A novel embryonic stem cell line derived from the common marmoset monkey (*Callithrix jacchus*) exhibiting germ cell-like characteristics

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**Background:** Embryonic stem cells (ESC) hold great promise for the treatment of degenerative diseases. However, before clinical application of ESC in cell replacement therapy can be achieved, the safety and feasibility must be extensively tested in animal models. The common marmoset monkey (*Callithrix jacchus*) is a useful preclinical non-human primate model due to its physiological similarities to human. Yet, few marmoset ESC lines exist and differences in their developmental potential remain unclear.

**Methods:** Blastocysts were collected and immunosurgery was performed. cjes001 cells were tested for euploidy by karyotyping. The presence of markers for pluripotency was confirmed by immunofluorescence staining and RT–PCR. Histology of teratoma, *in vitro* differentiation and embryoid body formation revealed the differentiation potential.

**Results:** cjes001 cells displayed a normal 46,XX karyotype. Alkaline phosphatase activity, expression of telomerase and the transcription factors OCT4, NANOG and SOX2 as well as the presence of stage-specific embryonic antigen (SSEA)-3, SSEA-4, tumor rejection antigens (TRA)-1-60, and TRA-1-81 indicated pluripotency. Teratoma formation assay displayed derivatives of all three embryonic germ layers. Upon non-directed differentiation, the cells expressed the germ cell markers VASA, BOULE, germ cell nuclear factor and synaptosomal complex protein 3 and showed co-localization of VASA protein within individual cells with the germ line stem cell markers CD9, CD49f, SSEA-4 and protein gene product 9.5, respectively.

**Conclusions:** The cjes001 cells represent a new pluripotent ESC line with evidence for enhanced spontaneous differentiation potential into germ cells. This cjes001 line will be very valuable for comparative studies on primate ESC biology.

**Key words:** embryonic stem cell / common marmoset / germ cell / non-human primate / pluripotency

**Introduction**

Cell replacement therapy using pluripotent or multipotent stem cells holds great promise for regenerative treatment of a vast number of degenerative diseases. However, it is still disputed if embryonic stem cells (ESC), somatic stem cells or recently described induced pluripotent stem (iPS) cells are best suited for cell replacement studies in preclinical and possible future clinical applications (Meissner et al., 2007; Okita et al., 2007; Wernig et al., 2007; Yu and Silva, 2008). This open issue needs extensive further investigation with all cell types being considered as sources for cell replacement therapies. Therefore, the safety and potential of all respective cell types have to be tested in preclinically relevant animal models.

Mouse ESC differ from non-human primate and human ESC cells with respect to cell culture requirements, morphology, physiology and gene expression, reflecting significant differences between mouse and primate embryogenesis and physiology (Fougerousse et al., 2000; Ginis et al., 2004; Turnpenny et al., 2006). Therefore, it is of fundamental importance to study primate ESC. But beside ethical concerns as well as legal limitations in several countries using
human ESC, further doubts are related to the clinical safety of the potential therapies using pluripotent stem cells (Stojkovic et al., 2004). It has to be ensured that transplanted cells are not tumorigenic and that cell replacement therapy does not cause other harmful long-term side effects. Furthermore, a real benefit from cell replacement therapy has to be demonstrated for the treated individual in preclinical studies. Since primates and mice differ significantly and several neurological diseases, such as Parkinson’s or Alzheimer’s disease, cannot be properly mimicked, especially with regard to their cognitive deficits, non-human primate models are of great relevance. To provide the best preclinical test systems, non-human primate disease models are needed in combination with allogenic replacement cells. This ensures that all cell–cell and cell–matrix interactions as well as all ligand–receptor interactions probably needed during cell replacement therapy properly function in the respective preclinical non-human primate disease model. Hence, although previous results from mouse ESC have provided invaluable insight in stem cell biology, the potential of clinical stem cell applications in humans must be pioneered in non-human primate species (Wolf et al., 2004) such as macaques and marmoset monkeys. The common marmoset monkey (Callithrix jacchus) is readily available as a non-human primate model that exhibits many physiological similarities to humans (Michel and Mahouy, 1990; Mansfield, 2003; Zühlke and Weinbauer, 2003; Esllamboli, 2005). However, only a few ESC lines of this species exist to date. More than 10 years ago Thomson et al. (1996) created eight ESC lines from the common marmoset, but these lines are no longer available (J. Thomson, personal communication). We recently continued research with marmoset ESC by creating and characterizing the three lines: CMESC20, CMESC40 and CMESC52 (Sasaki et al., 2005). Nevertheless, the more ESC lines from one species that are available the better, and the more profound the knowledge about a certain stem cell type from a certain species will be. For instance, human and non-human primate ESC lines diverge in their karyotype, gene expression and differentiation potential (Heins et al., 2004; Chen et al., 2008). To have available a broad collection of ESC lines, isolated from embryos at different developmental stages and sex, will benefit research on the spectrum of intra- and inter-cell line-specific varieties and epigenetic stability.

In this article, we established and characterized a fourth marmoset ESC line (cjes001) that could be cultivated successfully for over 24 months (passage 84). Besides the potential to develop into somatic cell types, this cell line also revealed strong potential to develop into germ cells upon spontaneous differentiation.

Materials and Methods

Recovery of blastocysts and initial culture

All procedures were carried out according to German and Japanese Animal Experimentation Law and all animal experiments in Japan were approved by the institutional animal care and use committee, and were performed in accordance with institutional guidelines. Animals were housed according to standard German and Japanese Primate Centre practice for the common marmoset. The method of blastocyst recovery has been described in detail (Sasaki et al., 2005). Briefly, marmoset preimplantation embryos were recovered from adult marmosets kept in the marmoset colony at the Central Institute for Experimental Animals (Kawasaki, Japan) 8 days after putative ovulation (10.7 ± 1.3 days after progesterin F2a administration) by uterus-flush. Out of eight animal flushes, 15 embryos were collected and immunosurgically treated to receive 15 inner cell mass (ICMs) for further cultivation. Of these 15 ICMs, 3 could be expanded until passage 10. Two of these initial lines differentiated eventually, so that finally only the cjes001 line was established. The ratio from blastocyst to established non-human-primate embryonic cell line was in this case 15:1. cjes001 cells showed flat, packed and tight colony morphology and a high nucleus to cytoplasm ratio, corresponding to the morphology reported for other primate ESC, including humans, rhesus and cynomolgus monkeys. cjes001 cells were cultured as described (Sasaki et al., 2005).

Immunosurgery and maintenance of ESC

The immunosurgery, isolation and culture of ESC lines were performed as described in detail (Sasaki et al., 2005). The zona pellucida of the marmoset blastocyst was removed by digestion in 0.1% pronase in phosphate-buffered saline (PBS). To remove the trophoblast, the blastocysts were first incubated for 45 min at 37°C in 5% CO₂ with a 10-fold dilution of anti-marmoset fibroblast rabbit serum in Dulbecco’s modified Eagle’s medium (DMEM). After mechanical removal of the trophoblast by pipetting, the ICM was plated on 3500-μg/girradiated mouse embryonic fibroblast (MEF) feeder cells. First passaging of the ICMs was performed after 10–14 days by physical removal of the ICM outgrowth and dissociation by vigorously pipetting. The medium used in initial ESC culture contained 80% Knockout DMEM (Invitrogen) supplemented with 20% Knockout Serum Replacement (KSR; Invitrogen), 1 mM l-glutamine, 0.1 mM MEM non-essential amino acids, 0.1 mM β-mercaptoethanol (Sigma), 100 IU/ml penicillin, 100 μg/ml streptomycin sulfate, 250 ng/ml amphotericin B and 10 ng/ml leukemia inhibitory factor. Established cell lines were cultured without amphotericin B and leukemia inhibitory factor. For passaging, ESC colonies were treated with trypsin–EDTA (0.25% trypsin,1 mM CaCl₂, 20% KSR in DMEM) to remove them from feeder layer, mechanically dissociated into clumps of 10–50 cells and replated on a new irradiated MEF feeder layer. To date, the ESC line cjes001 line has been maintained under these culture conditions for 24 months (passage 84).

Immunofluorescence staining

The cjes001 colonies were grown on γ-irradiated MEF cells in foil-bottom 24-well plates (Lumox™, Greiner Bio-One, Stuttgart, Germany) for 2–5 days, fixed for 30 min in 4% paraformaldehyde, 0.04% Triton X-100 and then washed twice in PBS. The staining with primary antibodies was done according to the manufacturer’s recommendations. Antibodies were diluted in Tris-buffered saline supplemented with 5% bovine serum albumin. A complete list of all primary and secondary antibodies used in this study is provided in Table I. After 16 h incubation in first antibody dilution at 4°C, cells were washed twice in PBS, incubated for another 60 min with the respective secondary antibody covalently linked to Alexa dye A488 or A568, or the streptavidin–fluorescein isothiocyanate conjugate (STAR2B). Immunofluorescent double-stainings were performed by simultaneous incubation with both primary and secondary antibodies. Images were taken on a Zeiss Axio Observer Z1 microscope. Counterstaining reagents were propidium iodine (1:10,000, 5 min) or Hoechst 33258 (Sigma-Aldrich).

Alkaline phosphatase staining

For alkaline phosphatase staining, the alkaline phosphatase staining kit (Dako Universal LSAB Kit, K0674 AP) was used according to the
Table I Antibodies used in the study to develop a novel embryonic stem cell line from the common marmoset monkey

<table>
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PGP9.5, protein gene product 9.5; SSEA, stage-specific embryonic antigen; TRA, tumor rejection antigen; AFP, α-Fetoprotein; FITC, fluorescein isothiocyanate.

manufacturer’s instructions. For histochemistry, cells were fixed with 4% paraformaldehyde for 30 s, washed twice in PBS and incubated with Fuchsین as substrate for 30 min at room temperature.

Karyotypic analysis

Confluent ESC colonies were incubated for 4 h in ESC medium containing 0.02 µg/ml Colcemid (Invitrogen GmbH, Karlsruhe, Germany), and then washed once with PBS and trypsinized (15 min, 37°C). After detachment, the colonies were centrifuged (200g, 10 min) and the pellet was resus- pended in 3 ml of prewarmed (37°C) 8 mM KCl/15 mM sodium citrate solution for 25 min at room temperature. After centrifugation (200g, 10 min), 2 ml of the KCl/sodium citrate solution was removed and 4 ml ice-cold MetOH/acetic acid (3:1) slowly added to the vial. After 5 min incubation at room temperature, the cells were pelleted again (200g, 10 min), 4.5 ml of supernatant removed, the remaining liquid carefully dropped on glass cover slips and dried overnight. For Giemsa staining ([Sigma, 0.4% (w/v) in buffered methanol solution, pH 6.8], the dye was added for 2 min on the coverslip, then washed 10 times withaq. bidest and dried again. For chromosome analysis, the cells were incubated after fixation on the coverslips for 5 min in McIlvaine-buffer (pH 4.6) including 0.01 µg /ml fluorescence dye (Hoechst 33258, Sigma), then washed 10 times withaq. bidest. McIlvaine-buffer containing 5 µg/ml Quinacrine mustard (Sigma Q2876) was added for 20 min. After the second staining, the cells were washed again 10 times withaq. bidest, incubated for 5 min in McIlvaine-buffer again and embedded in mounting media (Citifluor Ltd, London, UK). The chromosome analysis was performed with a Leica CW 4000 system with a modified chromosome template based on data from Sherlock et al. (1996).

Telomerase detection

cjes001 telomerase activity was determined by Biomax Telomerase detection kit (Biomax Inc., Ijamsville, MD, USA) according to the manufacturer’s references. Telomerase from the cell extract adds telomeric repeats onto a substrate oligonucleotide and the resulting extended product is sub- sequently amplified by PCR (http://www.biomax.us). Briefly, 1 × 10^6 cjes01 cells were lysed and the cell extract was added to a quantitative telomerase determination pre-mix in a real-time PCR reaction utilizing SYBR green for 37 cycles. As controls, MEFs, immortal green monkey kidney cells (COST) and SYBR green, exclusive of cell extract, were used.

Reverse transcriptase–polymerase chain reaction

RNA from cjes001 or teratoma was isolated by RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s recommendation. First-strand complementary DNA (cDNA) was synthesized with Omniscript RT Polymerase (Qiagen) and cDNA was amplified in 35 cycles (denaturation 95°C 1 min/annealing 60°C 30 s/elongation 72°C 60 s) with 2.5 U BiothermStar TAQ Polymerase (Genechem, Luedinghausen, Germany) in PCR reaction buffer [160 mM (NH₄)₂SO₄, 670 mM Tris–HCl, pH 8.8, 15 mM MgCl₂, 0.1% Tween 20], 0.2 mM dNTP and 0.5 mM of each primer. cDNA from MEF cells and mock reverse transcription without RT provided negative controls. A complete list of oligonucleotides used in this study is shown in Table II. If marmoset DNA sequences were unavailable, the expected sizes of the PCR products were deduced from alignments of the homologous human and mouse sequences. Selected RT–PCR products were verified by DNA sequencing (data not shown). Normal monkey tissues exhibiting considerable expression of the respective genes served as positive controls.

Embryoid body formation

To study embryoid body (EB) formation, undifferentiated ESC were removed from the MEF layer by graduated trypsinization using 0.25% trypsin that was supplemented with 1 mM CaCl₂ and 20% KSR until the colonies detached from the feeder layer, further dissociated using 0.25% trypsin in PBS with 20% KSR and 1 mM CaCl₂, and cultured in hanging drop cultures for 14–20 days in DMEM (10% fetal bovine serum) with a medium change every 3 days. The EBs were frozen in OCT Compound (Tissue-Tek, Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands) for cryosections, prepared for semi-thin sections according to previously published protocols (Godmann et al., 2008) or used in parallel for RT–PCR.

In vivo differentiation analysis and histology of teratoma

Eight weeks after subcutaneous injection of 1–3 × 10^6 cjes001 cells, tumor formation could be observed in non-obese diabetic/severely compromised immunodeficient (NOD/SCID) mice. The tumors were resected from the mice, fixed in Bouin’s fixative (0.9% picric acid, 9.6% formaldehyde and 4.8% acetic acid) for 5 h, further treated according to standard histological protocols for parafin-embedded tissues and sectioned at 5 µm for hematoxylin and eosin staining. Parts of the tumor were snap-frozen in liquid nitrogen for RNA analysis. RT–PCR analysis was performed as described above. As a positive control for teratoma formation,
murine ESC were utilized, whereas PBS was injected for the negative control.

**In vitro differentiation**

To spontaneously differentiate the cjes001 cells, the colonies were removed from the MEF layer by trypsinization, dissociated using 0.25% trypsin in PBS with 20% KSR and 1 mM CaCl\(_2\), and cultured on gelatin-coated petri dishes (Nunc) in MEF-medium. After 7–10 days, the cells had lost their characteristic undifferentiated morphology and were collected for RT–PCR or analyzed by immunofluorescence staining as described above.

**Western blot analysis**

Western blot analysis was performed as described (Quintana et al., 1993). Briefly, about 50 mg of testis tissue or stem cell culture material was mechanically homogenized (3 × 30 s) in 2.5 ml IMP buffer [0.15 M NaCl, 20 mM HEPES, 1 mM EDTA and a protease inhibitor cocktail 1:10 (Sigma #P8340), at pH 7.4] using a tissue homogenizer, centrifuged (10 min, 750 g, 4°C) and resuspended in 2.5 ml lysis buffer (IMP buffer + 0.5% Nonidet P40). The protein content was determined by BCA protein assay kit (Novagen #71285-3) and equal amounts of protein per lane were loaded onto a sodium dodecyl sulfate gel. A protein marker (Novex sharp prestained protein standard, Invitrogen) served as size standard. After electrophoresis, the gel content was transferred to a polyvinylidene difluoride membrane (Amersham Hybond-P) in an electrophoresis chamber (Roth, 100 V, 1.2 h, 300 mA). The membrane was washed in PBS, incubated for 1 h in blocking solution and stained with the primary antibody (1:500) overnight (4°C). After 3–5 min washes in PBS, the secondary, horse-radish peroxidase conjugated antibody (1:10 000) was added for 1 h and again washed 2 × 15 min. The detection was carried out with an enhanced chemiluminescence kit (Amersham #RPN2209) in an Ecomaxx X-ray Film developer.

**Results**

**cjes001 shows typical ESC morphology and a normal karyotype**

Out of 15 ICM initially cultured, the cjes001 line was established. This line conserved its typical ESC morphology (Fig. 1A and see Sasaki...
et al., 2005) and marker expression for 24 months (84 passages) and remained positive for alkaline phosphatase (Fig. 1B). The doubling time of cjes001 monitored by 5-bromo-2-deoxyuridine was roughly 19 h (data not shown). Karyotyping analysis after 64 passages showed a regular 46, XX chromosome set (Fig. 2).

**cjes001 cells express undifferentiated ESC marker molecules**

Immunofluorescence staining revealed the expression of the transcription factors OCT4, NANOG and SOX2 (Fig. 3), which serve as markers for pluripotency of undifferentiated ESC (Boyer et al., 2005). All factors localized to the nucleus. Upon spontaneous differentiation of marmoset ESC, the expression of the pluripotency markers OCT4 and NANOG is greatly diminished at the mRNA level but not completely abolished, whereas SOX2 could not be detected in differentiated cells by RT–PCR (Fig. 4). The pluripotent cell surface antigens (Lanctot et al., 2007) stage-specific embryonic antigen (SSEA)-3 and SSEA-4 (Fig. 5A–I) and keratan sulfate (tumor rejection) antigens TRA-1-60 and TRA-1-81 (Fig. 6A–F) were also strongly expressed by undifferentiated ESC. Quantitative real-time PCR analysis showed high levels of telomerase activity. The detected activities were 5-fold higher than in highly proliferative MEFs and approximately twice as high as in the SV40 virus-mediated immortalized monkey control cell line COS7 (Fig. 7).

**Figure 1** (A) Light microscopy shows tightly packed cell colonies with distinct boundaries. (B) Positive staining for alkaline phosphatase.

**Figure 2** cjes001 cells exhibit a normal 46, XX karyotype after 64 passages.
**Figure 3** Immunofluorescent detection of the transcription factors OCT4 (A–C), NANOG (D–F) and SOX2 (G–I).

B, E and H show red nuclear counterstaining using propidium iodide (PI); C, F and I show green Alexa 488 staining of the respective specific antigen. A, D and G show the merged pictures. All proteins were detected in the nucleus. J–L show the negative control for the detection of NANOG staining omitting the first antibody which was generated in goat and M–O the corresponding negative control for OCT4 and SOX2. The respective first antibodies were both generated in rabbit.

cjes001 cells can form different types of EB and teratoma

As shown in Fig. 8, cjes001 cells can form cystic as well as compact types of EB. Both developed to a size of ~1,000 μm in diameter. Immunofluorescent detection of germ layer markers on cryosections of compact type EBs revealed the presence of Brachyury (mesoderm), α-Fetoprotein (AFP, endoderm) and βIII tubulin (ectoderm). Semi-thin sections showed that the wall of the cystic bodies consisted of a flattened epithelium, whose apical surface was oriented to the lumen of the cyst (right surface of the tissue string in Fig. 8F).
The outer cells of the wall of the cyst had mainly mesenchymal appearance (Fig. 8F). In addition to in vitro differentiation in EBs, we also tested the pluripotency of the ESC in vivo by teratoma formation in NOD/SCID mice (Fig. 9A–E). This assay allowed prolonged differentiation of cjes001 cells. Histological sections of the subcutaneously developed encapsulated tumor exhibited fully differentiated tissues of different embryonic origins. Adenomatous and columnar epithelia, mesenchyme, neuroglia, chondrocytes and bone, including bone marrow, besides other cell types developed within the teratoma (Fig. 9A–E). Differentiation of cjes001 cells into derivatives of all three embryonic germ layers during teratoma formation was also confirmed by the detection of AFP (endoderm), Brachyury (mesoderm) and βIII tubulin (ectoderm) by RT–PCR (Fig. 9F). Upon spontaneous differentiation of cjes001 cells, we detected up-regulation of CD 34, a single-pass transmembrane sialomucin protein associated with early hematopoietic and vascular tissue (Fig. 10). Early trophoblast marker Bex1/Rex3 was detected as well as the intermediate filament protein Nestin, which is frequently used to trace neuronal differentiation. The absence of FoxD3 in undifferentiated cjes001 cells is identical to human ESC (Ginis et al., 2004), whereas its presence in differentiated ESC can be explained by its known antagonizing effect on the activity of OCT4 (Guo et al., 2002). By antagonizing the pluripotency factor OCT4, FoxD3 facilitates embryonic lineage-specific transcriptional specification. Neuronal differentiation also occurred very likely within spontaneously differentiating ESC. Beside its remarkable morphology, the cell shown in Fig. 10B was strongly positive for the neuronal marker βIII tubulin.

Evidence for germ cell specification in differentiating cjes001 cells

Interestingly, concurrent with the down-regulation of OCT4, SOX2 and NANOG during spontaneous ESC differentiation (Fig. 4), specific marker mRNAs for germ line/germ cell development such as VASA, Figure 4 Comparison of the mRNA expression the pluripotency transcription factors OCT4, NANOG and SOX2 in undifferentiated cjes001 ESC versus differentiated ESCs (ESCD). This figure demonstrates significant down-regulation of the respective mRNAs after 1 week of spontaneous differentiation. β-ACTIN was used to normalize data.

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The outer cells of the wall of the cyst had mainly mesenchymal appearance (Fig. 8F). In addition to in vitro differentiation in EBs, we also tested the pluripotency of the ESC in vivo by teratoma formation in NOD/SCID mice (Fig. 9A–E). This assay allowed prolonged differentiation of the cjes001 cells. Histological sections of the subcutaneously developed encapsulated tumor exhibited fully differentiated tissues of different embryonic origins. Adenomatous and columnar epithelia, mesenchyme, neuroglia, chondrocytes and bone, including bone marrow, besides other cell types developed within the teratoma (Fig. 9A–E). Differentiation of cjes001 cells into derivatives of all three embryonic germ layers during teratoma formation was also confirmed by the detection of AFP (endoderm), Brachyury (mesoderm) and βIII tubulin (ectoderm) by RT–PCR (Fig. 9F). Upon spontaneous differentiation of cjes001 cells, we detected up-regulation of CD 34, a single-pass transmembrane sialomucin protein associated with early hematopoietic and vascular tissue (Fig. 10). Early trophoblast marker Bex1/Rex3 was detected as well as the intermediate filament protein Nestin, which is frequently used to trace neuronal differentiation. The absence of FoxD3 in undifferentiated cjes001 cells is identical to human ESC (Ginis et al., 2004), whereas its presence in differentiated ESC can be explained by its known antagonizing effect on the activity of OCT4 (Guo et al., 2002). By antagonizing the pluripotency factor OCT4, FoxD3 facilitates embryonic lineage-specific transcriptional specification. Neuronal differentiation also occurred very likely within spontaneously differentiating ESC. Beside its remarkable morphology, the cell shown in Fig. 10B was strongly positive for the neuronal marker βIII tubulin.
synaptonemal complex protein 3 (SCP3) and germ cell nuclear factor (GCNF) were up-regulated (Fig. 11A). Additionally, the germ cell marker BOULE was expressed at relatively high levels in those colonies we defined as undifferentiated. In contrast, DAZL (Deleted in Azoospermia-like) could not be detected in spontaneously differentiating ESC.

To substantiate VASA expression at the protein level in differentiating ESC and to ensure that the VASA antibody obtained from Abcam used in immunofluorescence (Fig. 12) detects a protein of the correct size (72 kDa) also in the marmoset, we performed western blot analysis with protein from marmoset testis (Fig. 11B, left lane). In addition, in conventional immunohistochemistry, this VASA antibody purchased from Abcam, as well as the antibody obtained from R&D systems used in some double stainings shown in Fig. 12, exhibited the expected staining pattern for VASA (Castrillon et al., 2000) in adult human, macaque and marmoset testes (unpublished data). In western blot analysis, we obtained a specific and robust signal for VASA with differentiated cjes001 cells, further confirming germ line differentiation in cultures of these marmoset ESC. In control γ-irradiated MEF cells (which support the growth of cjes001 cells), we detected only a faint VASA signal (~25% of the signal intensity of the middle lane, normalized to β-ACTIN) probably originating from mouse primordial germ cells present in the feeder cell preparation. To further corroborate the development of germ line cells, we co-localized VASA protein (Castrillon et al., 2000) with the germ cell markers CD9 (Kanatsu-Shinohara et al., 2004), CD49f (Conrad et al., 2008), SSEA-4 (Mueller et al., 2008) and protein gene product 9.5 (PGP9.5) (Luo et al., 2006; Herrid et al., 2007), respectively, in individual cells (Fig. 12). These double-stainings revealed interesting results that suggest a budding process of germ cells from the ESC.

Figure 12A shows that possibly epithelial clusters of cells are VASA-positive (red in A and B) in differentiating ESC. Individual cells within these clusters also express CD49f, which is a marker for germ line stem cells within the testis (Conrad et al., 2008). There are also strongly VASA-positive cells within these clusters that are concomitantly CD9-positive (Fig. 12E–G). CD9 was successfully used for the enrichment of germ line stem cells from the mouse testis (Kanatsu-Shinohara et al., 2004). Figure 12I–K shows strong co-expression of VASA (green) and SSEA-4 in these cell clusters that are VASA-positive. We have recently shown that spermatogonial stem cells in the adult marmoset testis express high levels of SSEA-4 (Mueller et al., 2008). Our observations suggest that some cells bud off from these VASA-positive clusters. Figure 12M–O and Q–S shows cells in a plane above the VASA-positive ‘ground layer’ that strongly co-express VASA and SSEA-4 or VASA and PGP9.5, respectively. PGP9.5 has recently been described as a specific gonocyte and

### Analysis

**Figure 6** Positive staining for TRA-1-60 (A–C) and TRA-1-81 (D–F).

B and E show counterstaining by PI; C, F and I shows Alexa 488 staining, A and D the merged pictures.

**Figure 7** Telomerase activity quantified by real-time PCR.

To determine a normalized arbitrary value for the mRNA, every data point was normalized to the reference of an artificial molecule ‘TSR9’ from the kit. Data (mean ± SEM) are from triplicates. MEF, mouse feeder cells; COS7, immortalized green monkey kidney cell line; cjes001, marmoset ESC. Note the almost 2-fold increased number of telomerase mRNA molecules even compared with the immortalized COS7 cell line at cycle zero (C0).

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spermatogonial marker in porcine and cattle testes, respectively. Immunohistochemical detection of PGP9.5 in the non-human primate testis also specifically labels spermatogonia (unpublished results). Thus, it is likely that we detected early germ cell differentiation in colonies of differentiating ESC that became VASA-positive and, at least as a subset, also express the germ cell markers CD49f, CD9 and SSEA-4. Moreover, we show that a subpopulation of cells exhibiting a roundish shape strongly expressed VASA plus SSEA-4 or PGP9.5.

Discussion

Non-human primate ESC are an attractive tool to study aspects of early embryonic development (Rodda et al., 2002; Behr et al., 2005) and carry great hope for regenerative medicine (Murry and Keller, 2008). Creating new monkey ESC lines for characterization purposes in vitro and in vivo is an important step to improve the safety, performance and reproducibility of anticipated medical procedures prior to clinical trials. A wide range of different lines at hand will help to mimic epigenetic variation, because human and non-human primate ESC lines diverge in karyotype (Thomson et al., 1996), gene expression and differentiation potential (Heins et al., 2004; Chen et al., 2008; Dighe et al., 2008). In this study, we established and characterized a novel ESC line from the common marmoset monkey, named cjes001. As standards for successful establishment, we judged morphology and utilized a panel of molecular signatures, including transcription factors, surface antigens, lineage-specific gene expression and enzyme activity. Long-term cultivation up to passage 84 with normal karyotype demonstrates the reliability of culture conditions, media composition and MEF density.

The morphology of cjes001 matched those of other undifferentiated primate ESC colonies in other reports (Thomson et al., 1996; Sasaki et al., 2005), namely the distinct colony boundaries and the high nucleus:cytoplasm ratio with prominent nucleoli. However, we did not observe any ESC colony resembling morphologically an early embryo consisting of regularly structured tissues in terms of embryonic germ layer formation, as was described previously by Thomson et al. (1996). The strong histochemical staining for alkaline phosphatase is

Figure 8  cjes001 cells can form EB with a (A) cystic or (B) compact (solid) phenotype. Expressions of endodermal α-Fetoprotein (AFP, C), ectodermal (βIII tubulin, D) and mesodermal (Brachyury, E) markers (green with red nuclear counterstaining) suggest embryonic germ layer differentiation in compact EB. F shows a semi-thin section through the wall of a cystic EB. The inner surface of the cyst exhibited a flattened epithelium (right boundary of the tissue string), whereas the outer surface consisted mainly of mesenchymal cells. (G) Cystic EBs expressed almost no AFP, whereas βIII tubulin and Brachyury were clearly detectable.
also characteristic of undifferentiated ESC. The surface antigen composition with strong expression of SSEA-3, -4 and keratan sulfate antigens TRA1-60 and TRA1-81 matched other reports of undifferentiated non-human primate and human ESC and human iPS cells (Thomson et al., 1995, 1996, 1998; Sasaki et al., 2005; Takahashi et al., 2007). SSEA-1, which is primarily present in rodent ESC (Lanctot et al., 2007), was not detected. As for transcription factors, the strong presence of OCT4, NANOG and SOX2 indicates the pluripotency of ESC, which is also reflected by the enhanced levels of telomerase activity detected at passage 64.

All pluripotency transcription factors tested were down-regulated upon ESC differentiation as revealed by RT–PCR; other
differentiation-specific genes were switched on, such as CD34 for hematopoietic progenitors, NESTIN for neuronal progenitors, as well as FOXD3 (Fig. 10). Interestingly, this forkhead transcription factor, which is required in the mouse for the establishment of the epiblast from the ICM and hence also a factor representing differentiation (Hanna et al., 2002), is not expressed in both undifferentiated human and marmoset ESC, but appears later in differentiation with its antagonistic effect on OCT4 (Guo et al., 2002). Subcutaneous injection of cjes001 cells into immunodeficient mice resulted in tumors expressing marker mRNAs representing all three embryonic germ layers [βIII tubulin for differentiated neural cells (ectoderm), Brachyury for mesoderm and AFP for endoderm]. Also, histological evaluation of the teratoma revealed tissues indicative of a tumor derived from pluripotent cells, such as chondrocytes, bone tissue, bone marrow, mesenchyme, muscle, nerves and epithelia. Altogether, the data show that this novel marmoset ESC line can form teratoma and, thus, is pluripotent.

Interestingly, RT–PCR analysis of differentiated ESC revealed the presence of genes considered specific for germ cells (Fig. 11). VASA can be detected in vivo in migrating and post-migratory primordial germ cells as well as in gonocytes in the fetal testis and in premeiotic, meiotic and post-meiotic testicular germ cells (Castrillon et al., 2000). In contrast, in undifferentiated human ESC, VASA mRNA and protein could not be detected (Clark et al., 2004), thus being presently the best and most reliable marker for germ cell development in cultures of pluripotent cells. Since we have also not detected VASA mRNA in undifferentiated ESC, but it was present in differentiated ESC, this suggested that germ cells spontaneously develop in cultures of cjes001 cells. To substantiate this finding, we have also demonstrated the presence of VASA protein in differentiated ESC by western blot analysis. To provide further evidence for spontaneous germ cell development from cjes001, we also confirmed expression of the germ cell marker mRNAs SCP3, BOULE and GCNF. SCP3 is a specific structural component of the meiotic synaptonemal complex and is essential for male fertility and for proper oogenesis in mice, and serves as an excellent marker for meiotic germ cells (Di Carlo et al., 2000; Yuan et al., 2000). Expression of SCP3 strongly indicates the presence of germ cells in early meiotic stages in spontaneously differentiating marmoset
ESCs, DAZ, DAZL and BOULE are germ cell-specific RNA-binding proteins essential for gametogenesis in several species (Xu et al., 2001). Although DAZ is lacking in the marmoset (Gromoll et al., 1999), DAZL and its ancestral pendant BOULE are expressed in the common marmoset testis in late spermatocytes/early spermatids and in early meiotic germ cells, respectively (Gromoll et al., 1999; Wistuba et al., 2006). Here, the germ cell marker BOULE was expressed at relatively high levels even in those colonies we defined as undifferentiated. Possibly, these colonies already contained some early differentiating germ cells which started expressing BOULE. Alternatively, BOULE is already expressed in cells that are still in a pluripotent state. We think it is conceivable that an individual cell can switch from an embryonic stem cell state (which is an artificial cell type that has no in vivo equivalent since pluripotent cells of the embryoblast do not self-renew and proliferate indefinitely) to an early primordial (pluripotent) germ cell state and possibly vice versa. Currently, we have no solid explanation for the absence of DAZL from the ESC. Since DAZL is, at least in human fetal germ cells, expressed in both sexes, even the female karyotype of our line cannot serve as an explanation for this. Possibly, this finding simply reflects that the germ cells differentiating in ESC cultures outwith their natural environment are not totally in concordance with their natural counterparts. However, in addition to VASA, SCP3 and BOULE, we also detected GCNF at high levels in differentiating cells, whereas this mRNA was almost absent from
undifferentiated cells (Fig. 11). Although GCNF is not absolutely germ cell-specific (Chung and Cooney, 2001), these transcript data altogether suggest that the cjes001 ESCs cannot only differentiate into cell types representing the three embryonic germ layers but also into germ line cells. This was strongly substantiated by the co-localization of VASA with several germ cell markers within individual cells. Since we could clearly distinguish between germ cell marker-expressing cells that were part of a presumably epithelial cellular association and cells supposedly budding off the previous mentioned layer of cells, we suggest that the germ cells that develop within these spontaneously differentiating ESC colonies are first specified when still associated with their neighboring cells and then detach from these cells, as shown in Fig. 12M–S. Altogether, our data further strengthen the view that the cjes001 cells are indeed pluripotent. Moreover, this culture system will allow interesting studies on the developmental control points distinguishing somatic differentiation from germ line maintenance. Future studies will also reveal the potential to develop post-meiotic gametes from these marmoset ESCs, applying directed differentiation protocols as already established for mouse ESCs (Hubner et al., 2003; Geijsen et al., 2004).

In conclusion, we have established and characterized a novel primate ESC line from the common marmoset which exhibits not only the potential to develop into many different somatic lineages but also the capacity to spontaneously develop into germ cells.

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