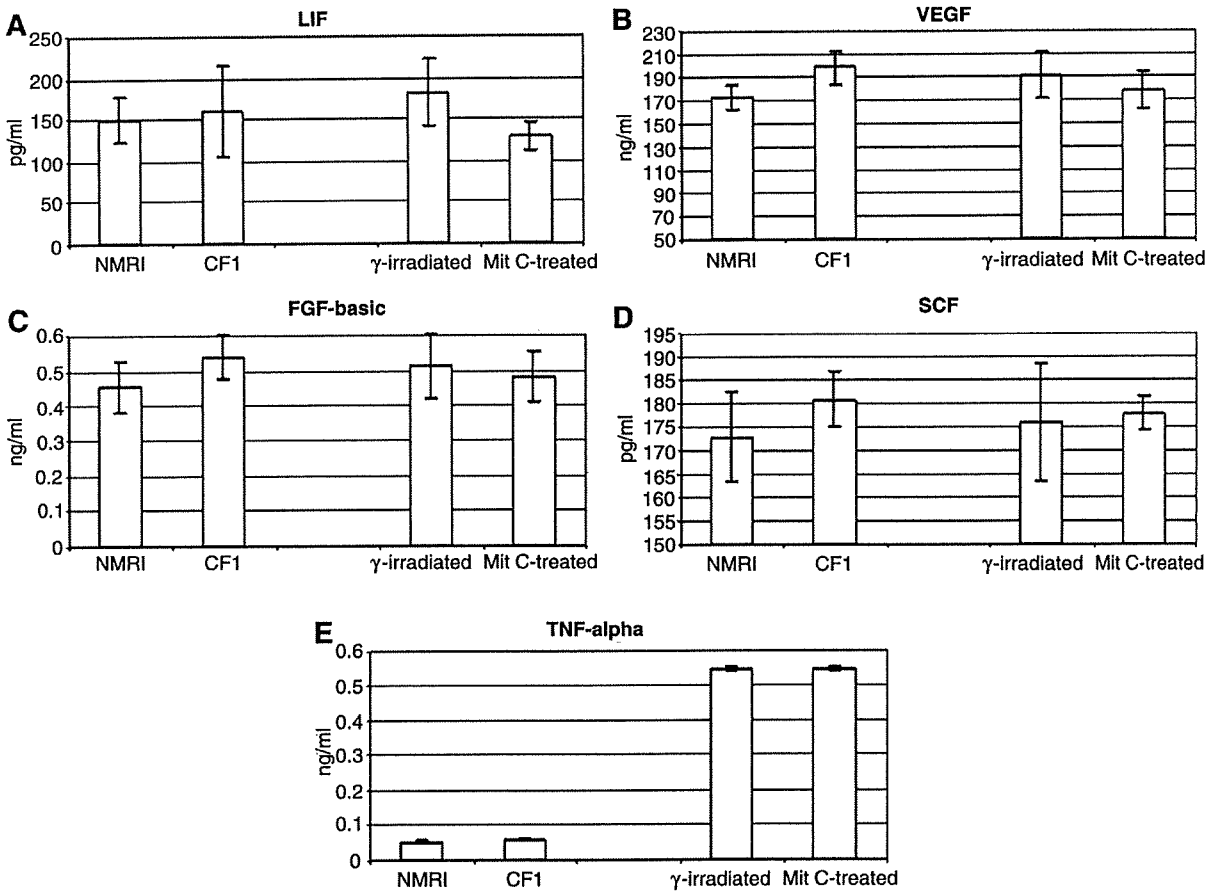

FIG. 3. Comparison of five soluble factors in NMRI mice versus CF1 mice and γ-irradiated versus Mitomycin C-treated, respectively. Significantly higher concentrations of FGF-basic are found in the supernatant of CF1-MEF ($p < 0{,}05$) (C) and a trend to higher concentrations of LIF, VEGF, and SCF in supernatant of CF1-MEF (**A,B,D**) compared to NMRI–MEF. Furthermore, the concentrations of LIF and VEGF are significantly higher in irradiated MEF ($p < 0{,}05$) (**A,B**) and FGF-basic shows a trend to a higher concentration on irradiated MEF than in chemically treated MEF (**C**).

of factors increasing in the beginning of time and remain on a plateau after 12 to 18 h. For example Fibrinogen and Il-2 (Interleukin-2) increase continuously over time (Fig. 4A and B). Il-7 (Interleukin-7), SCF, VEGF, and LIF increase in the first 12 to 18 h and then remain on a plateau (Fig. 4C–F). Some others factors decrease over time. A decrease is monitored in the case of FGF-basic and NGAL (Lipocalin-2) (Fig. 4G, and H). For Fibrinogen, Il-7, SCF, VEGF, and FGF-basic there is no difference in comparison of time response between Mitomycin C treatment and irradiation. In both cases of inactivation the soluble factors show the same response over time (Fig. 4A, C–E, and G). For Il-7, the irradiated feeder cells show a higher concentration than the chemical treated ones (Fig. 4C). For Fibrinogen, SCF, and FGF-basic the concentrations of Mitomycin C-treated MEF is higher (Fig. 4A, D, and G). The response of concentrations is equal in both inactivation methods. For Il-2, LIF, and NGAL trends of concentrations for the different inactivation methods is not equal. After 12 h the different inactivated MEF vary in their behavior (Fig. 4B, F, and H).

Effect on ESC

Although the differences in secretion of soluble factors in each feeder cell treatment group suggest quick effects on ESC growth rate, we just detected no significant difference between Mitomycin and irradiated MEF over 5 weeks (Fig. 5). This experiment was performed two independent times. There are nearly same cell numbers after 5 weeks of ESC on irradiated MEF and chemically treated MEF by Mitomycin C. The number of ESC on irradiated MEF (black line) is a bit higher than the number of ESC on Mitomycin C-treated MEF (gray line) (Fig. 5). In addition, there also appeared to be slightly, but not significantly, more differentiated ESC colonies on Mitomycin C-treated feeder cells than on γ-irradiated feeder cells (Fig. 6, statistics not shown). On Mitomycin C-treated MEF are larger ESC colonies with blurred borders (Fig. 6A, and C). The white arrows in panel A show the beginning of differentiation in the middle of the colony. The dashed arrow shows at the border show the undifferentiated morphology of cells in this colony. In comparison, ESC

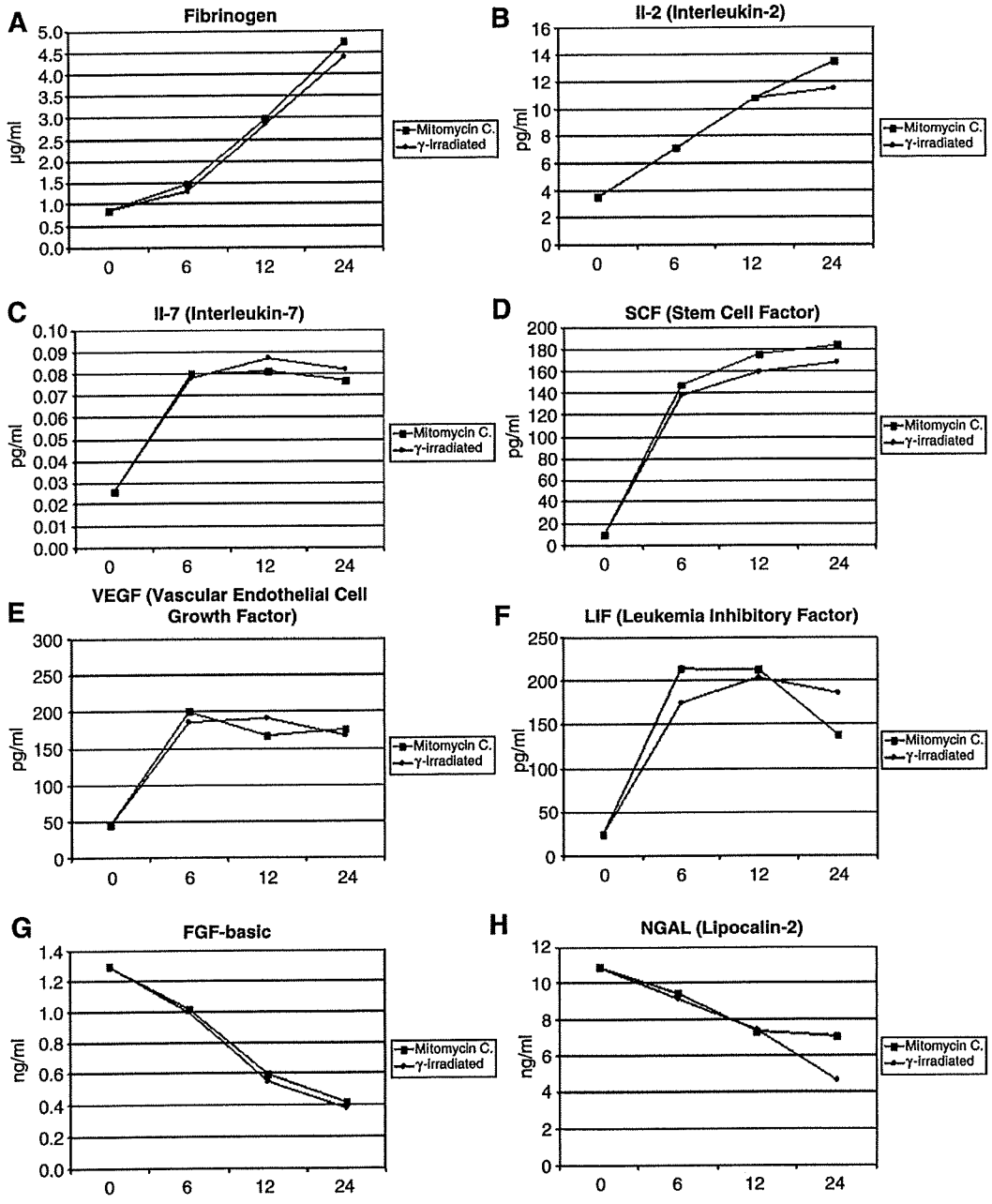

FIG. 4. The level of soluble factors secreted by MEF (NMRI) in comparison of inactivation methods monitored over time. Some factors such as Fibrinogen and IL-2 increase continuously (**A,B**) while others, such as Il-7, SCF, VEGF, and LIF increase very rapidly and then remain on a plateau after 12–18 h (**C–F**). On the other hand, some factors like FGF-basic and NGAL (Lipocalin-2) have a high concentration in the beginning and decline in their concentrations over time (**G,H**). The changes in concentration in comparison of Mitomycin C-tretaed and γ-irradiated MEF are very low. In cases of Fibrinogen, Il-7, SCF, and FGF-basic both feeder cell types behave the same over time (**A,C,D,G**). Both lines look the same even if they run parallel. For IL-2 and NGAL one can see a difference in concentration and response after 12 h (**B,H**).

growing on irradiated MEF look "shiny" with distinct borders (Fig. 6B, and D). The arrows demonstrate the homogenous cells in the colonies grown on γ-irradiated MEF (Fig. 6B). On Mitomycin C-treated MEF (Fig. 6C) the border is in comparison to the border of the colony in picture D of a colony on irradiated MEF not shiny. The dark arrows indicate single cells to show the difference between ESC on different inactivated MEF.

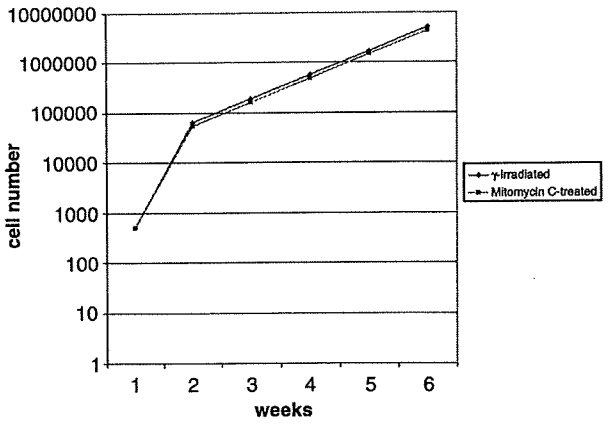

FIG. 5. The graph shows the proliferation rate of ESC in comparison to colonies grown on Mitomycin C-treated MEF (grey line) and on γ-irradiated MEF (black line).

Discussion

ESC are commonly maintained and expanded on inactivated feeder cell layers. The method of inactivation and the utilized mouse strain is thought to have an effect on growth rate and differentiation status of the ESC (Ponchio et al., 2000). Moreover the secreted cytokines, soluble factors, and the metabolic activity of MEF should have an effect on growth and differentiation of ESC (Prowse et al., 2007). In this study differences between the inactivation methods and the two mouse strains CF1 and NMRI were examined.

As expected, untreated MEF in general display a higher metabolic activity in a MTT assay, than either irradiated or chemically treated cells. Apparently, the inactivation by γ-irradiation leaves the cells with a 10% higher basic metabolic activity compared to Mitomycin C; thus, it seems to the more "gentle" method for MEF inactivation. Whether a higher metabolic activity is essential for enhanced growth of the ESC or the opposite, remains unclear. On the one hand, enhanced cytokine levels could stimulate ESC growth; on the

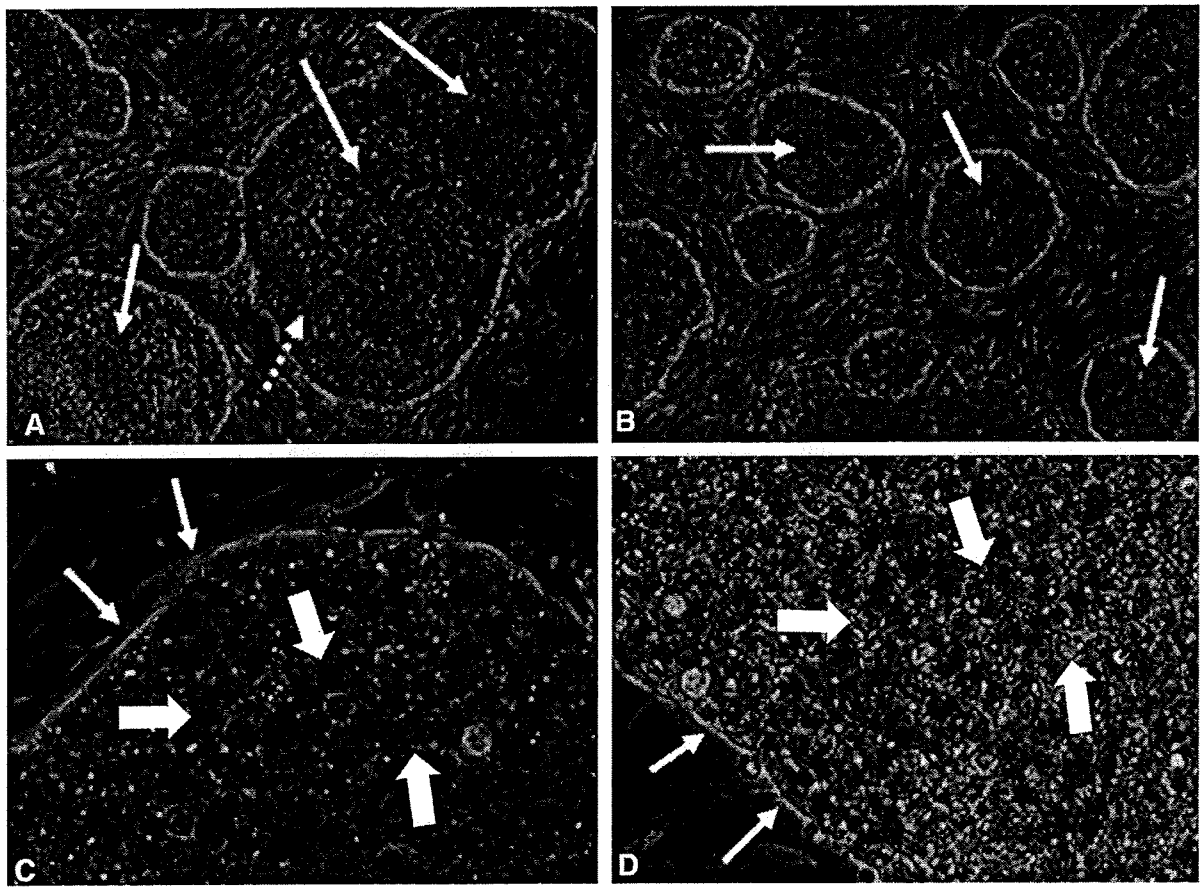

FIG. 6. (A–D) Larger ESC colonies with blur borders on Mitomycin C-treated MEF (A,C). The thin arrows in A show the beginning of differentiation in the middle of the colony. The dashed arrow at the border shows the undifferentiated morphology of cells in this colony. In comparison, B shows "shiny" ESC colonies with distinct borders grown on γ-irradiated MEF (B,D). The arrows demonstrate the homogenous cells in the colonies grown on γ-irradiated MEF. The lower pictures (C,D) show enlargement of colonies. Left C shows an ESC colony on Mitomycin C-treated MEF. The thin arrows point to the border of the colony on Mitomycin C-treated MEF. It is in comparison to the border of the colony in D, which shows an ESC colony on irradiated MEF, not shiny. The thick arrows point on single cells to show the difference between ESC on different inactivated MEF (C,D).

other, enhance spontaneous differentiation. For other cells it has previously been published that the treatment makes no difference for cultivation (Ponchio et al., 2000). However, there is evidence in the literature that for other cell types, such as B lymphocytes, γ-irradiation of the feeder layer is much more effective in terms of cell expansion than Mitomycin C treatment (Roy et al., 2001). As a side note, MEF with a higher metabolic rate persist longer as a coherent feeder layer on the plastic well and hence improve the overall handling and splitting of ESC.

The information obtained from the cytokine array, namely, the low metabolic activity of Mitomycin C-treated MEF, is consistent with the MTT results, because it can be expected that cells with high metabolic activity also secrete higher levels of cytokines and other soluble factors. Especially, the levels of LIF, FGF-basic, and VEGF are higher after irradiation. LIF is described as cytokine for maintaining both proliferation and the developmental potential of nonprimate stem cells (Fry, 1992; Li et al., 2007; Metcalf, 1991), and it is known as an additive for media of human and mouse ESC. Interestingly, LIF is higher in irradiated MEF than in Mitomycin C treated. A high level of LIF secreted by MEF may be beneficial for cultivation of nonprimate ESC in an undifferentiated status; we see no effect of LIF in marmoset ESC culture. FGF is a growth factor and regulatory protein. Both factors also play an important role in embryonic development (Dvorak and Hampl, 2005; Lavine et al., 2005; Marie, 2003; Yu and Ornitz 2008; Xu et al., 2005). It can be speculated, that due to that increased concentrations of FGF-basic and LIF expressed by irradiated MEF, the ESC display a slight tendency to less spontaneous differentiation.

Another key player being described to play an important role in differentiation of ESC is VEGF (Sone et al., 2007). In our case, VEGF has also a much higher metabolic activity in irradiated MEF than in Mitomycin C-treated MEF. It has been described to have an effect on differentiation process from ES cells to vascular cell components. For keeping ESC in undifferentiated status the concentration of secreted VEGF likely should be minimized. SCF is thought to influence the undifferentiated status of the ESC likewise. That could be an advantage of inactivation by treatment with Mitomycin C, because concentration of SCF is slightly higher in Mitomycin C-treated MEF. Level of TNF-alpha are almost the same in Mitomycin C-treated and irradiated MEF.

The comparison of the different mouse strains displayed higher concentrations of almost all factors like, for example LIF, VEGF, FGF-basic, SCF, in CF-1 mice MEF. All key players known to affect ESC differentiation like VEGF and also the factors that are important to maintain the undifferentiated status are higher. For ESC lines in need of high levels of cytokines, we clearly recommend CF-1 feeder cells, for ESC with marginal cytokine requirements NMRI MEF would be appropriate. The concentration of TNF-alpha is almost the same in both mouse strains. For this factor there seems to be no preference to use NMRI or CF1 MEF such as Mitomycin C treatment or irradiation.

In cytokine arrays, the concentration of most important factors promoting differentiation like VEGF (Hehlgans and Pfeffer, 2005; Sone et al., 2007) increased over time and remain on a plateau. SCF, which promotes an undifferentiated status, reaches a plateau after 18 h.

From this background, the empirically discovered strict media change each 24 h in stem cell cultures and becomes comprehendible. On the other hand, the levels of LIF with its ability to prevent differentiation abilities likewise reaches a plateau after 12 h, thus possibly diminishing differentiation induced by other factors. The imbalance of pluripotency-supporting factors versus differentiation-inducing factors accumulating in ESC media older than 24 h may explain the rapid spontaneous differentiation of ESC on older feeder layers. It would, therefore, be interesting to monitor these factors over a longer period of time or artificially increase concentrations of single factors immediately after splitting of ESC.

Interestingly, the morphology of ESC seems to be different depending on the feeder cells. From the results about monitoring cell proliferation cultivation of ESC especially for expansion seems to be more effective on irradiated MEF. On Mitomycin C-treated MEF there are 15% less ESC than on irradiated MEF over a time frame of 5 weeks. From visual assessment there was a higher number of blur bordered, "nonshiny," that is differentiated ESC colonies on the Mitomycin C-treated feeder cells than on the γ-irradiated. Maybe this observation is caused by the lower level of cytokines, or maybe by other soluble factors not being measured here being secreted by the Mitomycin C-treated MEF. From this point of view, cultivation of cjes001 seems to be more effective on irradiated feeder cells to get high numbers of undifferentiated ESC colonies.

In conclusion, a clear-cut general solution for cultivation of stem cells cannot certainly be deduced from this study. However, we find significant differences in radiation and Mitomycin treatment of MEF, namely, significantly higher cytokine and metabolite levels in irradiated cells. For the cell line cjes001 from *Callithrix jacchus* we prefer cultivation on γ-irradited MEF because of higher proliferation rate of ESC, less differentiation, and higher concentrations of soluble factors.

For optimizing the cell culture conditions for each ESC line, we encourage researchers to test if the specific line used in culture is a "high-level cytokine" or "low-level cytokine" line and thereby improving ESC performance from the beginning.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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Preimplantation Development of Somatic Cell Cloned Embryos in the Common Marmoset (*Callithrix jacchus*)

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Abstract

The somatic cell nuclear transfer technique has been applied to various mammals to produce cloned animals; however, a standardized method is not applicable to all species. We aimed here to develop optimum procedures for somatic cell cloning in nonhuman primates, using common marmosets. First, we confirmed that parthenogenetic activation of *in vitro* matured oocytes was successfully induced by electrical stimulation (three cycles of 150 V/mm, 50 μsec×2, 20 min intervals), and this condition was applied to the egg activation procedure in the subsequent experiments. Next, nuclear transfer to recipient enucleated oocytes was performed 1 h before, immediately after, or 1 h after egg activation treatment. The highest developmental rate was observed when nuclear transfer was performed 1 h before activation, but none of the cloned embryos developed beyond the eight-cell stage. To investigate the causes of the low developmental potential of cloned embryos, a study was performed to determine whether the presence of metaphase II (MII) chromosome in recipient ooplasm has an effect on developmental potential. As a result, only tetraploid cloned embryos produced by transferring a donor cell into a recipient bearing the MII chromosome developed into blastocysts (66.7%). In contrast, neither parthenogenetic embryos nor cloned embryos (whether diploid or tetraploid) produced using enucleated oocytes developed past the eight-cell stage. These results suggest that MII chromosome, or cytoplasm proximal to the MII chromosome, plays a major role in the development of cloned embryos in common marmosets.

Introduction

SOMATIC CELL NUCLEAR TRANSFER (SCNT) techniques are powerful tools in the fields of basic biology concerned with elucidating the mechanisms of early stage development and nuclear reprogramming. They have been especially well developed as a productive method for producing hereditary identical animals, so-called cloning techniques (Willadsen, 1986; Wilmut et al., 1997). Application to the production of superior domestic animals, maintenance of mutant animals with low fertility, and preservation of endangered species is also predicted in the fields of stock raising and animal experimentation.

Recently, cloning techniques have also been applied to various life science research fields such as biomedical science, and not simply for the manufacture of copy animals. For example, in animal experimentation studies, cloning techniques have been used in gene manipulation of donor cells (Cibelli et al., 1998; Rideout et al., 2000; Schnieke et al., 1997). Generally, knock-in/-out mice are produced using chimeric mice with embryonic stem (ES) cells through homologous recombination (Bradley et al., 1984; Robertson et al., 1986; Thomas and Capecchi, 1987). However, because mice (Bradley et al., 1984) and rats (Li et al., 2008) are the only animals for which ES cells that contribute to germ line cells have so far been confirmed, gene manipulation of other animal species in a similar manner remains difficult. In the field of medical science, cloning techniques are also expected to be applicable to regenerative medicine and gene therapy through the establishment of embryonic stem cells from cloned embryos (Byrne et al., 2007; Rideout et al., 2002).

As laboratory animals, mice and rats have been highly developed regarding developmental and genetic characteristics biotechnologically, contributing significantly to

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medical research as representatives of human disease models. However, because rodents are phylogenically distant from *Homo sapiens*, knowledge provided by such animal experiments cannot always be extrapolated directly to humans. In contrast, the common marmoset, a small primate belonging to the *suborder Haplorhini*, is a favored laboratory animal compared with other primates due to its high breeding rate. In addition, similarity of cytokines and hormones, and drug metabolism with humans has also been shown (Hibino et al., 1999; Mansfield, 2003). The marmoset is a potential model animal for such gene therapies, and if it were to be introduced as a preclinical model, the above-mentioned application of cloning techniques could also be adapted for primates. Recently, marmoset ES cell lines have been established and used in preclinical studies for regenerative medicine (Kurita et al., 2006; Sasaki et al., 2005; Thomson et al., 1996). To elucidate the pathogenic mechanisms of various diseases or the safety and efficacy of ES cell therapies, genetically manipulated human disease animal models using nonhuman primates are required. However, genetically manipulated nonhuman primate models for human disease have not yet been established, except in the recent report of transgene-mediated overexpression of polyglutamine-expanded human huntingtin in the rhesus macaque as a model for human Huntington's disease (Yang et al., 2008). Accordingly, if application of developmental and genetic biotechnological procedures such as genetic manipulation were to be achieved, it is expected that marmosets could increase the utility of primates as a human model animal.

Cloning techniques have been examined in various mammals for the provision of cloned animals, but suitable methods vary depending on the species. For example, the method and timing of activation of the recipient cytoplasm and transplantation of donor nuclei varies with respect to the optimal conditions of reprogramming. In addition, especially in rats, maturation-promoting factor (MPF) activity, which is related to the reprogramming of donor nuclei, is decreased immediately in the recipient cytoplasm after removal of the female genome (Hirabayashi et al., 2003; Ito et al., 2005). So it should be necessary possible to investigate whether the presence of the oocyte genome in recipient ooplasm has an effect on the developmental potential, by creating polyploid SCNT or parthenogenetic embryos using the intact oocytes. To date, whole animal cloning of a monkey species has yet to be reported. In addition, there are few reports concerned on the reproductive engineering of marmosets (Gilchrist et al., 1997; Lopata et al., 1988; Marshall et al., 1998; Nayudu et al., 2003; Wilton et al., 1993). As a preliminary experiment to establish ES cell lines and produce cloned individuals from somatic cell cloned embryos in the present study, we examined the optimum procedure for SCNT and the *in vitro* development of SCNT embryos in common marmosets.

Materials and methods

Animals

We used common marmosets obtained from CLEA Japan (EDM: C. Marmoset (Jic), CLEA Japan, Inc.) and maintained the animals at the Central Institute for Experimental Animals. To obtain oocytes, 60 female marmosets, which were older than 2 years of age, were used for the experimental measures, and some of them were used repeatedly. All experiments were carried out after obtaining permission from the Institutional Animal Care and Use Committee at the Central Institute for Experimental Animals and the Committee of Animal Experimentation at Hiroshima University.

Preparation of matured oocytes

To collect germinal vesicle-stage (GV) oocytes, female marmosets were subjected to ovarian stimulation and oocyte collection procedures, with reference to previous reports (Marshall et al., 1998, 2003; Wilton et al., 1993) and our preliminary experiments. The estrous cycle was assessed by measuring serum concentration of progesterone using an automated immunoassay system (AIA360, Tosoh Corp., Tokyo, Japan). The females at the diestrus stage were treated by intramuscular injection of 50 IU FSH at 10:00 for 11 consecutive days. At 17:30 on the day following the final FSH injection, 75 IU hCG was administered by intramuscular injection. At 9:30 on the day following the hCG administration, animals were anesthetized with an intramuscular injection of 0.025 mg atropine sulfate (atropine sulfate injection 0.5 mg; Tanabe Seiyaku Co., Ltd., Osaka, Japan) and 70 mg/kg of ketamine hydrochloride (veterinary Ketalar 50; Sankyo Lifetech Co., Ltd., Tokyo, Japan) for immobilization. Immobilized animals were inhalation anesthetized using isoflurane (Forane; Abbott Japan, Tokyo, Japan), and the ovaries were exteriorized by midline laparotomy. Then, cumulus cell–oocyte complexes (COCs) were surgically collected from the ovarian follicles using a disposable syringe with a 23-G needle. The COCs were transported from the Central Institute for Experimental Animals to Hiroshima University by cargo service. During the 22- to 24-h period of transportation, they were kept in maturation medium at 38°C using a portable oven (Cell Transporter; Fujihira Industry Co., Ltd., Tokyo, Japan). The maturation medium was Waymouth's MB 752/1 Medium (Gibco, 11220-035, Carlsbad, CA) supplemented with 20% fetal bovine serum (Gibco, 16141-079), 1 μg/mL estradiol, 0.5 mM sodium pyruvate, 10 mM sodium lactate, 4 mM hypotaurine, and 1 mM glutamine. Upon arrival, the COCs were placed in PBI medium containing 300 units/mL hyaluronidase and cumulus cells were removed by pipetting. Oocytes with a polar body were confirmed as matured at metaphase II (MII) and used for subsequent experiments.

TABLE 1. ACTIVATION OF *IN VITRO* MATURED MARMOSET OOCYTES

Activation method[a]	*Number of oocytes (%)*			
	Examined	*Survived*	*Activated*	*Cleaved*
EP	57	57	57 (100)	57 (100)
EP + DMAP	35	33	33 (100)	33 (100)
$SrCl_2$	14	14	0 (0)	0 (0)

[a]EP = two electrical pulses of 150 V/mm, lasting 50 μsec each, were applied **for 3 cycles** to the oocytes every 20 min. DMAP: cultured in medium containg 2 mM DMAP for 6 h, $SrCl_2$ = cultured in medium containing 10 mM strontium chloride for 6 h.

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.

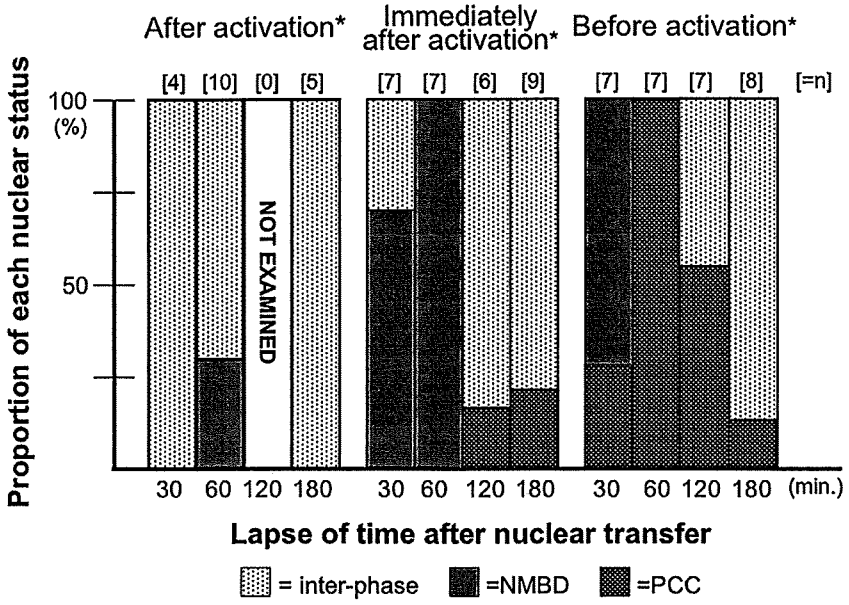

FIG. 1. Status of donor nuclei after transfer to recipient cytoplasm. Light-colored boxes show nuclei at interphase; gray boxes show nuclei that have undergone nuclear membrane breakdown; dark-colored boxes show nuclei that have undergone premature chromosome condensation. *Timing of nuclear transfer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

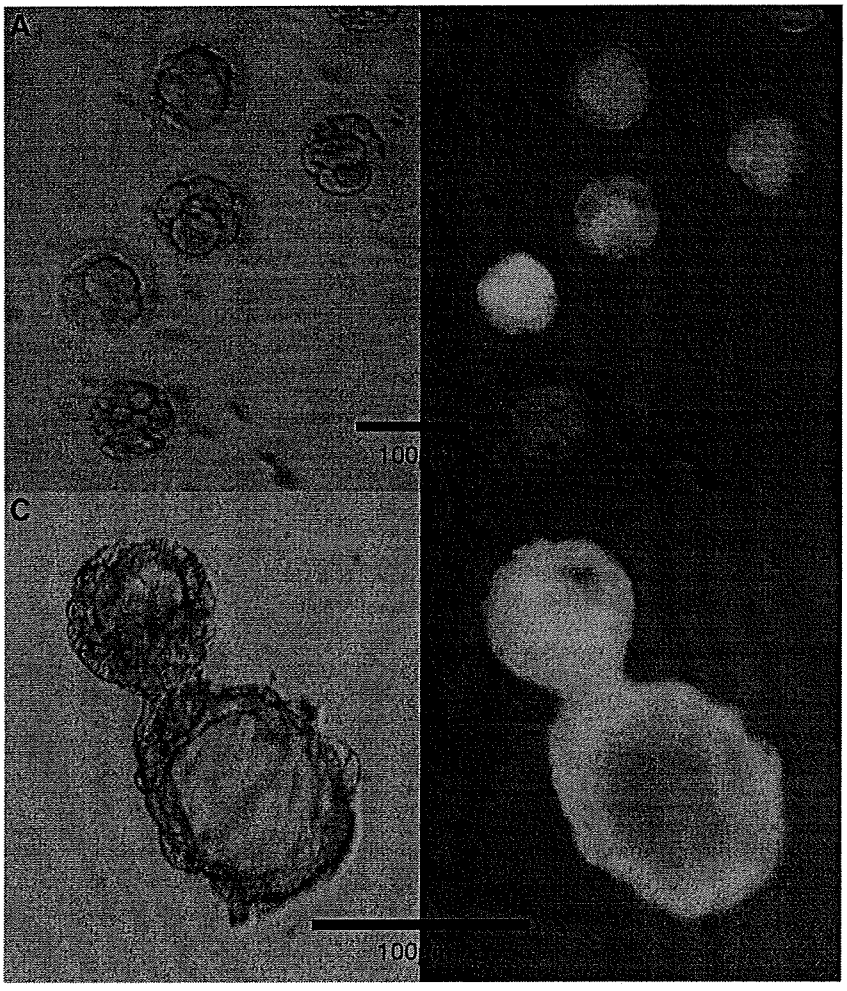

FIG. 2. Tetraploid SCNT [NT-tetra(od)] embryos at the four- to six-cell stage on day 3 (**A, B**) and at the blastocyst stage on day 8 (**C, D**), under bright-field and fluorescence microscopy, respectively. NT-tetra(od) embryos were produced by transferring an EGFP-gene-transfected MBMMNCs cell into 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.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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ARTICLES

Generation of transgenic non-human primates with germline transmission

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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.

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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.

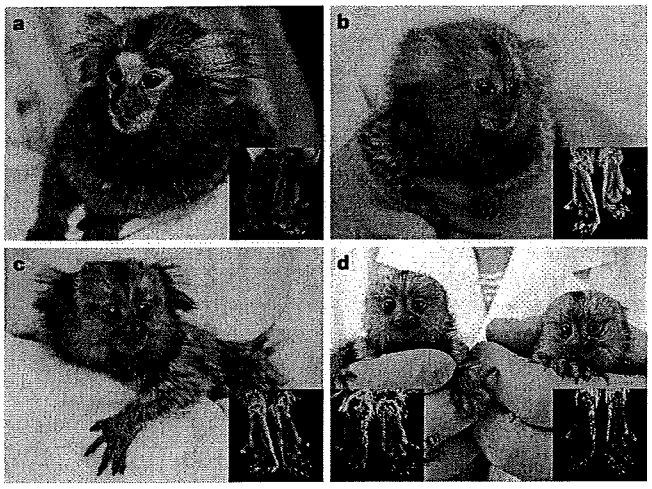

Figure 1 | Self-inactivating lentiviral vector-derived EGFP transgenic marmosets. a–d, The transgenic marmoset infants are shown. Shown are 584 (Hisui) (**a**), 587 (Wakaba) (**b**), 588 (Banko) (**c**), and twin infants 594 (Kei)/666 (Kou) (**d**). 584, 587 and 588 contained CAG-EGFP and 594/666 carried 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).

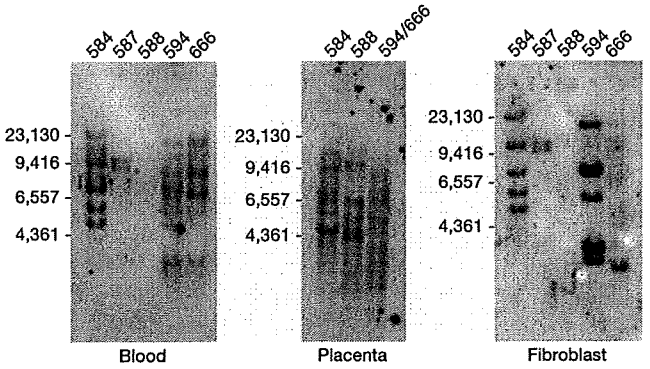

Figure 2 | Transgene insertions in several infant tissues. Southern blot analysis. All infants except 588 showed transgene integration in the skin fibroblast cells and blood, whereas 588 showed transgene integration in the placenta. The lane markers on the left of each gel represent base pairs.

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