hypercholesterolaemia, hypertension, and bone and cartilage disorders (Rudert *et al.*, 2002; Weekers *et al.*, 2002; Bösze *et al.*, 2003; Shiomi *et al.*, 2004). Many of these diseases are lifestyle related, and their prevalence is increasing rapidly in developed countries. Rabbit models are important because the disease aetiologies exhibited by humans are more similar to those exhibited by rabbits than those exhibited by mice. Rabbits are larger than mice, which makes surgery easier and enables large samples to be obtained. Another advantage of the rabbit as a laboratory species is that reproductive techniques such as superovulation, IVF (Chang *et al.*, 1959), sperm injection (Hosoi *et al.*, 1998; Ogonuki *et al.*, 2005), embryo cryopreservation (Kasai *et al.*, 1992), sperm freeze-drying (Liu *et al.*, 2004), and nuclear transfer cloning (Chesné *et al.*, 2002; Inoue *et al.*, 2002; Yang *et al.*, 2007) are well established. Rabbit ES cells would be invaluable for the study of human diseases using gene-targeted technology and for testing stem cell therapies for human applications. Although many attempts have been made to derive ES cell lines from rabbits, none has been successful (Cole *et al.*, 1964, 1966; Graves and Moreadith, 1993; Fang *et al.*, 2006; Wang *et al.*, 2007).

This study was undertaken to determine whether an efficient and reproducible technique for the establishment of rabbit ES cells could be developed by optimizing culture conditions. It was found that the density of feeder cells determines the fate of rabbit ES cells and that stable rabbit ES cell lines can be derived and propagated if the density of feeder cells is optimized.

Materials and methods

Derivation of rabbit ES cell lines

Mature Japanese White rabbits were purchased from Kitayama Labes (Nagano, Japan). Fertilized rabbit embryos were obtained from mature females that had been treated with 75 IU of FSH (Fertinorm P; Serono, Tokyo, Japan) and 100 IU of human chorionic gonadotrophin (HCG; Gonatropin; Teikoku Zoki, Tokyo, Japan) at an interval of 48 h or 72 h. The does were mated with fertile males immediately after HCG treatment. Twenty-two hours after mating, fertilized embryos (zygotes) were flushed from the oviducts using warmed HEPES-buffered RD medium (Carney and Foote, 1991) containing 4 mg/ml of bovine serum albumin (ICN Biomedicals, Irvine, CA, USA). The embryos were cultured in microdrops (16 µl, five embryos per drop) of fresh RD medium covered with mineral oil at 38°C under 5% O_2 and 6% CO_2 in air. In preliminary experiments, a number of embryos failed to hatch, or hatched only after a considerable delay; therefore, in subsequent experiments hatching was assisted by making a hole in the zona, using a Piezo micromanipulator, near the space between two blastomeres when the embryos reached the 2-cell stage. This facilitated hatching of blastocysts (see subsequent discussion). They were then cultured for 48 h until the early to middle blastocyst stages. Blastocysts escaped from the zona pellucida through the hole when medium was introduced into the perivitelline space using a Piezo micromanipulator (Yamagata *et al.*, 2002). With this method, zona pellucida-free blastocysts were easily collected and could be used immediately for ES cell derivation. They were transferred to a four-well dish (Nunc, Roskilde, Denmark) and cultured on mitomycin C-treated ICR mouse embryonic fibroblast (MEF) cells at a concentration of 36 × 10^3 cells/cm^2. The culture medium (rESM) consisted of 78% DMEM/F-12 (Invitrogen, Carlsbad, CA, USA), 20% knockout serum replacement (KSR; Invitrogen), 2 mmol/l GlutaMax (Invitrogen), 1% non-essential amino acids, 0.1 mmol/l β-mercaptoethanol, 10^3 IU/ml ESGRO (murine leukaemia inhibitory factor; Invitrogen), and 8 ng/ml human recombinant basic fibroblast growth factor (Wako, Osaka, Japan). Six to 8 days after the initial plating, the outgrowth of the ICM was removed, dissected mechanically, and transferred to a four-well dish containing fresh feeder cells. Passage of ES-like cells was performed by incubating the cells with 0.05% trypsin for 1 min at room temperature, and mechanically disaggregating the resulting small clumps into single cells. Cells were then counted in a haemocytometer, resuspended, and plated at a density of 3 × 10^3 cells/cm^2 in culture medium. ES cell lines were obtained after five to eight passages. Fresh medium was added daily and cells were passaged every 3–4 days.

Detection of undifferentiated markers

Marker expression was analysed by fixing rabbit ES cells attached to the bottom of the culture plates in 4% paraformaldehyde for 15 min at room temperature, followed by and washing twice (5 min each) in phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST). For permeabilization, cells were treated with 0.1% Triton X-100 in PBS for 10 min and washed twice with PBST. Cells were incubated in blocking solution (3% bovine serum albumin in PBS) for 30 min. The following primary antibodies were used: anti-SSEA1, anti-SSEA3, and anti-SSEA4 from Developmental Studies Hybridoma Bank (Iowa City, Iowa, USA), anti-Oct4 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-NANOG from COSMOBIO (Tokyo, Japan). All antibodies were diluted in blocking solution (SSEA1 and Oct4, 1:500; SSEA3, SSEA4, and NANOG, 1:100) and incubated with samples overnight at 4°C. The next day, cells were washed three times with PBST and incubated with secondary antibodies at 4°C for 3 h. The following secondary antibodies were used: FITC-conjugated goat anti-mouse IgG from Zymed laboratories (San Francisco, CA, USA) and FITC-conjugated goat anti-mouse IgM, FITC-conjugated goat anti-rabbit IgG, and FITC-conjugated rabbit anti-goat IgG from Sigma (Saint Louis, Missouri, USA). Cells were washed three times with PBST, after which fluorescent signals were analysed.

To detect alkaline phosphatase activity, rabbit ES cells were stained using an alkaline phosphatase kit (Sigma) according to the manufacturer's protocol.

Karyotype analysis

Rabbit ES cells in the log growth phase were incubated with colcemid (final concentration = 100 ng/ml) for 4 h at 37°C in 6% CO_2. Cells were trypsinized and pelleted at 120 *g* for 3 min, resuspended in 6 ml of 75 mmol/l KCl, and incubated at 37°C for 15 min. Cells were centrifuged at 120 *g* for 3 min and then fixed using a 50% Carnoy's solution (acetic acid to methanol ratio = 1:3). The centrifugation and fixing steps were repeated three times. During the last repeat, cells were kept in the Carnoy's solution and dropped onto glass slides. Chromosome spreads were stained with Giemsa solution. At least 20 metaphase spreads were counted for each rES-like cell line.

Telomerase activity

Telomerase activity was detected using a TRAPeze telomerase detection kit (Chemicon, Temecula, CA, USA) according to the manufacturer's protocol. The samples were separated using Tris-buffered EDTA-based 8% acrylamide non-denaturing gel electrophoresis. The gels were stained with SYBR Green I (1:10000; TaKaRa, Shiga, Japan).

Lentivirus transduction

For expression of GFP, the self-inactivating lentiviral vector construct pCS-CDF-UbC-GFP-PRE was used, which contains the *EGFP* gene under the control of the human ubiquitin C (UbC) promoter. Lentiviral vectors pseudotyped with the vesicular stomatitis virus G glycoprotein were generated as described previously (Tahara-Hanaoka *et al.*, 2002). Rabbit ES cells were cultured overnight with rESM-containing lentiviruses at a multiplicity of infection (MOI) of 5, 50, or 200 in flat-bottomed 48-well plates, at 37°C in 6% CO_2 in air. The total volume of culture medium per well was less than 110 µl. Fourteen hours after transduction, 100 µl of rESM was added. Two days after transduction, ES cells were harvested by trypsinization and were re-plated into 12-well plates. The medium was changed daily. Five days after transduction, GFP-positive colonies were removed, mechanically dissociated into small clumps, and then cultured in flat-bottomed six-well plates. GFP-positive cells were subsequently propagated under standard ES cell-passaging conditions.

Cell cloning

EGFP-expressing rabbit ES cell colonies were dissociated into single cells using 0.05% trypsin. The dissociated cells were seeded onto an MEF feeder layer ($6 \times 10^3/cm^2$) in flat-bottomed 96-well plates at clonal density (one cell/well). The colony-forming wells were counted 5–7 days later and cloning efficiencies were calculated. These colonies were amplified by passaging to four-well plates. After they had been passaged several times, expanded ES cells were analysed for alkaline phosphatase activity.

Differentiation *in vitro* and *in vivo*

For embryoid body (EB) formation, ES cells were digested with 0.05% trypsin, resuspended in a solution containing 78% DMEM/F-12, 20% fetal bovine serum (FBS), 2 mmol/l GlutaMax, 1% non-essential amino acids, and 0.1 mmol/l β-mercaptoethanol, and cultured in hanging drops. EB were collected after 4–7 days in suspension culture and transferred to plastic dishes coated with gelatin to promote adherence. Culture was continued for an additional 7–14 days in order to promote differentiation. The outgrowths were then fixed and stained using haematoxylin and eosin.

To induce differentiation of ES cells into specific cell lineages, these cells were cultured in several differentiation media. The culture medium for neural differentiation was DMEM/F-12 containing 0.5% FBS, 2 mmol/l GlutaMax, 1% non-essential amino acids, 0.1 mmol/l β-mercaptoethanol, and 10 ng/ml human platelet-derived growth factor-BB (R and D Systems, Minneapolis, USA). The culture medium for epithelial differentiation was DMEM/F-12 containing 10% FBS, 2 mmol/l GlutaMax, 1% non-essential amino acids, 0.1 mmol/l β-mercaptoethanol, and 10^3 IU/ml ESGRO. Fourteen days after induction, differentiated cells were fixed in paraformaldehyde and stained with the following antibodies: mouse anti-β-tubulin III (Sigma) for neurons, mouse anti-glial fibrillary acidic protein (GFAP, Santa Cruz) for astrocytes, and rat anti-collagen IV (Novotec, Saint Martin La Garenne, France) and anti-human mucin (MUC1, Abcom, Cambridge, UK) for epithelial cells. For visualization of cells, F-actin was stained using phalloidine.

For teratoma formation, $2–5 \times 10^6$ ES cells (rES8–2 and rES9–2) were injected under the kidney capsule of 5- to 8-week-old severe combined immunodeficient (SCID) mice. After 10–12 weeks, teratomas were dissected and fixed in paraformaldehyde. Paraffin sections were stained with haematoxylin and eosin (H & E).

RNA extraction and quantitative PCR

Total RNA was isolated from rabbit ES cells cultured in the presence of KSR or FBS, using ISOGEN (Nippon Gene, Toyama, Japan). First-strand cDNA was synthesized using a Takara RNA PCR kit (Takara, Shiga, Japan) with an oligo(dT)-3 site adaptor primer. Synthesized cDNA was subjected to quantitative PCR. The ABI Prism 7900HT was used to determine the mRNA expression levels using the QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany), and a cycling programme of 94°C for 10 min, 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. The forward and reverse primers used were as follows: rabbit *Oct4*: TTCCCAACGAGAGGATTTTG and GAACTTCACCTTCCCCACCAA; rabbit *G3*pdh: GGAGCCAAACGGGTCATCATCTC and GAGGGGCCATCCACAGTCTTCT. Data were normalized relative to *G3*pdh amplification.

Production of chimeric embryos

In order to attempt the production of chimeric rabbits, GFP-expressing lines (rES8–2, rES9–2, and rES9–7) of ES cells were trypsinized to dissociate them as single cells or small clumps of cells. Recipient embryos were recovered from superovulated (see above) females at the eight-cell or blastocyst stage following natural mating. Five to 20 ES cells were injected into the cavities of the blastocysts or into the perivitelline spaces of the eight-cell embryos using a piezo-driven micromanipulator. Blastocysts shortly after injection, or blastocysts derived from injected eight-cell embryos were transferred into the uteri of day 3 pseudopregnant females that had been treated with 100 IU HCG and finger stimulation 3 days before transplantation. The chimerism of the newborn pups was determined by the presence of GFP fluorescence.

Animal experiments

All animals were maintained and used for experiments in accordance with the guidelines for animal experimentation of the RIKEN Bioresource Centre.

Results

The fate of ES cells was determined by feeder cell density. Zona-free blastocysts were readily obtained by applying pressure in the perivitelline space. The blastocysts thus collected were normal in appearance and the trophectoderm was completely free of zona components (**Figure 1A**). Three to 6 days later, outgrowth of ICM cells was observed on the culture plate (**Figure 1B**). In an initial series of experiments, these emerging ES-like cells were passaged onto feeder cells (36×10^3 cell/cm^2), as is the routine practice for mouse ES cells. The ES cell-like colonies all disappeared within four or five passages. However, it was observed that ES cell-like colonies reappeared over the feeder-free areas on the culture plates from which colonies had been removed. Therefore, it was assumed that a high concentration of feeder cells represses ES cell growth.

To assess the correlation between ES-like cell growth and feeder cell concentration, ES cells were seeded onto varying concentrations of feeder cells (0, 3, 6, 12, or 36×10^3/cm^2). As expected, when ES cells were seeded onto a high concentration of feeder cells (36×10^3/cm^2), ES cell colonies disappeared within several passages. On the other hand, ES cells cultured under feeder-free conditions spread sparsely on the bottom as flattened cells and did not form any colonies (**Figure 2A**). The ES cell colonies formed most efficiently when they were cultured at a feeder cell density of 6×10^3/cm^2. This density also improved colony morphology (densely packed cells of homogenous sizes), alkaline phosphatase activity, and cell proliferation (**Figure 2A,B**). Therefore, this feeder cell density was used for the next series of experiments. Eleven ES cell lines were established from 91 blastocysts (**Table 1**). Rabbit ES cells grew as monolayer colonies with a doubling time of 13–15 h (**Figure 2C**). ES cell lines that were passaged 20 times or more were used for detailed characterizations (**Table 2**). A rabbit ES cell line, rES 8–2, was cultured for more than 200 days (up to the 56th passage) using a conventional trypsin digestion protocol and was then cryopreserved (**Table 1**). The majority of cells (>70%) from this line showed a normal karyotype during early passages (around passage 7), and even at passage 55, about 60% of cells maintained the normal karyotype. Five ES cell lines were examined for pluripotency and proliferative ability and it was found that these qualities were maintained after freezing and thawing (data not shown). As expected from their proliferation potentials, the ES cells exhibited high levels of telomerase activity at all passages (**Figure 2D**).

Expression of marker proteins

Expression of ES-cell marker proteins was examined using antibodies against SSEA-1, SSEA-3, SSEA-4, OCT4, and NANOG. Rabbit ES cells were weakly positive for SSEA1 and SSEA4, strongly positive for NANOG and OCT4, and negative for SSEA3 (**Figure 3**). The cells were negative or very weakly positive for Tra-1–60 and Tra-1–81 (data not shown). These marker proteins were associated with the pluripotency of cultured cells: a high fluorescent intensity of anti-OCT4 was observed in closely packed colonies, whereas no fluorescence was detected in sparsely diffused ES cells (**Figure 3**).

Gene transduction and clonal expansion

To examine whether the rabbit ES cells could be genetically modified using exogenous genes, gene transduction experiments were performed using a lentiviral vector. Five

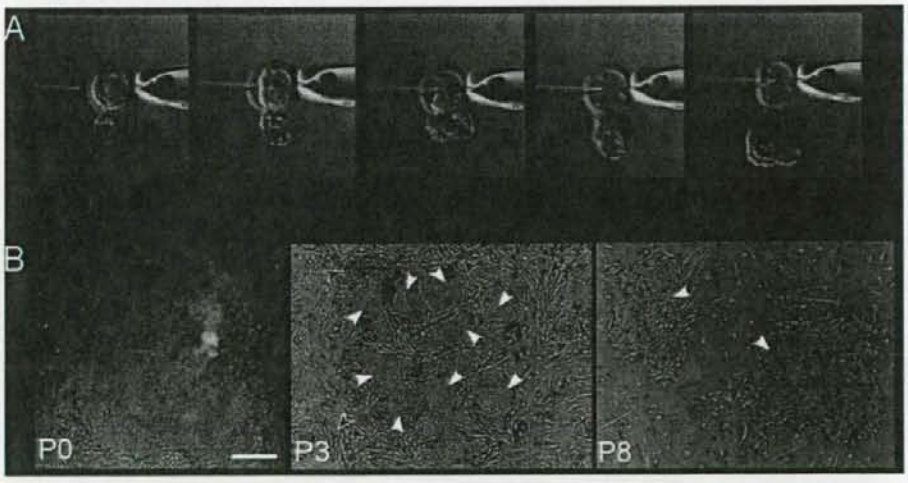

Figure 1. Derivation of primary outgrowth from rabbit blastocyst. (A) Artificial zona-shedding of a blastocyst using a Piezo micromanipulator. The blastocyst was smoothly flushed through a slit by introducing medium into the perivitelline space from the opposite side. (B) The appearance of cell colonies at different stages, from the outgrowth of ICM cells to the emergence of stable ES colonies. P0: primary rabbit ES cell colony grown on feeder cells 7 days after plating. P3: mixed populations of stem-cell-like (black arrowheads) and differentiating (white arrowheads) colonies at passage 3. P8: only stem-cell-like colonies remained after the eighth passage (black arrowhead). Scale bar: 50 μm.

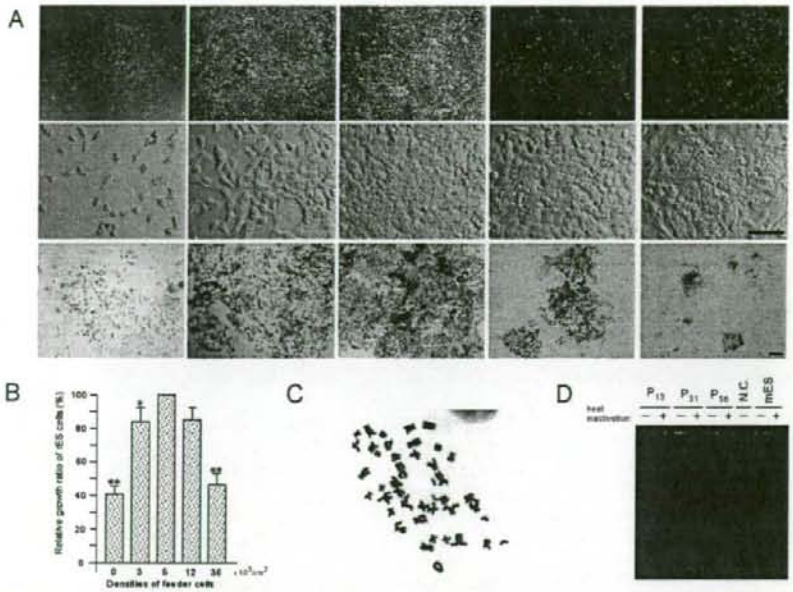

Figure 2. Effects of feeder cell density on rabbit ES cell colonies. (**A**) Feeder cell density is critical for colony formation from ES cells. Rabbit ES cells were seeded onto various concentrations of feeder cells (0, 3, 6, 12, or 36 × 10^3/cm²) and examined under a phase-contrast microscope (lower magnification, upper panel) or Hoffman optics (higher magnification, middle panel), and for alkaline phosphatase activity (lower panel). Large colonies of densely packed cells that had the highest alkaline phosphatase activity were formed when ES cells were seeded onto a concentration of feeder cells of 6 × 10^3/cm². Under other feeder cell conditions, ES cells differentiated but did not form colonies (0 and 3 × 10^3/cm²), or formed small colonies (12 × 10^3/cm² and 36 × 10^3/cm²). Scale bars: 200 μm, 100 μm, and 200 μm respectively. Representative images of rES9–7 at passage 13. (**B**) Relative growth ratios of rabbit ES cells exposed to different densities of feeder cells. The highest proliferation ratio was observed when ES cells were seeded onto 6 × 10^3/cm² feeder cells (= 100%). Asterisks indicate significant differences (*$P < 0.05$ and **$P < 0.01$) compared with the 6 × 10^3/cm² group. Cell numbers were counted at the time of passage (passaged more than five times) and the average growth ratios were calculated for each group. (**C**) The normal number ($2n = 44$) of metaphase chromosomes in a rabbit ES cell from rES8–2 at passage 20. (**D**) Detection of the telomerase activity of rabbit ES cells using the telomeric-repeat amplification (TRAP) protocol. The cells had high telomerase activity at all passages. Heat-inactivated (+) samples were used as negative controls. N.C., lysis buffer only; mES, mouse ES cells used as a positive control.

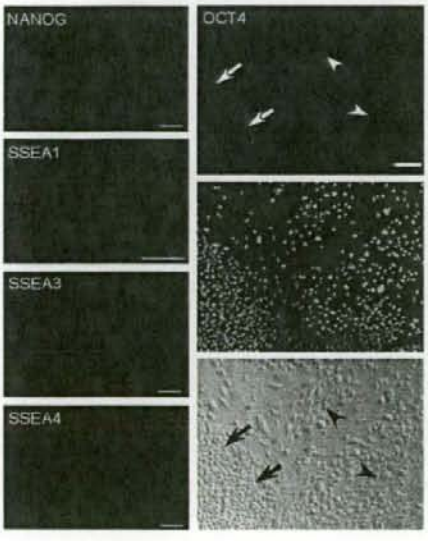

Figure 3. Detection of pluripotency markers in rabbit ES cells. Cells were positive for NANOG, SSEA-1, SSEA-4, and OCT4 but not for SSEA-3. In the panel on the right, OCT4-positive undifferentiated cells are exclusively localized within the compacted colony (arrows), whereas OCT4-negative differentiating cells are sparsely distributed (arrowheads). From the top: anti-OCT4 staining, Hoechst dye staining, and Hoffman optics image. Representative images of rES8–2 cells at passage 8. Scale bar: 100 μm.

Table 1. Results of rabbit embryonic stem (ES) cell line derivation.

Series	*No. blastocysts used*	*No. (%) outgrowth*	*No. (%) ES cell lines*	*Line*	*No. passages*[a]	*Days of culture*[a]
rES8	22	7 (31.8)	4 (18.2)	rES8-1	6	33
				rES8-2	60	227
				rES8-3	6	33
				rES8-4	6	33
rES9	30	8 (27.6)	4 (13.3)	rES9-1	30	113
				rES9-2	32	120
				rES9-6	20	70
				rES9-7	20	70
rES10	39	10 (25.6)	3 (7.7)	rES10-1	12	44
				rES10-2	12	44
				rES10-3	12	44
Total	91	25 (27.4)	11 (12.1)	–	–	–

[a]Before freezing for cryopreservation.

Table 2. Characterization of rabbit embryonic stem cell lines that had been passaged 20 times or more.

Cell line	*AP activity*	*Immuno-staining*	*EB formation*	*In-vitro differentiation*	*Tera-toma*	*Karyo-type*	*RT-PCR*	*Telom-erase (Oct4)*	*Cloning*	*Gene modification*
rES8-2	+	+	+	+	+	+	+	+	+	+
rES9-1	+	+	+	+	+	+	+	nd	+	+
rES9-2	+	nd	+	nd	nd	+	+	nd	nd	+
rES9-6	+	nd	nd	nd	nd	+	nd	nd	nd	+
rES9-7	+	nd	+	nd	nd	+	+	nd	nd	+

AP = alkaline phosphatase; EB = embryoid body; + = detected; nd = not determined.

ES cell lines were infected with a lentiviral vector containing the EGFP gene and were cultured for 2 days. Although many GFP-positive colonies formed in all lines at an MOI of 200, very few cells were transfected at an MOI of 50. No GFP-positive cells were observed at an MOI of 5 (data not shown). The GFP gene was successfully integrated into the genome of all cell lines, as indicated by consistent expression of GFP fluorescence over many passages.

The cloning efficiency of rabbit ES cells was also examined using GFP-positive cells. Single cells from the rES8–2 (passage 12) and rES9–1 (passage 9) lines were seeded onto 96-well plates at clonal density and their growth was recorded daily using a fluorescence microscope (**Figure 4**). More than one-fifth (21.2 ± 2.3%) of the individual cells formed colonies within 5–10 days and could be passaged successfully. These cells exhibited alkaline phosphatase activity and were indistinguishable from their parental cell lines in morphology.

Differentiation *in vitro* and *in vivo*

Rabbit ES cells readily started to differentiate when FBS (instead of KSR) was added to the culture medium, as indicated by the reduction in *Oct4* expression and alkaline phosphatase activity (**Figure 5B,C**). They further differentiated into EB in suspension culture (**Figure 5D**). Plating of EB onto a gelatin-coated culture dish resulted in outgrowths of cells of various types, including multiple cystic structures (**Figure 5E**). These heterologous cell populations included cobblestone-like cells, neuron-like cells, lipid-bearing cells, and eosinophilic granule-producing cells (**Figure 5F–I**). It was also examined whether ES cells could be induced to differentiate into specific cell lineages. In a neural differentiation medium, ES cells differentiated into β-tubulin III-positive neurons and GFAP-positive astrocytes (**Figure 5J**). In the epithelial differentiation medium, they differentiated into MUC1-postive or collagen IV-positive epithelial cells (**Figure 5K–L**).

To examine the differentiation potency of rabbit ES cells *in vivo*, two ES cell lines (rES8–2 and rES9–2) were transplanted into SCID mice. At 10–12 weeks after transplantation, both lines formed teratomas consisting of tissues derived from three germ layers: hair follicles (ectoderm), skeletal muscle fibres (mesoderm), and glands (endoderm) (**Figure 6A–D**).

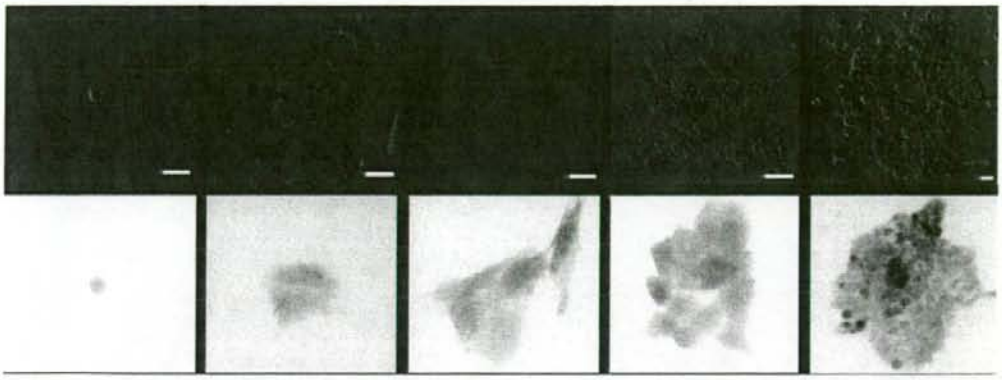

Figure 4. Clonal expansion of rabbit ES cells. Time course of colony formation from a single rabbit ES cell that was transfected with the *EGFP* gene by a lentiviral vector (rES8–2 cells at passage 12). The ES cell was seeded onto feeder cells and cell proliferation was scored at 2, 12, 24, 48, and 120 h. Scale bar: 20 μm.

Development of chimeric embryos

The ability of rabbit ES cells to contribute to fetal development was tested by the production of chimeric embryos. After the transfer of 47 embryos into recipient females, 17 pups were born in four different experiments using three ES cell lines (rES8–2, rES9–2, and rES9–7). However, examination of GFP fluorescence revealed that the ES cells made no contribution to any of their tissues.

Discussion

Human ES cells are potentially useful for regenerative medicine, developmental biology, tissue regeneration, disease pathology, and drug discovery (Thomson *et al.*, 1998). However, the use of human ES cell lines is limited because the destruction of developing human embryos is required for their establishment (Lerou *et al.*, 2008). Recently, induced pluripotent cells (iPS cells) have been generated from human somatic cells, which is expected to overcome the ethical problems associated with cell lines derived from embryos (Takahashi *et al.*, 2007; Park *et al.*, 2008). However, the safety and reliability of their use for tissue regeneration in humans is still to be determined (Stojković and Phinney, 2008). Many attempts have been made to use animal models to overcome limitations associated with human ES cells (Familari and Selwood, 2006). However, stable animal ES cell lines have only been generated from mice and monkeys (Thomson *et al.*, 1995). The use of monkey cell lines may hinder progress because of the limitations of transplantation trials. Mouse ES cells are easily generated from blastocysts and used in regenerative experimental models. However, extrapolation of results obtained using mice to humans is limited because of fundamental differences in colony morphologies, growth requirements, and biochemical characteristics between murine and human ES cells (Koestenbauer *et al.*, 2006).

It has been proposed that rabbit ES cells represent an alternative small animal model for human ES cells, and several reports on their derivation have been published since the first attempt by Cole *et al.* in 1964. However, so far as is known, no stable rabbit ES cell lines have been developed. This is probably because of their limited propagation potential during conventional culture. Although a recent report indicated that rabbit ES cells may be passaged for more than a year (Fang *et al.*, 2006; Wang *et al.*, 2007), it has been difficult to maintain good cell lines for long periods (Jianglin Fan, personal communication). The present report describes an easy and reproducible technique for the establishment of rabbit ES cell lines with indefinite proliferative ability. Derivation of this ES cell line was achieved primarily by optimization of feeder cell conditions and secondarily by an improved zona-shedding method for blastocysts.

Feeder cell layers are extensively used for derivation and maintenance of ES cells or ES-like cells in many species. Feeder cells, mostly of murine or homologous origin, are thought to support ES cells by supplying nutrients and growth factors in a paracrine fashion and by providing scaffolds for ES cell colonization (Choo *et al.*, 2006; Stacey *et al.*, 2006). It was found that high densities of feeder cell layers repressed proliferation of rabbit ES cells, and that rabbit ES cells spread onto areas that were free of feeder cells during culture. This suggests that the feeder cells inhibit proliferation of rabbit ES cells by competing for surface area, or through a contact-mediated mechanism. Nevertheless, feeder cells are apparently indispensable for the maintenance of the pluripotency of rabbit ES cells, as they transformed into fibroblast-like cells in the absence of feeder cells. Thus, feeder cell density was found to be critical for derivation and culture of rabbit ES cells. The optimal window was narrower than expected; density was optimal at 1/6 of confluency and inferior at 1/12 and 1/3 of confluency. Identification of the feeder cell factors necessary for ES cell pluripotency is warranted so that a feeder-free culture system can be established for rabbit ES cells.

Another important finding was that damage to blastocysts must be minimized for successful outgrowth culture. Rabbit blastocysts are surrounded by a strong zona pellucida and a very thick outer mucin coat. *In vivo*, hatching blastocysts escape from these two protein layers through a hole made by trophoblastic

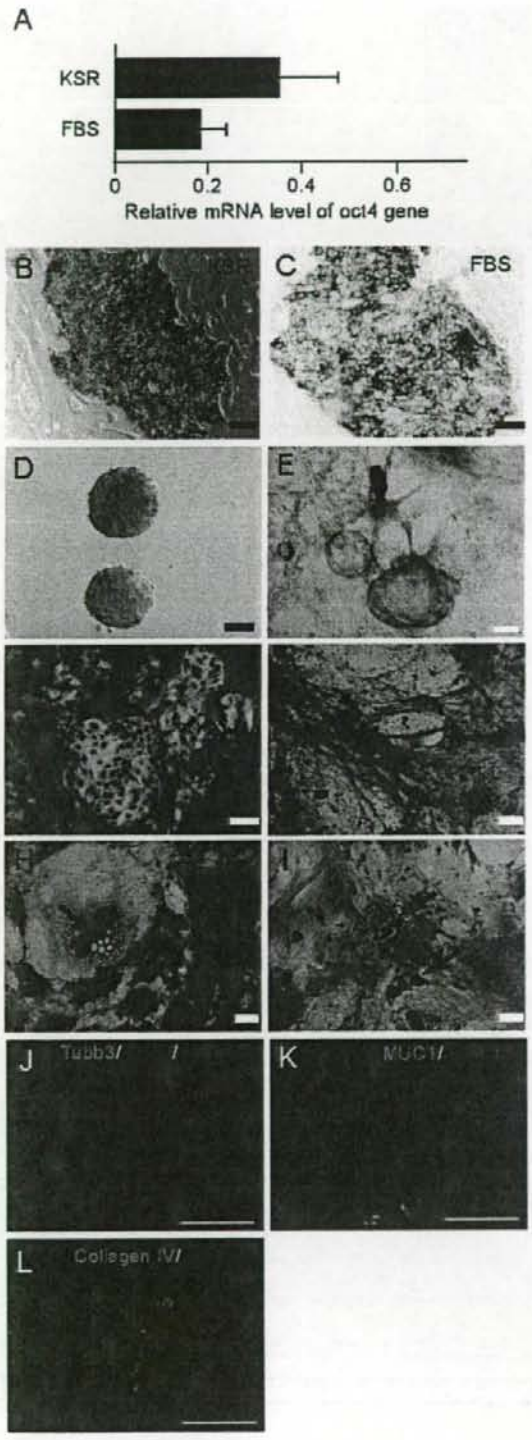

Figure 5. In-vitro differentiation of rabbit ES cells. **(A)** Relative expression levels of rabbit *Oct4* in the presence of KSR or FBS. Reduced expression was observed when rabbit ES cells were cultured in the presence of serum. **(B, C)** Alkaline phosphatase activity of rabbit ES cell colonies cultured in the presence of KSR or FBS. The reduced and uneven distribution of alkaline-phosphatase-positive cells is noted in the FBS group. **(D)** Typical EB in hanging drop culture. **(E)** EB forming cystic structures on a tissue culture dish. **(F–I)** H & E staining of EB-derived outgrowths. The cells are cobblestone-like cells **(F)**, neuron-like cells **(G)**, lipid-bearing cells (arrow in **H**), and eosinophilic granule-containing cells (arrows in **I**). **(J–L)** Direct differentiation *in vitro* of ES cells in differentiation media. Markers specific for neurons (β-tubulin; Tubb3), astrocytes (GFAP), and epithelial cells (Muc1 and collagen IV) are evident. Scale bars: 200 μm in **(E)** and **(J)**; 100 μm in **(B)**, **(C)**, **(D)**, **(F)**, **(K)** and **(L)**; and 20 μm in **(G–I)**.

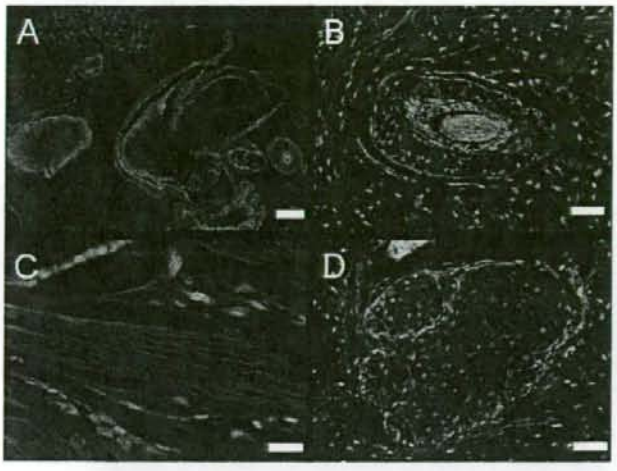

Figure 6. In-vivo differentiation of rabbit ES cells. (**A**) Histology of a teratoma at low magnification. A variety of cell types are evident in the section. (**B–D**) Teratomas at high magnification. A hair follicle (**B**), muscle fibres (**C**), and a gland (**D**) are evident. Scale bars: 200 µm in (**A**); 50 µm in (**B**) and (**D**); and 20 µm in (**C**).

knobs (Steer, 1970). Therefore, exogenous mechanical or enzymatic treatment of these layers may damage blastocysts. In preliminary experiments, the mucin coat and zona pellucida of rabbit blastocysts flushed from the uteri of naturally mated does on day 3.5 post-coitus were removed either chemically using acidic Tyrode's solution (pH 2.4) or enzymatically using pronase (0.5% in PBS). However, because of damage to the blastocysts, it was not possible to establish ES cell lines (unpublished). In this study, embryos were collected before mucin was deposited and a method was used that mimicked natural blastocyst hatching. By applying positive pressure inside the zona, blastocysts easily escaped from the zona through a hole. The blastocysts were healthy in appearance, attached to the bottom of the culture vessel, and formed ICM colonies.

In addition to their usefulness as a regeneration therapy model, ES cells have great potential for the production of genetically modified animals, especially gene-targeted animals. However, the mouse is the only animal species in which germline-contributing chimeras have been produced using ES cells. Attempts have been made to generate chimeric rabbits by ES cell aggregation or injection, but there is no evidence of germline contribution of ES cells (Schoonjans *et al.*, 1996). Recently, murine pluripotent cell lines that have several characteristics in common with human ES cells have been generated from epiblast cells (Brons *et al.*, 2007; Tesar *et al.*, 2007). One of the most intriguing findings was that very few chimeras were obtained from them and germline transmission was not observed. These studies suggest that human ES cells are eventually equivalent to the early post-implantation epiblasts, rather than to their ICM progenitors (Rossant, 2008). If this is also true of rabbit ES cells, it may be difficult to generate germline chimeras in rabbits. Indeed, it has been found that bFGF, the growth factor known to promote human ES cell self-renewal, was also essential for the maintenance of rabbit ES cells (unpublished), whereas the effect of LIF remains unclear. An alternative strategy involves the application of nuclear transfer technology. Fortunately, ES cells are easier to clone using nuclear transfer than other differentiated somatic cells (mouse, bovine) (Saito *et al.*, 2003; Wakayama 2007). As nuclear transfer cloning has been successful in rabbits, knockout rabbits could be produced by combining nuclear transfer and ES cell gene targeting.

The advantages of the ES cell derivation technique are its ease and high reproducibility. Rabbit ES cells with indefinite self-renewing potential can be generated consistently from about 10% of embryos (**Table 1**). This has been confirmed by others (T Kishigami, personal communication). Consequently, rabbit ES cells should be added to the list of stable ES cells in mammalian species. Rabbit ES cells are suitable for use in small animal models to study human cell-transplantation therapy and human diseases and might be combined with gene-targeting techniques in the future.

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Maternal Behavior of Laboratory-born, Individually Reared Long-tailed Macaques (*Macaca fascicularis*)

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To investigate maternal behavior in laboratory-born, individually reared monkeys, we carried out a statistical analysis of 896 long-tailed macaques (*Macaca fascicularis*) based on breeding records of the Tsukuba Primate Research Center (National Institute of Biomedical Innovation, Ibaraki, Japan). Data were obtained from 3266 cases of normal delivery between 1982 and 2004. In each case, maternal behavior was classified as either adequate or inadequate. We examined the effects of parity and the sex of the infant on maternal behavior. We also investigated the similarity of maternal behavior between mothers and their daughters and the effect of quality of maternal care received in infancy on maternal behavior as an adult. The results showed that only the mother's number of deliveries had a significant effect on maternal behavior. The greatest improvement of maternal behavior was observed at second delivery, and the incidence of improvement kept being above 0 thereafter. Our results suggest that, as reported previously, parity is an important factor in the adequacy of maternal behavior in individually reared monkeys.

Abbreviations: IR, improvement ratio; IA, individual adequacy; SPF, specific pathogen-free; TPRC, Tsukuba Primate Research Center

The Tsukuba Primate Research Center (TPRC; National Institute of Biomedical Innovation, Ibaraki, Japan) was established in 1978. The original purpose of monkey breeding in the TPRC was to supply the laboratory-bred monkeys for national vaccine safety testing performed in the National Institute of Infectious Diseases. All monkeys in the TPRC breeding colony were free from measles virus by 1982, and the breeding colony became free *Shigella*, *Salmonella*, *Mycobacterium tuberculosis*, *Mycobacterium bovis*, simian varicella virus, and herpes B virus by 2002.

The TPRC now has expanded its function to provide monkeys to medical researchers in a broad range of fields, such as infection and hyperimmunization restraint. These research areas often need monkeys that are specific-pathogen free (SPF) at its highest level. To effectively maintain and supply SPF monkeys and monkey fetuses that are appropriate for each experiment, the TPRC uses individual rearing. Monkeys are separated from their mothers 5 to 6 mo after birth and receive pair-rearing with an age-matched cagemate until 2 y of age. Thereafter, they are kept in individual cages except during mating periods. Even though TPRC monkeys always have visual, auditory, and olfactory contact with their conspecifics living in the same room, their social experience is quite limited compared with that of wild or group-reared captive monkeys.

Many previous studies indicated that monkeys with limited or no social experience show abnormal social behavior. The most extreme cases are reported in a particular series of studies.[1,7,8,19,20] For the duration of their infancy, monkeys were housed without their mothers and with little or no opportunity to interact with other monkeys. As a result, they had difficulty forming normal social relationships in many interactive situations, such as playing,[1,8,20] mating,[7] and mothering.[1,7,20] The abnormal maternal behavior of these monkeys evoked interest among researchers. Some of the females that had poor social experiences as infants abused or neglected their own infants, in some cases so severely that the infants died.[20]

Many of the cited studies were observations of monkeys reared under more severe circumstances than those in a normal breeding colony. Even in normal breeding colonies, most researchers observe only a portion of the colony animals.[12,14-17,21] No large-scale investigation has examined the effects of individual rearing on the maternal behavior of the monkeys. Here we report the statistical analysis of data accumulated over a period of 20 y on the maternal behavior of more than 3000 cases involving approximately 900 monkeys that were individually reared for most of their lives.

Materials and Methods

Rearing and breeding conditions of the TPRC. The rearing and breeding conditions of the TPRC have changed somewhat over time, depending on prevailing regulations and practices. However, what has never changed is the individual rearing of monkeys and the length of time during which monkeys are reared with their mothers or age-mates.

The rearing and breeding conditions were fixed as follows from 2005. Monkeys in the TPRC breeding colony are reared in individual cages (0.5 m wide × 0.8 m high × 0.9 m deep; stainless steel mesh). The breeding rooms are rectangular, and the individual cages are installed on the long sides of the room. Each room contains at least 90 cages. Most (90% to 95%) cages are occupied continuously. Therefore, monkeys can always make visual, auditory and olfactory contact with their room-mates. Ambient temperature in the rooms is kept about 25 °C, and humidity is set at 50% to 60%. The air is replaced 12 times hourly.

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Lighting is on for 12 h, from 0700 to 1900. Monkeys are provided with apples in the morning, and monkey chow is given to them twice in the afternoon. Water is available ad libitum.

Monkeys in the breeding colony are inspected daily by experienced animal technicians. When any abnormality is found, a veterinarian examines the monkey promptly and applies the appropriate treatment. Moreover, the monkeys are medically examined under anesthesia at least once every 2 y. The medical examination consists of body weight measurement, tuberculin test, blood sample, stool test, examination of the fundus, and a medicated bath.

Monkeys are separated from their mothers 5 to 6 mo after birth and receive peer-rearing with an age-mate until 2 y of age. After that, they are reared individually except during the mating period. The mating period begins 11 d after menstrual bleeding is observed, which is 1 d before the estimated ovulation. The monkey is anesthetized and moved to the cage next to the mating partner at least 1 d before the start of the mating period. The mating period is begun by removing a partition between the cages. After 3 d the partition is replaced.

Pregnancy diagnosis is conducted by the ultrasonography under anesthesia 5 wk after the end of the mating period. If the female is not pregnant, she begins the next mating period around the presumed ovulation day. During pregnancy, females are reared individually. After delivery, the dam and her infant are reared together for about 6 mo, after which they are separated. The offspring begin peer-rearing with an age-mate, and dams are reared individually again.

These rearing and breeding conditions are approved by the Institutional Animal Care and Use Committee of National Institute of Biomedical Innovation, Japan.

Laboratory procedures. Subjects were 896 laboratory-born long-tailed macaques (*Macaca fascicularis*) in the breeding colony of the TPRC. Data were obtained from breeding records collected between March 1982 and March 2004. Individual monkeys gave birth from 1 to 12 times (mean ± SD, 3.7 ± 2.3). The total number of normal births was 3266.

Animal technicians determined the adequacy of maternal behavior on the basis of daily inspection from the infant's birth to separation of mother and infant, about 6 mo.

Breeding records reported whether a dam showed inadequate maternal behavior, regardless of duration or frequency. Moreover, even if a mother that was once judged to be 'inadequate' never again showed inadequate behavior to the same offspring, the record was never changed. In short, only the cases in which inadequate maternal behavior was *never* observed by the animal technicians were recorded as adequate.

Inadequate maternal behavior was defined as rejection of the infant, holding an infant incorrectly, refusal to nurse, or violence against the infant. The dam's avoidance of, or escape from, physical contact with her infant was considered rejection of the infant. Holding an infant incorrectly means that the dam held her infant on her ventral side upside down. Refusal to nurse means that the dam held her infant correctly but prevented the infant's access to her nipples. Violence against the infant was physically hitting or stepping on her offspring.

When a dam showed inadequate maternal behavior, she and her infant were separated, and food and medical treatment were given to the infant as needed. If the infant regained his or her health, he or she was returned to the mother. In most cases, the infant was returned to the dam only once. The animal technician observed the pair for about 10 min, and if the dam took care of her infant normally, the infant was allowed to remain. If inadequate maternal behavior was observed at that time or at the inspection thereafter, the infant was separated from the mother again and was never returned to her.

If the infant was in poor physical condition for more than 2 to 3 d, or if the dam showed inadequate mothering after the infant's return, the infant was reared by artificial nursing or by foster mothers.

Consistency in adequacy of maternal behavior. Multiparous monkeys were classified into 4 groups based on changes in their adequacy of maternal behavior: good, improved, poor, and inconsistent. Monkeys that showed adequate care for all their offspring were classified as having good maternal behavior. The monkeys belonging to the improved group showed inadequate maternal behavior at the first delivery or between the first and a certain delivery number, but they never had inadequate behavior thereafter. Note that our definition of improvement is based on the change in maternal behavior between deliveries, not on a change with the same infant. Monkeys that showed inadequate maternal behavior with all their infants were classified as having poor maternal behavior. The remaining monkeys were classified as having inconsistent maternal behavior.

Primiparous monkeys were classified into 2 groups (good and poor), based on their maternal behavior.

Effects of parity on maternal behavior. To determine whether adequacy of maternal behavior improves with increasing parity, we compared the proportion of 'adequate care' cases at each number of deliveries. As shown in Figure 1, adequacy of maternal behavior appears to improve after giving birth at least twice. However, this analysis may not reflect the natural tendency for improvement of maternal behavior, as monkeys showing inadequate maternal behavior may have been preferentially been excluded from the breeding colony as a means of colony management. Therefore, in a second analysis, we evaluated dams that gave inadequate maternal care to their first offspring based on the adequacy of mothering toward the second offspring. The monkeys that did not improve their maternal behavior at the second delivery were classified again, this time according to their maternal adequacy toward the third offspring. Data from the inconsistent group were excluded from this analysis. We then calculated the improvement ratio (IR) for each delivery number as $N_s / (N_s + N_i)$, where N_s is the number of monkeys that improved their maternal behavior and N_i is the number of monkeys that continued inadequate maternal behavior. If all monkeys that showed inadequate mothering toward the previous offspring improved, the value of IR was 1. Conversely, if none improved, the value of IR was 0.

Relationship of the maternal behavior of the mother to that of the daughter. A total of 340 pairs of mothers and daughters among the monkeys were included in the study. Because the subjects in the present study consisted of the monkeys belonging to various generations, some subjects were represented both as mother and as daughter. When a monkey had several daughters who experienced delivery, the dam was paired with each daughter. In other words, the number of times that a monkey was represented as mother was the same as the number of her daughters who had experience of delivery. Two analyses were conducted using the data from these pairs..

First, to investigate similarity of maternal behavior between mother and daughter, we calculated the ratio of the number of observations of adequate maternal behavior to the total number of deliveries for each monkey. We defined this value as individual adequacy (IA). We then examined the correlation of IA between daughter and mother by Spearman rank correlation coefficient.

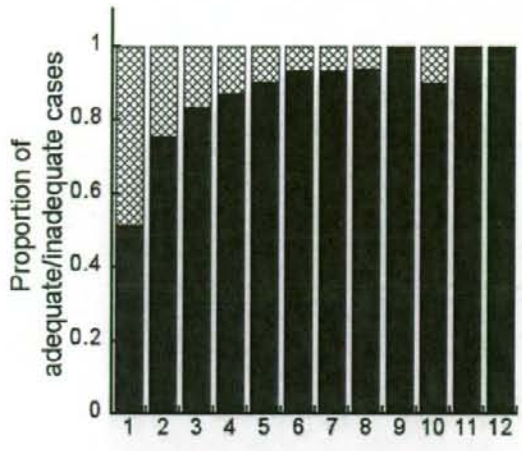

Figure 1. Apparent improvement of maternal behavior with childbirth. We classified each case by the number of deliveries and calculated the ratio of adequate to inadequate maternal behavior. Because there was a possibility that monkeys that showed inadequate behavior were excluded preferentially from the breeding colony, we conducted another analysis (see Figures 2 and 3).

Second, to investigate whether the quality of maternal care received in infancy influenced the maternal behavior of the daughter, we divided the daughters into 2 groups according to the quality of maternal care they received (adequate and inadequate). We then compared the IA value and the consistency in adequacy of maternal behavior in the 2 groups of daughters by *t* test.

Effects of sex of the infant on maternal behavior. We divided all cases by the sex of the infant. We then calculated the incidence of adequate and inadequate maternal behavior in each group and compared them by χ^2 test.

All statistical analyses were performed using StatView statistical software (version 5.0; SAS Institute, Inc., Cary, NC).

Results

Consistency in adequacy of maternal behavior. Of 3266 normal deliveries, 2459 (75.3%) were associated with adequate maternal behavior, and the remaining 807 (24.7%) were categorized as having inadequate maternal care. The severity and continuity of inadequate maternal behavior differed. As soon as inadequate behavior was observed by an animal technician, the infant was separated from the mother for treatment as needed. In 390 of the 807 (48.3%) cases with inadequate maternal behavior, the physical condition of the infant was good, and the dam showed signs of accepting her infant; therefore, the infant was returned to his or her biological mother and was reared by her until their separation. However, if the mother continued displaying inadequate behavior or if the physical condition of the infant was impaired, the infant was reared by artificial nursing (266 of the 807 cases, 33.0%) or by foster mothers (151 cases, 18.7%).

The results of classifying monkeys by the consistency in adequacy of maternal behavior are shown in Table 1. Of primiparous monkeys (n = 187), 42.2% were categorized as having good maternal behavior, and 57.8% were categorized as having poor maternal behavior. Of multiparous monkeys (n = 709), 52.0% were categorized as good; most of the remaining monkeys had maternal behavior that improved with subsequent births (28.8%) or that remained poor regardless of parity (15.0%). Only 4.2% of the multiparous monkeys were classified as having inconsistent maternal behavior regardless of parity.

Table 1. Distribution of monkeys, arranged by the consistency of adequacy of maternal behavior

	No. of subjects				
Parity	Good	Improved	Poor	Inconsistent	Total
1	79	na	108	na	187
2	77	30	44	2	153
3	56	58	29	6	149
4	67	34	17	5	123
5	56	24	11	3	94
6	43	22	0	7	72
7	32	15	2	5	54
8	20	11	3	1	35
9	12	7	0	0	19
10	5	2	0	1	8
11	0	1	0	0	1
12	1	0	0	0	1
Primiparous	79	na	108	na	187
Multiparous	369	204	106	30	709
Total	448	204	214	30	896

na, not applicable

In the improved group, the number (mean ± SD) of times that dams continued to show inadequate behavior from the first delivery was 1.5 ± 0.9, with 4.5 ± 2.1 deliveries. The total number of normal deliveries which dams in this group experienced was 909. In 614 of these cases, their maternal behaviors were determined as adequate. In the other 295 cases, the maternal behaviors were determined as inadequate. In the inconsistent group, the mean number of deliveries was 5.1 ± 1.9, and the total number of normal deliveries was 152. In 84 of these cases, maternal behavior of the dams was determined as adequate. In the other 68 cases, the maternal behavior was determined as inadequate.

Effects of parity on maternal behavior. As shown in Figures 2 and 3, the incidence of adequate maternal behavior at the first delivery was 51.7%. The highest IR was observed at the second delivery. After that, the value of IR was low but nonvanishing (Figure 3). However, only a few dams required more than deliveries to develop adequate maternal behavior (Figure 2).

Relationship of the maternal behavior of a mother to that of her daughter. The correlation between the IA of mothers and daughters was not statistically significant (Figure 4; Spearman's rank correlation coefficient, n = 340 pairs, $P > 0.1$). Furthermore, the IA value of the daughters that received adequate maternal care in infancy was not significantly different from that of the daughters that received inadequate care in infancy (*t* test, $t(338) = -0.1$, $P > 0.10$; IA of daughters that received adequate care, 0.5 ± 0.4; IA of daughters that received inadequate care, 0.5 ± 0.4). Moreover, consistency in adequacy of maternal behavior did not differ between the 2 groups of daughters (Figure 5).

Effect of sex of the infant on maternal behavior. In total, 1709 infants were male and 1557 were female. For 75.0% (1282) of the male infants and 75.6% (1177) of the female, mothers showed adequate maternal behavior. The quality of maternal care the infants received did not differ according to their sex (χ^2 test, $P > 0.1$).

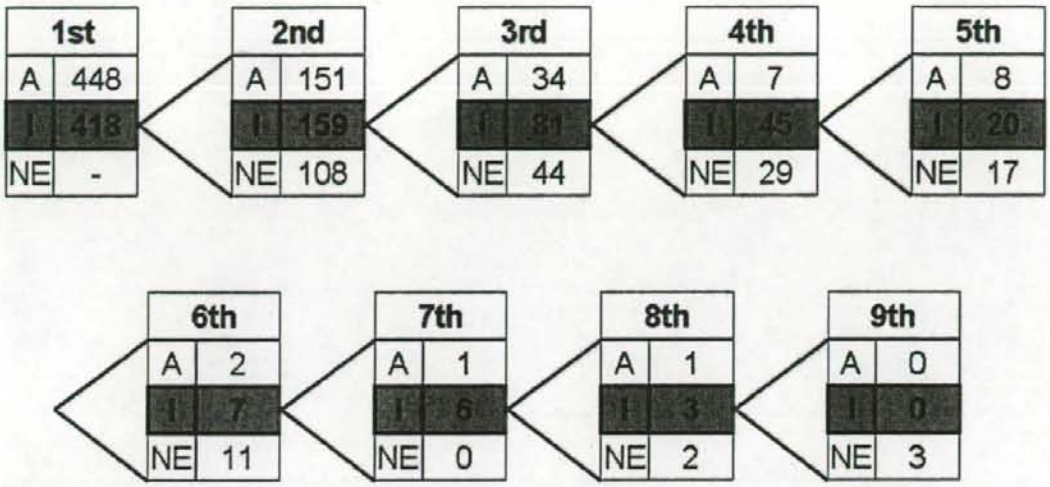

Figure 2. Schema for calculation of improvement ratio (IR) for each delivery number. The ordinal number at the top of each box is the number of deliveries. Inside each box is the number of subjects in each category: A, monkeys that showed *adequate* maternal behavior at delivery; I, monkeys that showed *inadequate* maternal behavior at delivery; NE, monkeys that *did not experience* that number of deliveries. The total number of monkeys at each delivery number is equal to the number of monkeys categorized as I at the previous delivery number. Because the number of monkeys belonging to I became 0 at the ninth delivery, we stopped the analysis at that step.

Discussion

One of the most important questions we asked is whether monkeys in our breeding colony show inadequate maternal behavior at a higher rate than do group-reared or free-ranging monkeys. The incidence of inadequate maternal behavior in the TPRC was 24.7% (807 of 3266 cases). However, our assessment of inadequacy is quite strict: a dam defined as having inadequate maternal performance as soon as she displays a single suboptimal rearing behavior. Moreover, this classification is never revised, even if the dam improves her maternal behavior by the time of protocol-defined separation from her infant. In approximately half (390 of 807) of the cases defined as having inadequate maternal behavior, the dam showed the suboptimal behavior only transiently and then reared her infant successfully without any intervention until the separation. Therefore, we consider the actual percentage of inadequate maternal behavior to be 12.8% (417 of 3266 cases).

In 1 study, the incidence of inadequate maternal behavior in group-reared monkeys was 1.9% to 12.2%.[13] However, the authors stated that the actual incidence might be higher because they counted only severe cases of neglect and abuse. We are aware that not all infant deaths are the result of inadequate maternal care, and for comparison, infant mortality in a free-ranging troop was 6.7% to 16.2%.[9,10,18] Compared with these data, the incidence of inadequate maternal behavior in the TPRC is not unreasonably high.

However, our results do not suggest that the social experience of dams has no effect on their maternal behavior. In our study, the incidence of adequate maternal behavior by individually reared monkeys at the first delivery was 51.7%. In a 1981 study,[21] among 6 long-tailed macaques that received peer-rearing with 6 to 7 age-mates until sexual maturation, all but 1 showed normal maternal care to the first offspring. In a 1989 study of 10 long-tailed macaques reared in different social conditions,[11] 1 group consisted of monkeys that were born and reared in family groups until the first delivery. Another group consisted of monkeys that were born in family groups but were reared with 6 to 7 age-mates. All monkeys in both groups showed adequate maternal behavior. In other studies,[1,20] most of the monkeys that had limited social experience could not rear their first infants, but these authors observed only 4 to 8 monkeys. Of 50 rhesus monkeys that were reared without mothers, 34 (68.0%) abused or neglected their first infants.[19]

The monkeys that showed increased incidence of adequate maternal behavior[11,21] than that in the current study received peer-rearing for a longer time and with more age-mates than did those in the TPRC. In contrast, those that showed poorer rearing skills[1,19,20] were reared in a poorer social environment than those in the TPRC. These results suggest that the duration and complexity of social rearing affect maternal behavior at the first delivery.

As already mentioned, about half the monkeys in our study showed inadequate mothering at their first delivery, but about half of those monkeys improved their maternal behavior by their second delivery. Moreover, few monkeys showed inconsistent maternal behavior overall (Table 1). Overall, about 75% of the monkeys we studied had developed adequate maternal behavior by the time of their second delivery. These results suggest that the maternal behavior can be improved through the delivery experience. Regardless of whether the monkeys are captive or wild, many investigators have suggested that previous deliveries help the dam learn how to treat her infant and contribute to the overall improvement of infant survival rates.[6,9,10,13-15] Even monkeys that received no maternal care in infancy were able to improve their maternal behavior with repetition of delivery.[7,19]

We identified no factors other than parity that affected maternal behavior. Previous studies in group-living monkeys suggested that infant abuse, not neglect, tended to be observed only in particular matrilines.[12,13] We cannot know all the details regarding inadequate maternal behavior at the TPRC in the past, because the breeding records were designed to record

Figure 3. Improvement ratio (IR) for each delivery number. The open circle indicates the incidence of adequate maternal behavior at the first delivery for all subjects (n = 896). The closed circles indicate the IR. We calculated the IR for each delivery number by the formula $N_a / (N_a + N_i)$, where N_a is the number of monkeys that improved their maternal behavior, and N_i is the number of monkeys that continued inadequate maternal behavior. The actual values of N_a and N_i for each delivery number are shown in Figure 2.

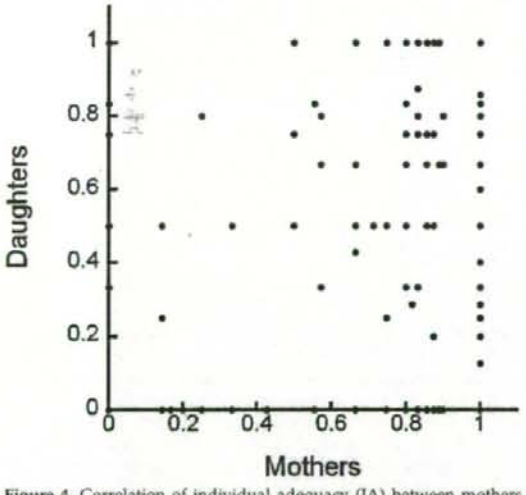

Figure 4. Correlation of individual adequacy (IA) between mothers and daughters (n = 340 pairs). Because some dots overlapped, the number of dots in the figure is less than the number of the pairs. There was no significant relationship between the IA of mothers and that of daughters.

maternal behavior only as adequate or inadequate. Perhaps we found no correlation in the adequacy of maternal behavior between mothers and daughters because of a limited choice of categories. We would need to examine the medical history for each infant until mother–infant separation to determine why maternal behavior was judged inadequate (for example, if the dam physically abused her infant). Moreover, although infant

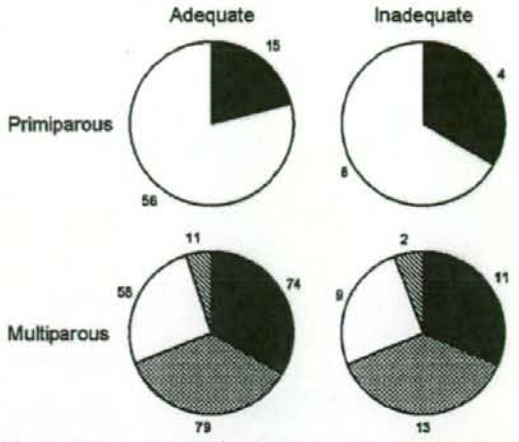

Figure 5. Relationship of maternal behavior of the mothers to that of their daughters. We divided the daughters into 4 groups according to the quality of maternal care they received (adequate or inadequate) and parity (primiparous or multiparous). Numbers show the number of subjects in each group. Black, good; cross-hatched, improved; white, poor; striped, inconsistent.

behavior between the sexes is quite different,[3,16] we did not find any effects of the infant's sex on maternal behavior. Supporting our finding, another study reported that sex of the infant was not a risk factor for abuse or neglect.[15]

Few previous reports about maternal behavior in long-tailed macaques are available; to our knowledge, there are only 2 observational experiments that compared maternal behavior in different rearing conditions.[11,17] Although another article[21] contains statistical data on maternal behavior in captive and peer-reared long-tailed macaques, only 6 subjects were assessed at their first delivery only. Most of the other studies we cite involve other *Macaca* species, and interspecies differences might not be negligible. Considering that long-tailed macaques are used in experiments as widely as other species, more information about their maternal behavior is needed.

Although the incidence of inadequate maternal behavior in the TPRC is not unreasonably high, we are apprehensive about the future. The maternal behavior of peer-reared or isolation-reared monkeys may differ from that of mother-reared monkeys,[4] and differences in maternal behavior may exist between laboratory-born and wild-born monkeys in the TPRC.[17] The differences might increase in each subsequent generation, as could the incidence of inadequate maternal behavior. Indeed, in the current study, the proportion of daughters in the group with good maternal behavior was lower than that overall and the ratio of the daughters in the group with poor maternal behavior was higher than that overall (Table 1 and Figure 5). Directly comparing the adequacy of maternal behavior between generations is difficult because the mean number of deliveries of each generation appears to differ. However, we need to determine whether the incidence of inadequate maternal behavior is likely to increase in future generations. Moreover, the SPF level required differs by experiment, and whether the level of social experience for each subgroup of monkeys can be changed depending on the purpose of the proposed research will be important to determine. Perhaps, for example, extension of the pair-rearing period[2] will decrease incidence of abnormal behavior in our monkeys. Further, modification of the cages may

make it possible to avoid disturbing the physical contact among the monkeys living in the adjoining cages.[5] For TPRC—and other facilities—to continue supplying high-quality monkeys, such possibilities need to be considered.

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AJA

Original Article

Intramanchette transport during primate spermiogenesis: expression of dynein, myosin Va, motor recruiter myosin Va, VIIa-Rab27a/b interacting protein, and Rab27b in the manchette during human and monkey spermiogenesis

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Abstract

Aim: To show whether molecular motor dynein on a microtubule track, molecular motor myosin Va, motor recruiter myosin Va, VIIa-Rab27a/b interacting protein (MyRIP), and vesicle receptor Rab27b on an F-actin track were present during human and monkey spermiogenesis involving intramanchette transport (IMT). **Methods:** Spermiogenic cells were obtained from three men with obstructive azoospermia and normal adult cynomolgus monkey (*Macaca fascieularis*). Immunocytochemical detection and reverse transcription-polymerase chain reaction (RT-PCR) analysis of the proteins were carried out. Samples were analyzed by light microscope. **Results:** Using RT-PCR, we found that dynein, myosin Va, MyRIP and Rab27b were expressed in monkey testis. These proteins were localized to the manchette, as shown by immunofluorescence, particularly during human and monkey spermiogenesis. **Conclusion:** We speculate that during primate spermiogenesis, those proteins that compose microtubule-based and actin-based vesicle transport systems are actually present in the manchette and might possibly be involved in intramanchette transport. *(Asian J Androl 2008 Jul; 10: 561–568)*

Keywords: intramanchette transport; manchette; spermiogenesis

1 Introduction

Teratozoospermia and maturation arrest during spermiogenesis are two forms of male infertility for which the causes remain unknown and there is currently no cure. Understanding the developmental mechanisms by which round spermatids evolve into elongated spermatids during normal spermiogenesis will help to treat such male infertility patients.

Immature round spermatids undergo dynamic morphological changes, acrosome formation, nuclear condensation, and elongation in the sperm head, and sperm tail

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formation during spermiogenesis. The manchette, a bundle of microtubules, is thought to play a role in those changes [1, 2]. The manchette, which is transiently formed in the distolateral region of the cytoplasm, radiates from the perinuclear ring of human Sb2 spermatids and monkey step 8 spermatids. The manchette appears at the time when the spermatid nucleus initiates elongation and disappears when elongation and condensation approach completion. The appearance and disappearance of the manchette are likely related to the dynamic morphological changes in the spermatids during spermiogenesis.

Sperm cells obtained from the *azh* mutant mouse have an abnormal head shape as a result of an abnormal shaping of the nucleus [3]. The microtubules of the manchette in this mouse display ectopic positioning, perhaps related to the abnormal head shape. Spermatids and sperm from the *azh* mutant mouse also have a lasso-like coiled tail and a high frequency of head dislocation and decapitation [4, 5].

The intramanchette transport (IMT) mechanism has been proposed to deliver molecules to the centrosome and the developing sperm tail during spermiogenesis [6–10]. The Golgi generates two types of vesicles, proacrosomal and non-acrosomal. Proacrosomal vesicles are transported to the acroplaxome, where they fuse and organize the acrosome [11]. Non-acrosomal vesicles are transported by the IMT mechanism. IMT might have two transport systems, microtubule-based and actin-based vesicle transport systems [12]. The former is analogous to intraflagellar transport, relying on the microtubule-based motor proteins kinesin/dynein and microtubules for transporting cargo proteins [13]. The latter uses the molecular motor proteins myosin Va and VIIa [14, 15], the motor recruiter myosin Va, VIIa-Rab27a/b interacting protein (MyRIP) [16, 17], and the vesicle receptor Rab27a/b [18, 19]. These proteins have primarily been studied in melanosome transport. A number of models have attempted to explain why two transport systems are necessary in developing spermatids and how they interact with each other. Some researchers have postulated that fast and long-range transport of molecules is mediated by the microtubule-based transport system, whereas short-range local transport is mediated by the actin-based system [20]. Intramanchette cargos might switch from a microtubule track to an actin track by exchanging a microtubule-based molecular motor, such as kinesin or dynein, for the actin-based molecular motor myosins Va/VIIa [21]. This process might involve the motor recruiter MyRIP/melanophilin to determine a cargo's final destination, and the vesicle receptor (Rab27a/b) might facilitate binding of a motor recruiter to enable microtubule-to-actin track switch of the cargo vesicle on the microtubule track.

The role of the molecular motors kinesin/dynein, as part of a microtubule-based vesicle transport system in IMT in primates, has not been investigated. Actin is present in the acroplaxome and along microtubule bundles of the manchette [21], and detected in immunoblotting of fractionated manchette [4, 22]. With the exception of the molecular motor myosin Va in rats [21], no evidence has shown that the molecular motors myosin Va/VIIa, the motor recruiter MyRIP, and the vesicle receptor Rab27a/b are involved in vesicular transport by way of F-actin (track) along microtubules of the manchette during spermiogenesis. In this report, we show that dynein, myosin Va, MyRIP, and Rab27b are localized to the manchette during primate spermiogenesis, speculating that these factors might possibly be involved in IMT.

2 Materials and methods

2.1 Collection of human spermatogenic cells

Sample collection and procedures were approved by the Ethics Committee of Tohoku University School of Medicine (Miyagi, Japan) and Suzuki Memorial Hospital (Miyagi, Japan). Informed consent was obtained from the subjects. Samples were obtained from three men with obstructive azoospermia in whom testicular sperm aspiration was carried out for diagnostic purposes. Normal spermatogenesis was confirmed in the specimen prior to use for these experiments. Evaluation of spermatogenic ability involved histologic examination of the testicular sperm aspiration sample; a Johnson score [23] of nine or greater was judged as normal spermatogenesis. The mean age of the patients was 36 years. The mean follicle-stimulating hormone (FSH) level in the patients was 2.8 mIU/mL.

2.2 Animals

The right testis and epididymis of a 17-year-old normal adult cynomolgus monkey (*Macaca fascicularis*) was obtained from the Tsukuba Primate Research Center, National Institute of Biomedical Innovation (Tsukuba, Japan). The animal, who weighed 5.8 kg, was fed fruits and a commercial monkey diet (type AS; Oriental Yeast,

Tokyo, Japan). All experiments were carried out according to the guidelines for animal experimentation of the National Institute of Biomedical Innovation.

2.3 Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from the testis of the monkey using an RNeasy mini kit (Qiagen, Tokyo, Japan). Total RNA (1 μg) was used as the template for first-strand cDNA synthesis using Superscript III reverse transcriptase (Gibco BRL, Eggenstein, Germany). One microliter of each cDNA was used as the template for PCR reactions with the following primers: dynein (GenBank accession number XM_001092103) forward (F), CCGTATTTGGGTCTATGA and reverse (R), TGAGCTCTAGGACACAAAGTT; myosin Va (GenBank accession number XM_001084476) F, AGGTGTTGAATCTGTATACTCC, and R, AGAGTCTTTCCTGTCTCGTA; MyRIP (GenBank accession number XM_001115628) F, CTCCAAGGCTCCTCAACAAAC, and R, TTGGGTCAAGGCACTGTCG; and Rab27b (GenBank accession number XM_001083017) F, GGGAACTGGCTGACAAAT, and R, CCACCATTGACAGTATCG (Nihon Gene Research Laboratories, Sendai, Japan). The reaction was cycled for 33 cycles, each of which consisted of denaturation at 95°C for 30 s, annealing at 61°C for MyRIP and 53°C for dynein, Myosin Va, and Rab27b for 30 s, and extension at 72°C for 30 s, followed by a 7-min extension at 72°C.

2.4 Indirect immunofluorescence

We analyzed testes from obstructive azoospermic patients and a wild-type monkey. Isolated seminiferous tubules in modified human tubal fluid (HTF containing HEPES buffer; Irvine Scientific, Santa Ana, CA, USA) with 10% serum substitute supplement (Irvine Scientific, Santa Ana, CA, USA) were minced with two fine forceps. After filtering minced tissues through 70 μm mesh to remove tissue debris, cell suspensions were centrifuged for 5 min at 400 × *g*. The pellets were resuspended in an appropriate amount of modified HTF (approximately 2–3 mL) to achieve the proper cell density. After a second centrifugation, cell suspensions were allowed to stand at 37°C for 30 min to allow the spermatogenic cells to recover. Spermatogenic cells were allowed to adhere to coverslips coated in poly-L-lysine (Sigma, St. Louis, MO, USA) and fixed with 2% formaldehyde in microtubule-stabilizing buffer (50 mmol/L piperazine-1,4-bis(2-ethanesulfonic acid [PIPES], 5 mmol/L O,O'-bis(2-aminoethyl)ethyleneglycol-N,N,N',N'-tetraacetic acid (EGTA), and 5 mmol/L $MgSO_4$) for 1 h [24]. After rinsing the coverslips in phosphate-buffered saline, cells were permeabilized for 1 h with 1% Triton X-100 in phosphate-buffered saline (Sigma, St. Louis, MO, USA). Nonspecific antibody binding was prevented by incubation for 1 h with normal goat serum at 37°C. Cells were incubated with monoclonal antibodies against β-tubulin (T5293, diluted 1:100; Sigma), dynein (heavy chain) (D1667, diluted 1:50; Sigma), or polyclonal antibodies (sc9104, diluted 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) to detect microtubules, myosin Va (M4812, diluted 1 : 100; Sigma), MyRIP (ab10149, diluted 1:50; Abcam, Cambridge, MA, USA), or Rab27b (18973, diluted 1:100; IBL, Takasaki, Japan). Pre-immune mouse immunoglobulin (Ig)G1 antibody (diluted 1:20; Chemicon, Temecula, CA, USA) and rabbit IgG (diluted 1:100; Santa Cruz Biotechnology) were used for control experiments. Primary antibodies were detected with fluorescein-isothiocyanate-conjugated goat anti-mouse (Zymed Laboratories; San Francisco, CA, USA) and tetramethyl rhodamine isothiocyanate-conjugated antirabbit (Sigma) antibodies (both IgG, diluted 1:40). DNA was detected by labeling with Hoechst 33342 dye (Hoechst, Kumamoto, Japan). Coverslips were mounted in a drop of VectaShield mounting medium (Vector Laboratories, Burlingame, CA, USA).

2.5 Characteristics of spermatogenic cells and cell imaging

We compared the morphologic characteristics of fixed human spermiogenic cells with those of previously identified fixed cell types [25, 26] as described by Johnson *et al.* [27, 28]. We referred to descriptions of monkeys by Clermont and Leblond [29, 30] and Clermont [31] for this comparison. Coverslips were examined on a Leica DMRXA/HC epifluorescence microscope (Leica Microsystems, Heidelberg, Germany). Images were pseudocolored using Adobe Photoshop software (Adobe Systems, Mountain View, CA, USA) and printed on a color laser printer (Oki Microline 5300; Oki Data, Tokyo, Japan).

3 Results

3.1 RNA expression in monkey testis

RT-PCR was used to detect transcripts of the molecular motor myosin Va, the motor recruiter MyRIP,

and the vesicle receptor Rab27b in monkey testis. In addition to dynein, whose localization to sperm tails has been characterized, transcripts encoding myosin Va, MyRIP, and Rab27b were present in monkey testis (Figure 1). The RT-PCR results were not quantitative as co-amplification with a housekeeping gene was not included. To our knowledge, this result is the first report examining non-human primate testis, although Rab27b was detected in human testis [32] and myosin Va, MyRIP, and Rab27b had been previously seen in mouse testis [10, 21].

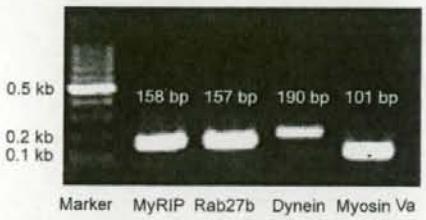

Figure 1. Gene expression of the molecular motor myosin Va, the motor recruiter MyRIP, and the vesicle receptor Rab27b and the molecular motor dynein in monkey testis were determined by RT-PCR. The numbers at the left indicate molecular marker size in kilobases (kb). The base pair (bp) size of each transcript is indicated.

3.2 Immunological localization of dynein on microtubule tracks in human and monkey spermatids

The immunolocalization of dynein was examined by immunofluorescence during human (Figure 2) and monkey (Figure 3) spermiogenesis. In elongating spermatids Sb2 and Sc (Figure 2A–C), and stage 8–10 spermatids (Figure 3A–C), the nuclei became elongated and condensed. The manchette, a bundle of microtubules that extends from the equatorial region of the nucleus toward the developing tail, began to materialize. More

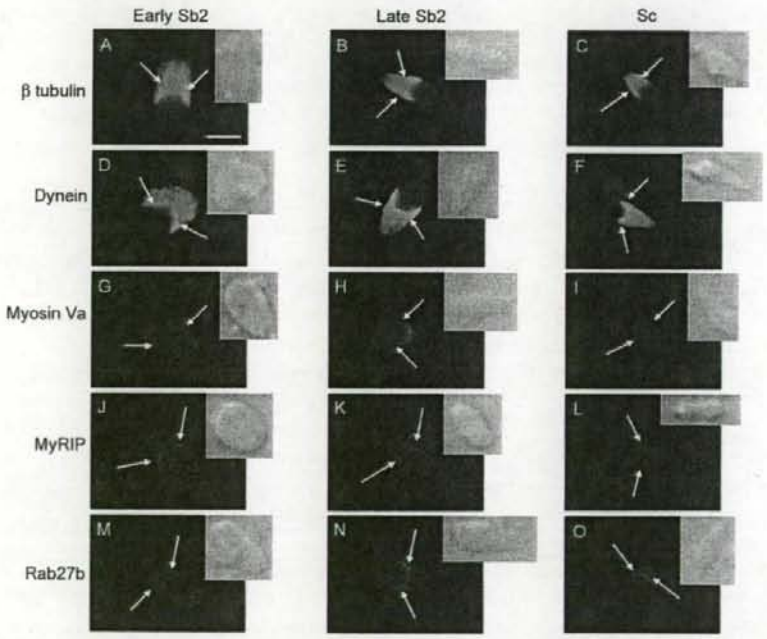

Figure 2. Immunolocalization of dynein, myosin Va, MyRIP, Rab27b and β tubulin in human spermatids with the corresponding phase contrast microsocopic images (insets). Green: β tubulin, dynein; Red: myosin Va, MyRIP, Rab27b; Blue: DNA. In elongating spermatids, Sb2 and Sc spermatids, the nuclei have become elongated; the manchette (A–C), a bundle of microtubules that extends from the equatorial region of the nucleus to the developing tail, is accompanied by strong dynein, myosin Va, MyRIP, and Rab27b staining (D–F, G–I, J–L, M–O, arrow). Scale bar = 10 μm.

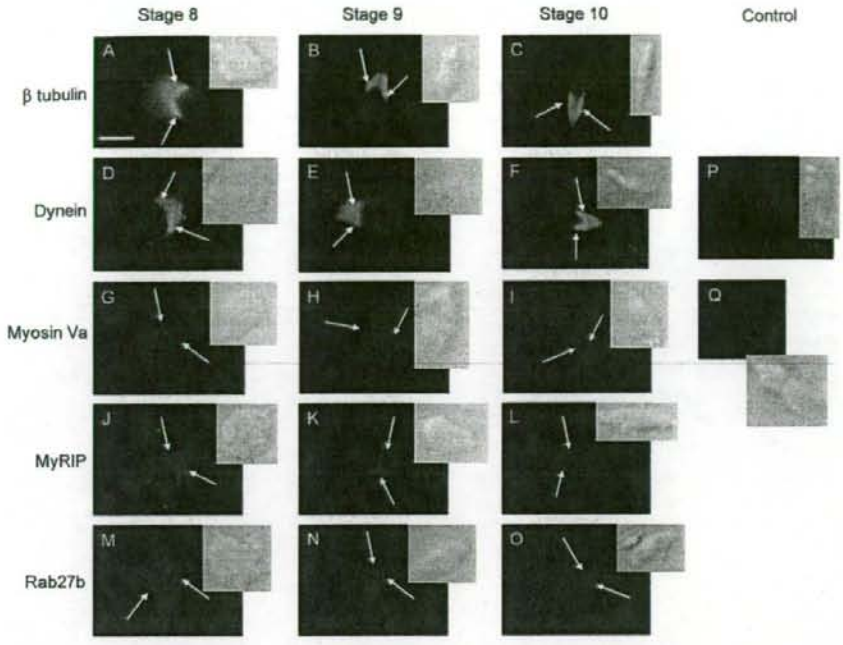

Figure 3. Immunolocalization of dynein, myosin Va, MyRIP, Rab27b and β tubulin in monkey spermatids with the corresponding phase contrast microsocopic images (insets). Green, β tubulin, dynein; Red, myosin Va, MyRIP, Rab27b; blue; DNA. In elongating spermatids; stage 8-10 spermatids, the nuclei have become elongated; the manchette (A–C), a bundle of microtubules that extends from the equatorial region of the nucleus to the developing tail, is accompanied by strong dynein, myosin Va, MyRIP, and Rab27b staining (D–F, G–I, J–L, M–O, arrow). Negative control (P, Q). Bar = 10 μm.

intense dynein immunoreactivity was localized to the manchette than cytoplasm (Figures 2D–F and 3D–F). When mouse IgG1 was applied as monoclonal primary antibody in place of antidynein antibodies, dynein could not be detected in the manchettes of stage 9 spermatids (Figure 3P). These results indicate that dynein colocalizes with microtubules that constitute the manchette during spermiogenesis.

3.3 Immunological localization of myosin Va, MyRIP, and Rab27b on F-actin tracks in human and monkey spermatids

In addition to dynein, the immunolocalization of myosin Va, MyRIP, and Rab27b were examined during human (Figure 2) and monkey (Figure 3) spermiogenesis. In elongating spermatids Sb2 and Sc (Figure 2A–C) and stage 8–10 spermatids (Figure 3A–C), nuclei become elongated and condensed. The manchette, a bundle of microtubules that extends from the equatorial region of the nucleus toward the developing tail, began to develop at this stage. Staining for myosin Va, MyRIP, and Rab27b was more intense in the manchette (Figures 2G–I, J–L, M–O and 3G–I, J–L, M–O) than that in the cytoplasm. When rabbit IgG was applied as primary antibody in place of antimyosin Va, antiMyRIP, and antiRab27b antibodies, no specific staining could be detected in the manchettes of stage 9 spermatids (Figure 3Q). We repeated each experiment at least three times.

4 Discussion

The timing of the appearance and disappearance of the microtubular manchette suggested a function in the dynamic morphological changes in spermatids throughout spermatogenesis. Therefore, the IMT mechanism, which contributes to vesicular transport events, is needed