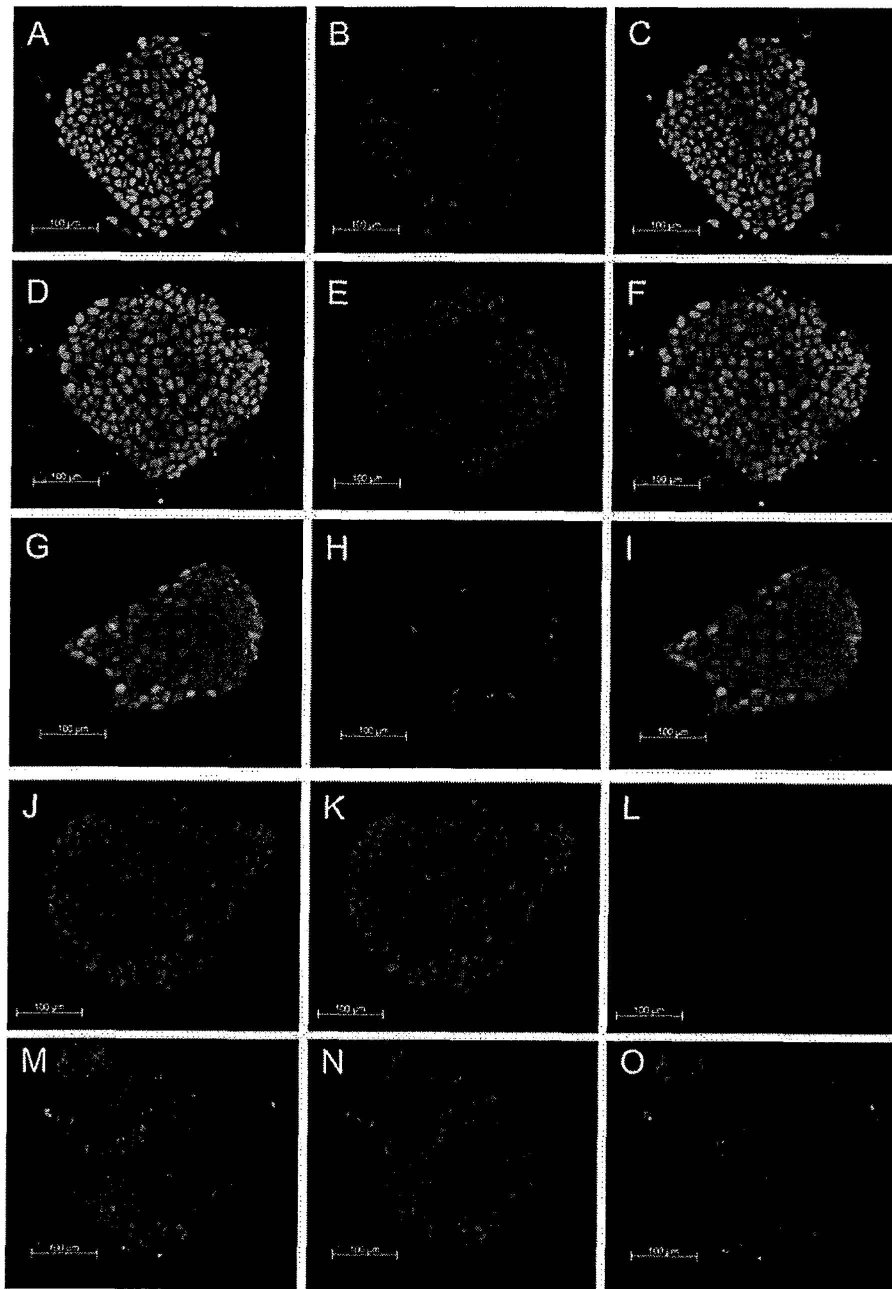

Figure 3 Immunofluorescent detection of the transcription factors OCT4 (**A–C**), NANOG (**D–F**) and SOX2 (**G–I**).
B, E and H show red nuclear counterstaining using propidium iodide (PI); C, F and I show green Alexa 488 staining of the respective specific antigen. A, D and G show the merged pictures. 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).

Downloaded from http://humrep.oxfordjournals.org at Kyushu University on May 27, 2010

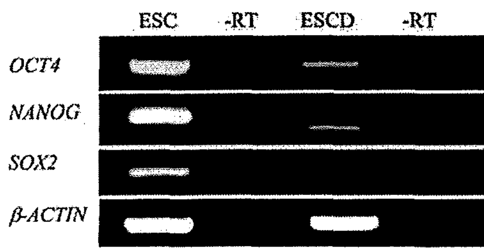

Figure 4 Comparison of the mRNA expression the pluripotency transcription factors *OCT4*, *NANOG* and *SOX2* in undifferentiated cjes001 ESC versus differentiated ESCs (ESCD).
This figure demonstrates significant down-regulation of the respective mRNAs after 1 week of spontaneous differentiation. β-ACTIN was used to normalize data.

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 trophoectoderm 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*,

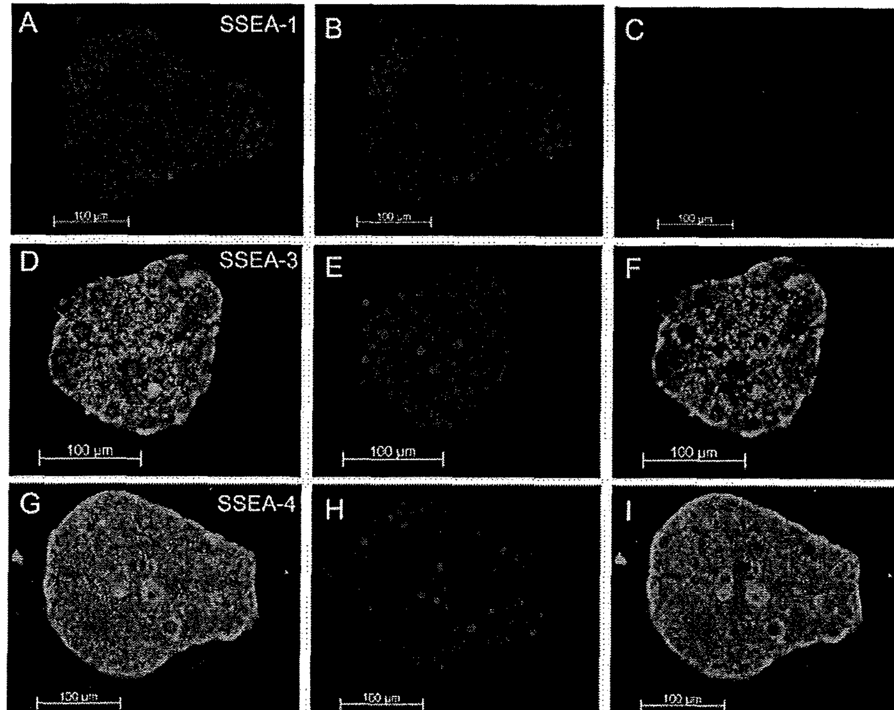

Figure 5 Stage-specific embryonic antigens.
No staining for SSEA-1 (**A–C**), but positive signals for SSEA-3 (**D–E**) and SSEA-4 (**G–I**). B, E and H show counterstaining by PI; C, F and I show Alexa 488 staining and A, D and G the merged pictures.

Downloaded from http://humrep.oxfordjournals.org at Kyushu University on May 27, 2010

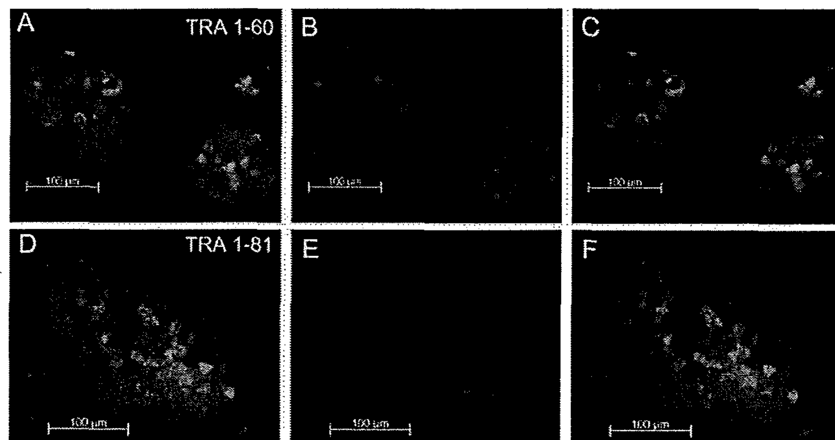

Figure 6 Positive staining for TRA-1-60 (**A–C**) and TRA-1-81 (**D–F**).
B and E show counterstaining by PI; C, F and I shows Alexa 488 staining, A and D the merged pictures.

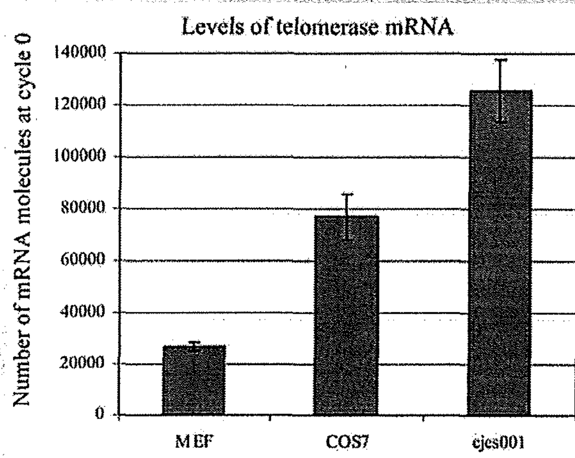

Figure 7 Telomerase activity quantified by real-time PCR.
To determine a normalized arbitrary value for the mRNA, every data point was normalized to the reference of an artificial molecule 'TSR9' from the kit. Data (mean ± SEM) are from triplicates. MEF, mouse feeder cells; COS7, immortalized green monkey 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).

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 (Müller 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 (Müller 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

Downloaded from http://humrep.oxfordjournals.org at Kyushu University on May 27, 2010

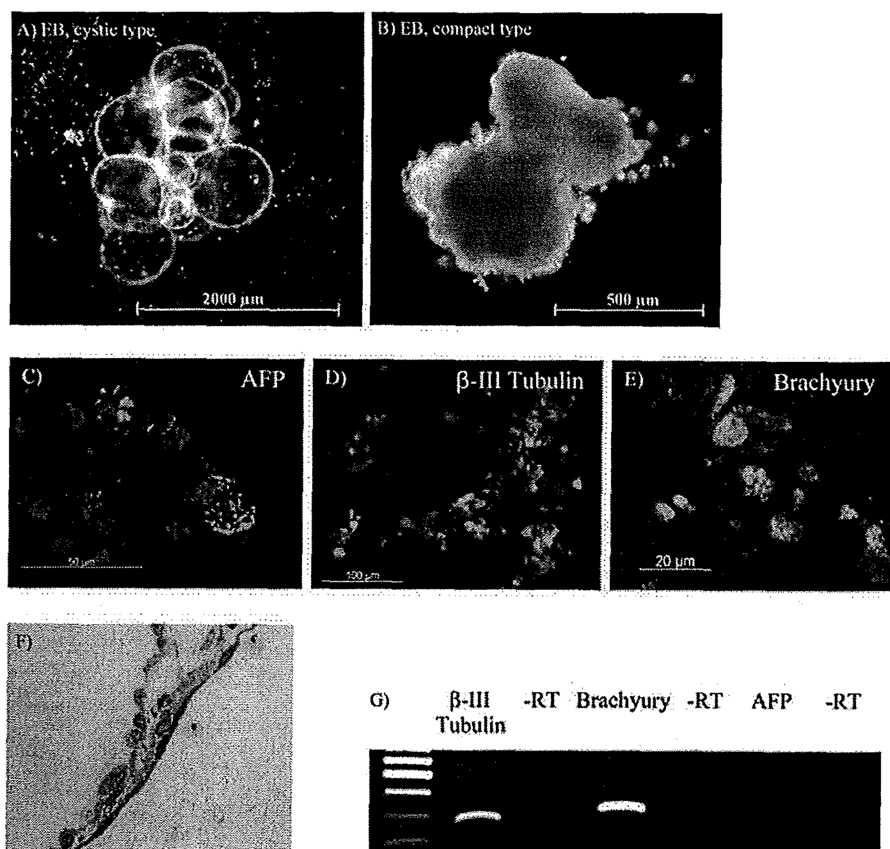

Figure 8 cjes001 cells can form EB with a (**A**) cystic or (**B**) compact (solid) phenotype.

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.

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

Downloaded from http://humrep.oxfordjournals.org at Kyushu University on May 27, 2010

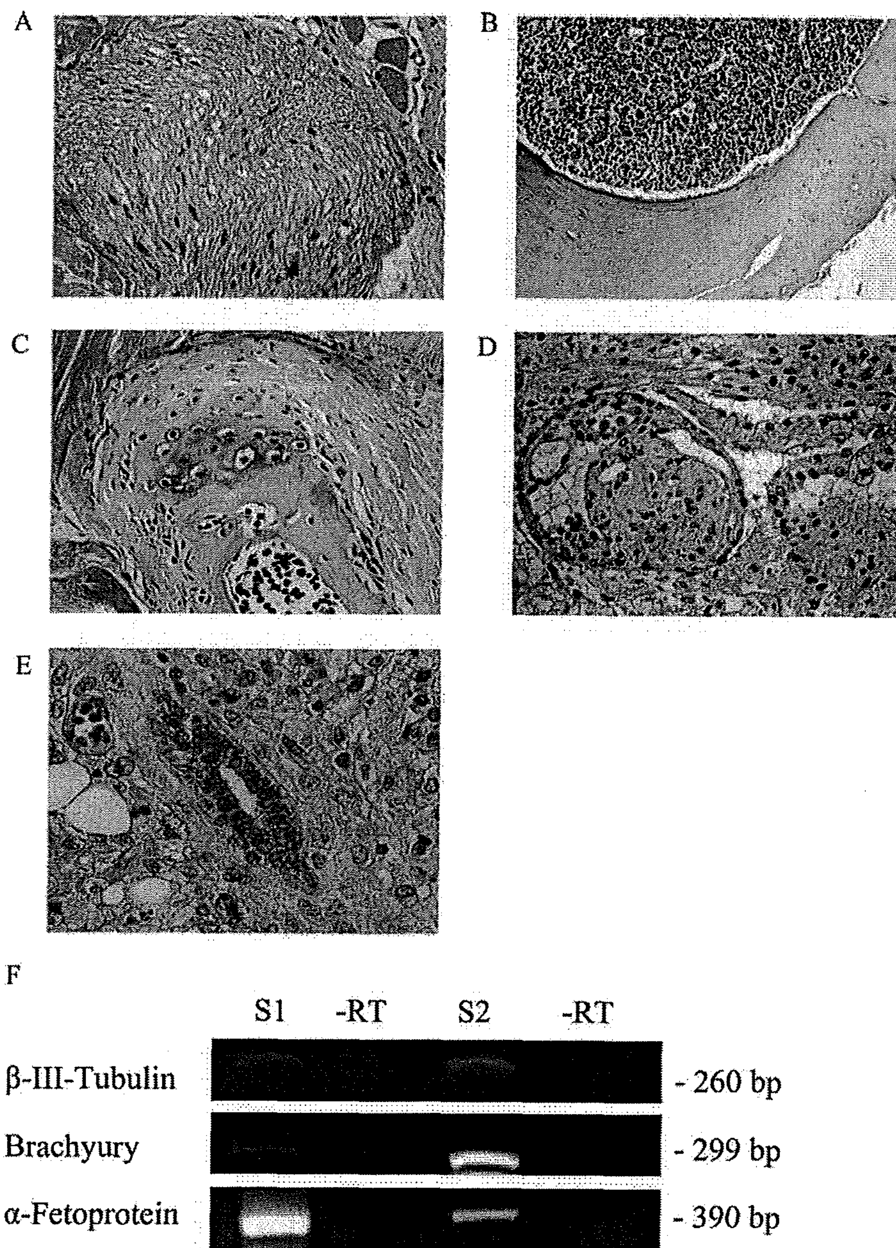

Figure 9 cjes001 cells form tumors containing derivatives of all three embryonic germ layers. (**A**) Neural structures within the tumor represent ectoderm. (**B**) Osteogenic and hematopoietic differentiation show mesoderm formation. (**C**) Chondrocytes and osteogenesis with adnate muscles tissue also represent mesoderm. (**D**) Adenomatous epithelium. (**E**) Columnar epithelium possibly representing endodermal differentiation. (**F**) RT–PCR analysis of two teratoma tissue samples (S1 and S2) showed expression of the marker mRNAs βIII tubulin, Brachyury and AFP, which represent ectoderm, mesoderm and endoderm formation, respectively.

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

Downloaded from http://humrep.oxfordjournals.org at Kyushu University on May 27, 2010

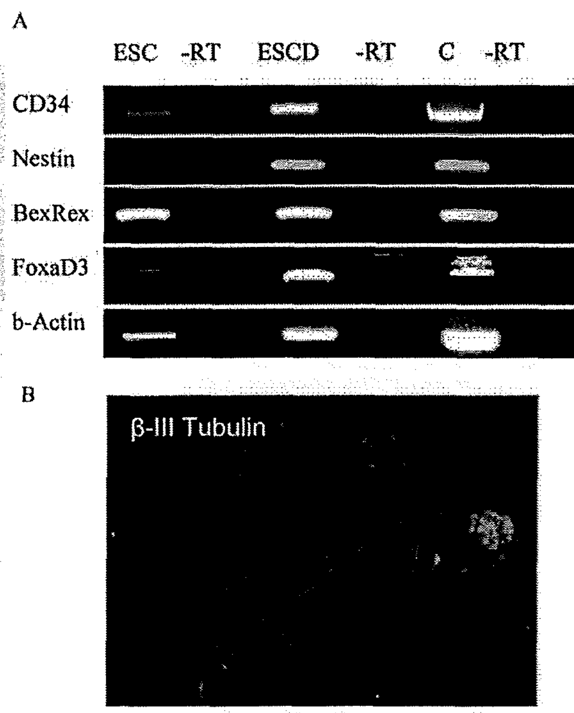

Figure 10 (**A**) RT–PCR expression analysis of selected differentiation markers, which are up-regulated during ESC differentiation.
For details, see Results section. (**B**) Immunofluorescent staining of a cell developed from differentiating ESC strongly resembling a neuronal cell. This cell was also strongly positive for the neuronal marker protein βIII tubulin.

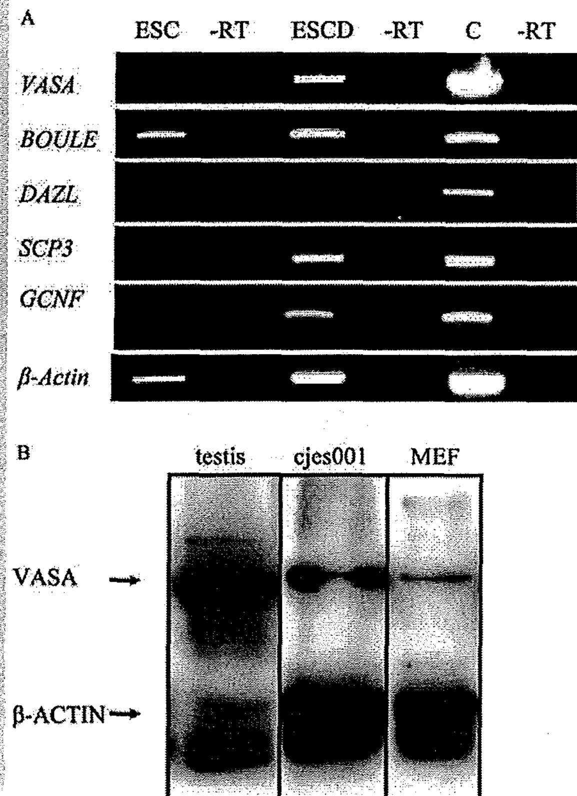

Figure 11 Analysis of germ cell-specific gene expression by RT–PCR (**A**) and western blot (**B**).
(A) The germ cell markers *VASA* and synaptonemal complex protein 3 (*SCP3*) are not detectable in undifferentiated ESC (ESC). In contrast, in differential ESC (ESCD), these germ cell markers are expressed at high levels almost reaching adult testicular expression levels (**C**, positive control). Also, germ cell nuclear factor (*GCNF*) was clearly up-regulated in differentiated ESC. In contrast to these regulated germ cell markers, *BOULE* was not found to be regulated during ESC differentiation and could be detected in both conditions. *DAZL* could be detected in neither condition. (B) Western blot analysis detecting specifically VASA protein in marmoset testis protein extracts (left lane) and in differentiated cjes001 cells. A significantly weaker signal was also obtained with a protein extract from mouse embryonic feeder cells alone, suggesting that the feeder cell population also contains mouse germ cells.

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

Downloaded from http://humrep.oxfordjournals.org at Kyushu University on May 27, 2010

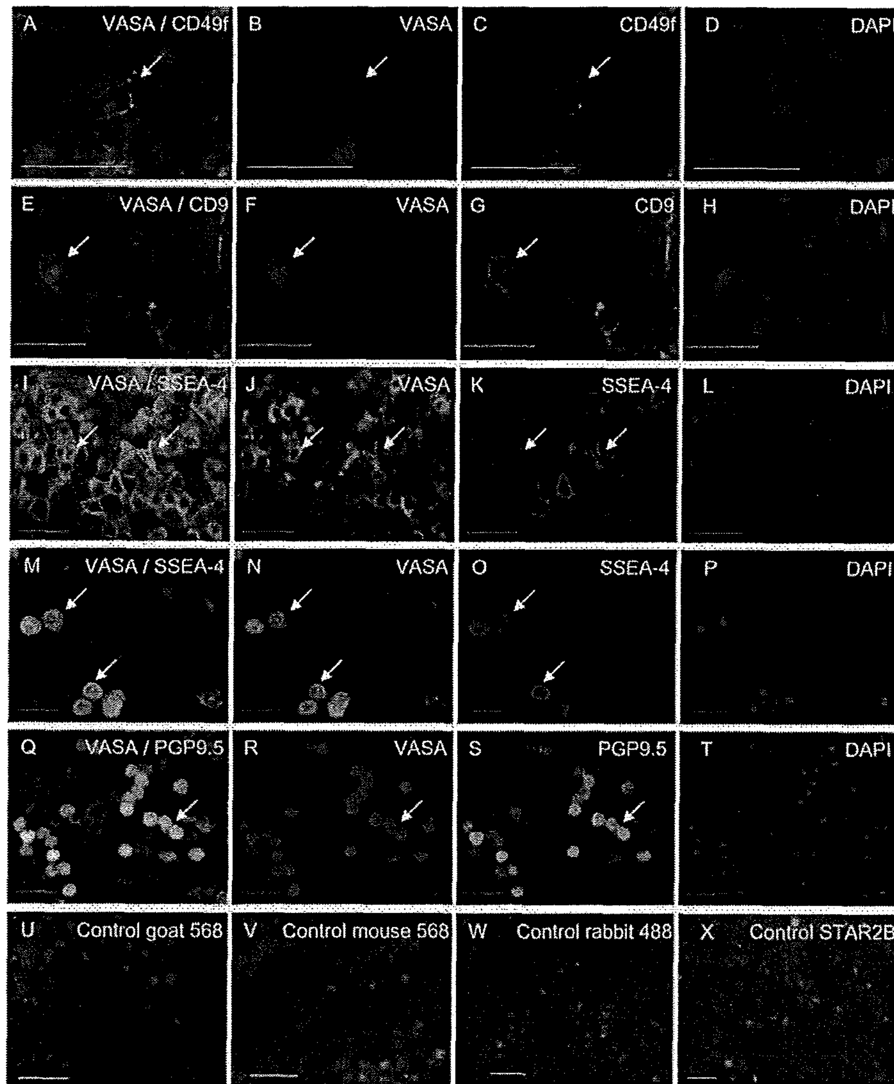

Figure 12 Co-localization of VASA together with different established germ cell markers in cells developing from differentiating ESC.
The left column shows the merged pictures that are shown in the second, third and fourth column in each lane (with the exceptions of **U–X**, which show the respective negative controls for all antibodies used in this experiments). **A–D** show co-localization of VASA together with CD49f. **E–H** show double-staining for VASA and CD9. **I–L** exhibit VASA expression in those cells that are also SSEA-4-positive. **M–P** demonstrate that VASA also co-localizes with SSEA-4 in roundish cells that are morphologically clearly different from the cells shown above. **Q–T** show the same roundish cell type co-expressing VASA and PGP9.5. DAPI, 4',6-diamidino-2-phenylindole.

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

Downloaded from http://humrep.oxfordjournals.org at Kyushu University on May 27, 2010

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.

Acknowledgements

We thank Reiko Hirakawa, Ayako Sugawara, Marion Niebeling and Kirsten Elger for excellent technical assistance. Request for cells should be addressed to E.S.

Funding

These studies were supported by an inhouse grant of the German Primate Center and the DAAD German-Japanese Exchange program (D/06/19948) and the German Federal Ministry of Education and Research (project induced pluripotent stem cells in large animal models (Ipsilam) as well as the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) for the Cluster of Excellence REBIRTH (From Regenerative Biology to Reconstructive Therapy). A part of this study was supported by funds from Technology Agency and from Special Coordination Funds for Promoting Science and Technology of Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government to E.S.

References

Behr R, Heneweer C, Viebahn C, Denker HW, Thie M. Epithelial–mesenchymal transition in colonies of rhesus monkey embryonic stem cells: a model for processes involved in gastrulation. *Stem Cells* 2005; **23**:805–816.

Boyer LA, Lee TI, Cole M F, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG *et al.* Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* 2005;**122**:947–956.

Castrillon DH, Quade BJ, Wang TY, Quigley C, Crum CP. The human VASA gene is specifically expressed in the germ cell lineage. *Proc Natl Acad Sci USA* 2000;**97**:9585–9590.

Chen H, Hattori F, Murata M, Li W, Yuasa S, Onizuka T, Shimoji K, Ohno Y, Sasaki E, Kimura K *et al.* Common marmoset embryonic stem cell can differentiate into cardiomyocytes. *Biochem Biophys Res Commun* 2008;**369**:801–806.

Chung AC, Cooney AJ. Germ cell nuclear factor. *Int J Biochem Cell Biol* 2001;**33**:1141–1146.

Clark AT, Bodnar MS, Fox M, Rodriquez RT, Abeyta MJ, Firpo MT, Pera RA. Spontaneous differentiation of germ cells from human embryonic stem cells in vitro. *Hum Mol Genet* 2004;**13**:727–739.

Conrad S, Renninger M, Hennenlotter J, Wiesner T, Just L, Bonin M, Aicher W, Bühring HJ, Mattheus U, Mack A *et al.* Generation of pluripotent stem cells from adult human testis. *Nature* 2008; **456**:344–349.

Di Carlo AD, Travia G, De Felici M. The meiotic specific synaptonemal complex protein SCP3 is expressed by female and male primordial germ cells of the mouse embryo. *Int J Dev Biol* 2000;**44**:241–244.

Dighe V, Clepper L, Pedersen D, Byrne J, Ferguson B, Gokhale S, Penedo MC, Wolf D, Mitalipov S. Heterozygous embryonic stem cell lines derived from nonhuman primate parthenotes. *Stem Cells* 2008;**26**:756–766.

Eslamboli A. Marmoset monkey models of Parkinson's disease: which model, when and why? *Brain Res Bull* 2005;**68**:140–149.

Fougerousse F, Bullen P, Herasse M, Lindsay S, Richard I, Wilson D, Suel L, Durand M, Robson S, Abitbol M *et al.* Human–mouse differences in the embryonic expression patterns of developmental control genes and disease genes. *Hum Mol Genet* 2000;**9**:165–173.

Geijsen N, Horoschak M, Kim K, Gribnau J, Eggan K, Daley GQ. Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature* 2004;**427**:148–154.

Ginis I, Luo Y, Miura T, Thies S, Brandenberger R, Gerecht-Nir S, Amit M, Hoke A, Carpenter MK, Itskovitz-Eldor J *et al.* Differences between human and mouse embryonic stem cells. *Dev Biol* 2004;**269**:360–380.

Godmann M, Katz JP, Guillou F, Simoni M, Kaestner KH, Behr R. Krüppel-like factor 4 is involved in functional differentiation of testicular Sertoli cells. *Dev Biol* 2008;**315**:552–566.

Gromoll J, Weinbauer GF, Skaletsky H, Schlatt S, Rocchietti-March M, Page DC, Nieschlag E. The old world monkey DAZ (Deleted in AZoospermia) gene yields insights into the evolution of the DAZ gene cluster on the human Y chromosome. *Hum Mol Genet* 1999;**8**:2017–2024.

Guo Y, Costa R, Ramsey H, Starnes T, Vance G, Robertson K, Kelley M, Reinbold R, Scholer H, Hromas R. The embryonic stem cell transcription factors Oct-4 and FoxD3 interact to regulate endodermal-specific promoter expression. *Proc Natl Acad Sci USA* 2002;**99**:3663–3667.

Hanna LA, Foreman RK, Tarasenko IA, Kessler DS, Labosky PA. Requirement for Foxd3 in maintaining pluripotent cells of the early mouse embryo. *Genes Dev* 2002;**16**:2650–2661.

Heins N, Englund MC, Sjoblom C, Dahl U, Tonning A, Bergh C, Lindahl A, Hanson C, Semb H. Derivation, characterization, and differentiation of human embryonic stem cells. *Stem Cells* 2004;**22**:367–376.

Herrid M, Davey RJ, Hill JR. Characterization of germ cells from pre-pubertal bull calves in preparation for germ cell transplantation. *Cell Tissue Res* 2007;**330**:321–329.

Hubner K, Fuhrmann G, Christenson LK, Kehler J, Reinbold R, De La Fuente R, Wood J, Strauss JF 3rd, Boiani M, Scholer HR. Derivation of oocytes from mouse embryonic stem cells. *Science* 2003;**300**:1251–1256.

Kanatsu-Shinohara M, Toyokuni S, Shinohara T. CD9 is a surface marker on mouse and rat male germline stem cells. *Biol Reprod* 2004;**70**:70–75.

Lanctot PM, Gage FH, Varki AP. The glycans of stem cells. *Curr Opin Chem Biol* 2007;**11**:373–380.

Downloaded from http://humrep.oxfordjournals.org at Kyushu University on May 27, 2010

Luo J, Megee S, Rathi R, Dobrinski I. Protein gene product 9.5 is a spermatogonia-specific marker in the pig testis: application to enrichment and culture of porcine spermatogonia. *Mol Reprod Dev* 2006;**73**:1531–1540.

Mansfield K. Marmoset models commonly used in biomedical research. *Comp Med* 2003;**53**:383–392.

Meissner A, Wernig M, Jaenisch R. Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. *Nat Biotechnol* 2007; **25**:1177–1181.

Michel JB, Mahouy G. The marmoset in biomedical research. Value of this primate model for cardiovascular studies. *Pathol Biol (Paris)* 1990; **38**:197–204.

Müller T, Eildermann K, Dhir R, Schlatt S, Behr R. Glycan stem-cell markers are specifically expressed by spermatogonia in the adult non-human primate testis. *Hum Reprod* 2008;**23**:2292–2298.

Murry CE, Keller G. Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. *Cell* 2008; **132**:661–680.

Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. *Nature* 2007;**448**:313–317.

Quintana J, Hipkin RW, Ascoli M. A polyclonal antibody to a synthetic peptide derived from the rat follicle-stimulating hormone receptor reveals the recombinant receptor as a 74-kilodalton protein. *Endocrinology* 1993;**133**:2098–2104.

Rodda SJ, Kavanagh SJ, Rathjen J, Rathjen PD. Embryonic stem cell differentiation and the analysis of mammalian development. *Int J Dev Biol* 2002;**46**:449–458.

Sasaki E, Hanazawa K, Kurita R, Akatsuka A, Yoshizaki T, Ishii H, Tanioka Y, Ohnishi Y, Suemizu H, Sugawara A *et al.* Establishment of novel embryonic stem cell lines derived from the common marmoset (*Callithrix jacchus*). *Stem Cells* 2005;**23**:1304–1313.

Sherlock JK, Griffin DK, Delhanty JD, Parrington JM. Homologies between human and marmoset (*Callithrix jacchus*) chromosomes revealed by comparative chromosome painting. *Genomics* 1996; **33**:214–219.

Stojkovic M, Lako M, Strachan T, Murdoch A. Derivation, growth and applications of human embryonic stem cells. *Reproduction* 2004; **128**:259–267.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007;**131**:861–872.

Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Becker RA, Hearn JP. Isolation of a primate embryonic stem cell line. *Proc Natl Acad Sci USA* 1995;**92**:7844–7848.

Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Hearn JP. Pluripotent cell lines derived from common marmoset (*Callithrix jacchus*) blastocysts. *Biol Reprod* 1996;**55**:254–259.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science* 1998;**282**:1145–1147.

Turnpenny L, Spalluto CM, Perrett RM, O'shea M, Hanley KP, Cameron IT, Wilson DI, Hanley NA. Evaluating human embryonic germ cells: concord and conflict as pluripotent stem cells. *Stem Cells* 2006;**24**:212–220.

Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* 2007;**448**:318–324.

Wistuba J, Luetjens CM, Wesselmann R, Nieschlag E, Simoni M, Schlatt S. Meiosis in autologous ectopic transplants of immature testicular tissue grafted to *Callithrix jacchus*. *Biol Reprod* 2006;**74**:706–713.

Wolf DP, Kuo HC, Pau KY, Lester L. Progress with nonhuman primate embryonic stem cells. *Biol Reprod* 2004;**71**:1766–1771.

Xu EY, Moore FL, Pera RA. A gene family required for human germ cell development evolved from an ancient meiotic gene conserved in metazoans. *Proc Natl Acad Sci USA* 2001;**98**:7414–7419.

Yu D, Silva GA. Stem cell sources and therapeutic approaches for central nervous system and neural retinal disorders. *Neurosurg Focus* 2008; **24**:E11.

Yuan L, Liu JG, Zhao J, Brundell E, Daneholt B, Hoog C. The murine SCP3 gene is required for synaptonemal complex assembly, chromosome synapsis, and male fertility. *Mol Cell* 2000;**5**:73–83.

Zuhlke U, Weinbauer G. The common marmoset (*Callithrix jacchus*) as a model in toxicology. *Toxicol Pathol* 2003;**31**(Suppl):123–127.

Submitted on July 16, 2008; resubmitted on November 12, 2008; accepted on January 13, 2009

Downloaded from http://humrep.oxfordjournals.org at Kyushu University on May 27, 2010

CLONING AND STEM CELLS
Volume 11, Number 2, 2009
© Mary Ann Liebert, Inc.
DOI: 10.1089/clo.2008.0064

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

Gesine Fleischmann,[1] Thomas Müller,[2] Rainer Blasczyk,[2] Erika Sasaki,[3,4] and Peter A. Horn[1]

Abstract

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

Introduction

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

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

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

[1]Institute for Transfusion Medicine, University Hospital Essen, Essen, Germany.
[2]Institute for Transfusion Medicine, Hannover Medical School, Hannover, Germany.
[3]Central Institute for Experimental Animals, Laboratory of Applied Developmental Biology, Department of Marmoset Research, Kawasaki, Japan.
[4]Center for Human Metabolomic System Biology, School of Medicine, Keio University, Tokyo, Japan.

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

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

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

Material and Methods

Cultivation of the embryonic stem cell line cjes001

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

Characterization of cjes001

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

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

Preparation of MEF

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

Inactivation of MEF

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

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

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

MTT Assay

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

Cytokine quantitation using a multiplexed immunoassay

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

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

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

Counting colonies and Morphology of cjes001

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

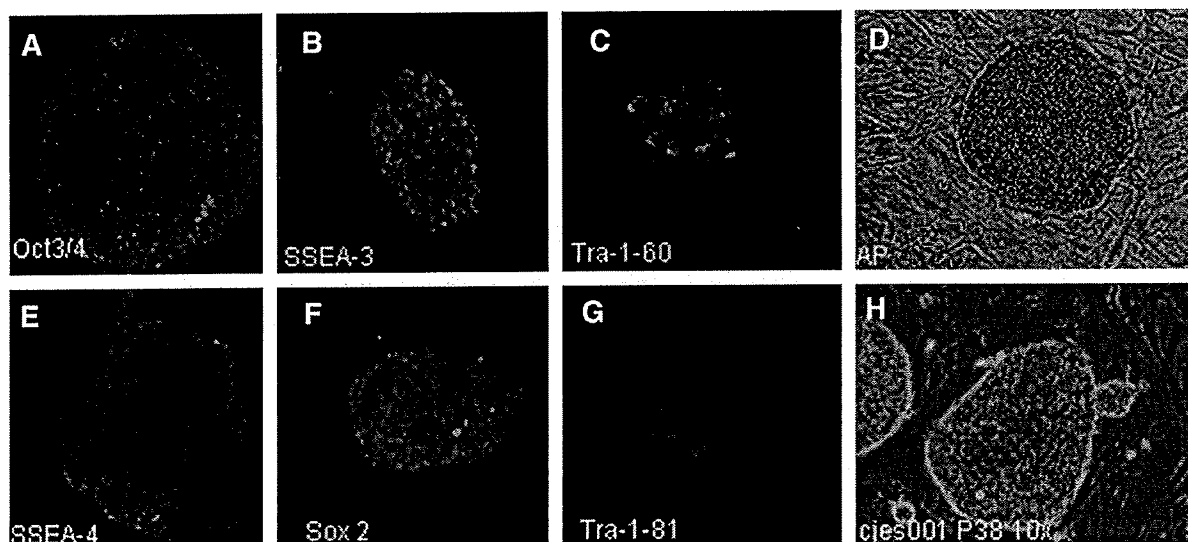

FIG. 1. Immunofluorescent staining with stem cell markers Oct4, SSEA-3, SSEA-4, Tra-1-60, Tra-1-81, and Sox-2 (A–F). Immunohistochemical staining of alkaline phosphatase (AP) (G). The positively staining underscores their undifferentiated status. Light microscopy picture of a cjes001 colony on inactivated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

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

Growth rate and differentiation status

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

Statistical analysis

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

Results

Characterization of cjes001

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

MTT assay

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

Soluble factors secreted by MEF

A bead-based multiplex quantitative analysis (Rules-Based Medicine, Inc.,) was performed to assess differences in concentrations of cytokines and other soluble factors secreted by MEF. Both the method of inactivation (either by

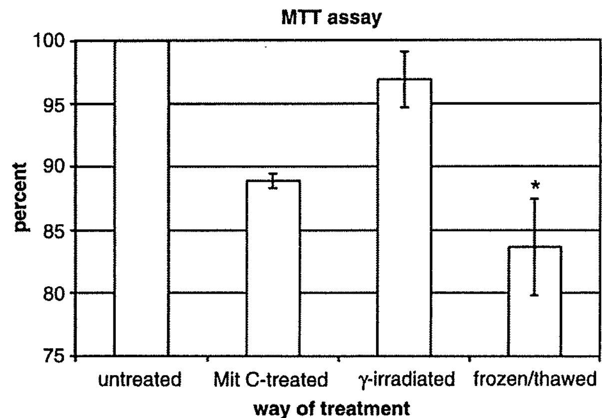

FIG. 2. Metabolic activity (%) of inactivated MEF, analyzed by MTT assay. Student's t-test unreveals a significant ($p < 0.02$) difference of metabolic activity between γ-irradiated versus chemically treated cells by Mitomycin C. Furthermore, freezing as well decreases the metabolic activity significantly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

Mitomycin C treatment or γ-irradiation), and MEF of two different mouse strains (NMRI vs. CF1) were compared. In addition, the kinetics of the release of 69 soluble factors by MEF over time by quantitation after 0, 6, 12, and 24 h was measured.

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

Mitomycin C treatment versus γ-irradiation

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

NMRI versus CF1

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

Time response

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

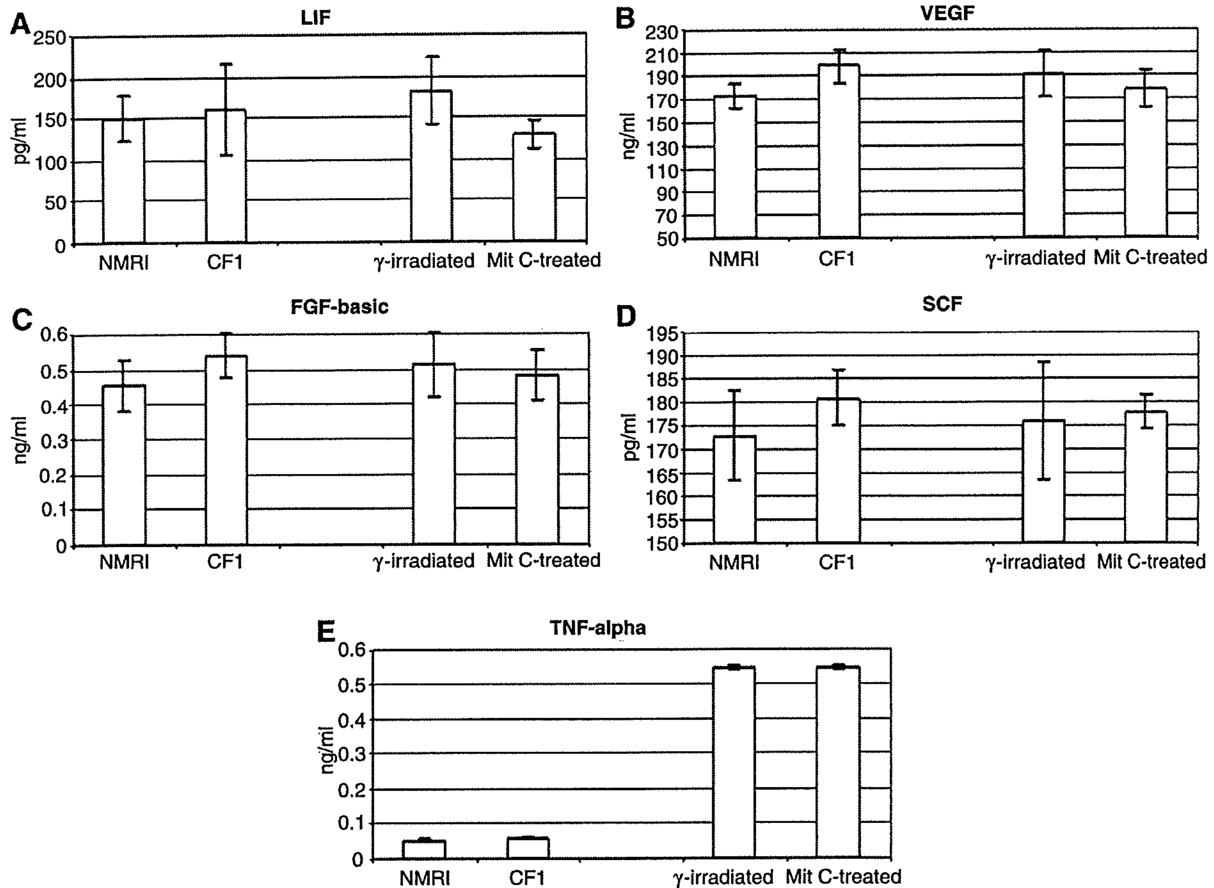

FIG. 3. Comparison of five soluble factors in NMRI mice versus CF1 mice and γ-irradiated versus Mitomycin C-treated, respectively. Significantly higher concentrations of FGF-basic are found in the supernatant of CF1-MEF ($p < 0{,}05$) (C) and a trend to higher concentrations of LIF, VEGF, and SCF in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

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

Effect on ESC

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

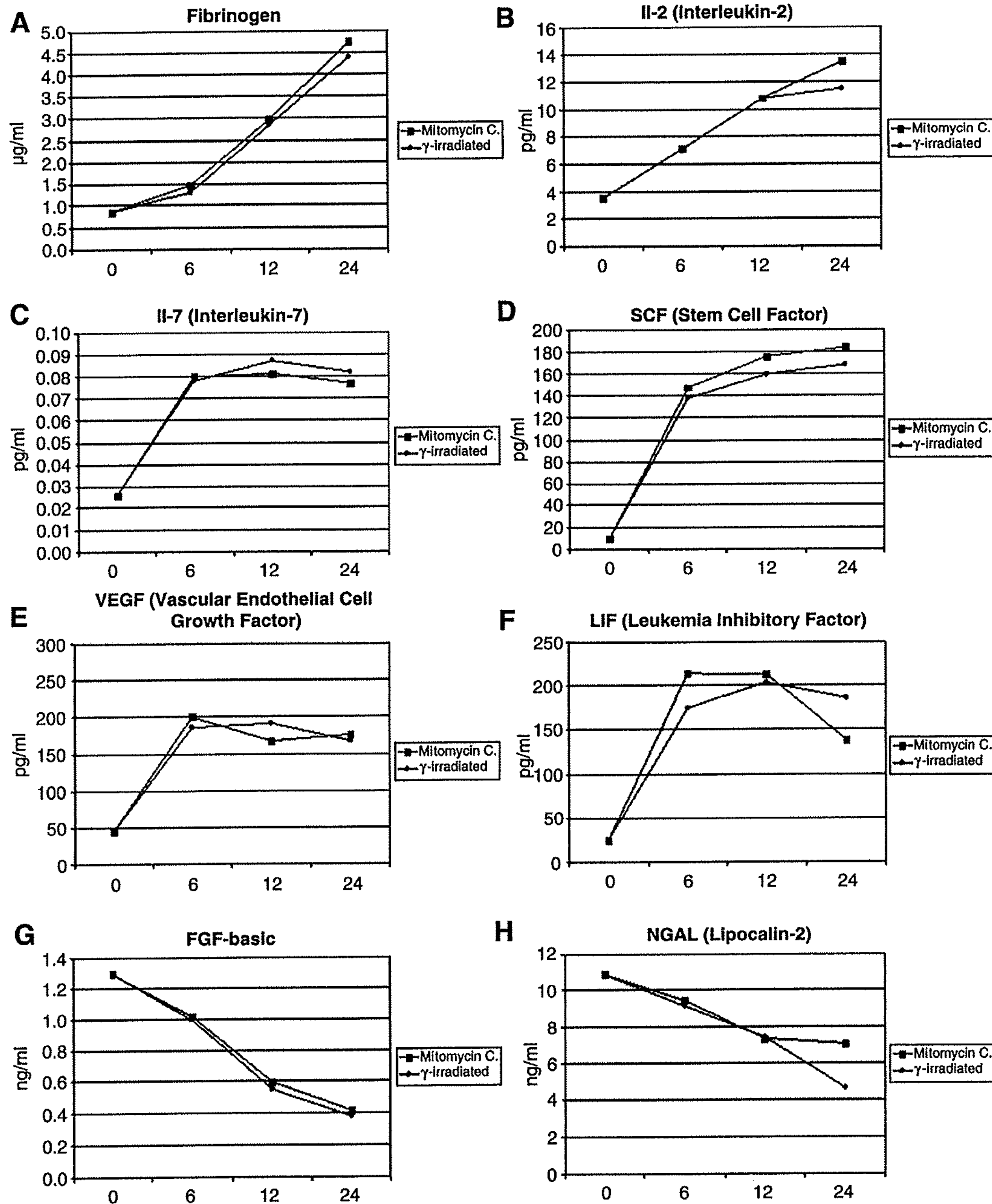

FIG. 4. The level of soluble factors secreted by MEF (NMRI) in comparison of inactivation methods monitored over time. Some factors such as Fibrinogen and IL-2 increase continuously (**A**,**B**) while others, such as Il-7, SCF, VEGF, and LIF increase very rapidly and then remain on a plateau after 12–18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

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

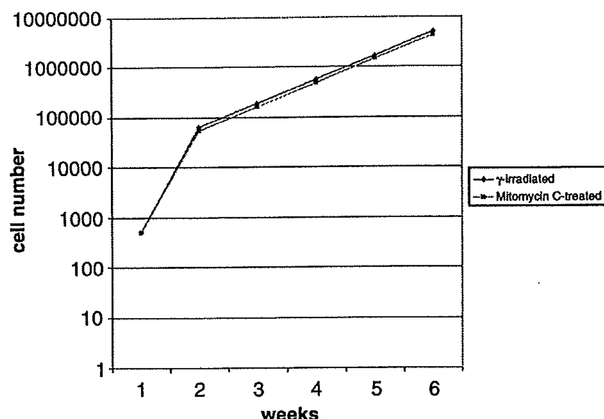

FIG. 5. The graph shows the proliferation rate of ESC in comparison to colonies grown on Mitomycin C-treated MEF (grey line) and on γ-irradiated MEF (black line).

Discussion

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

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

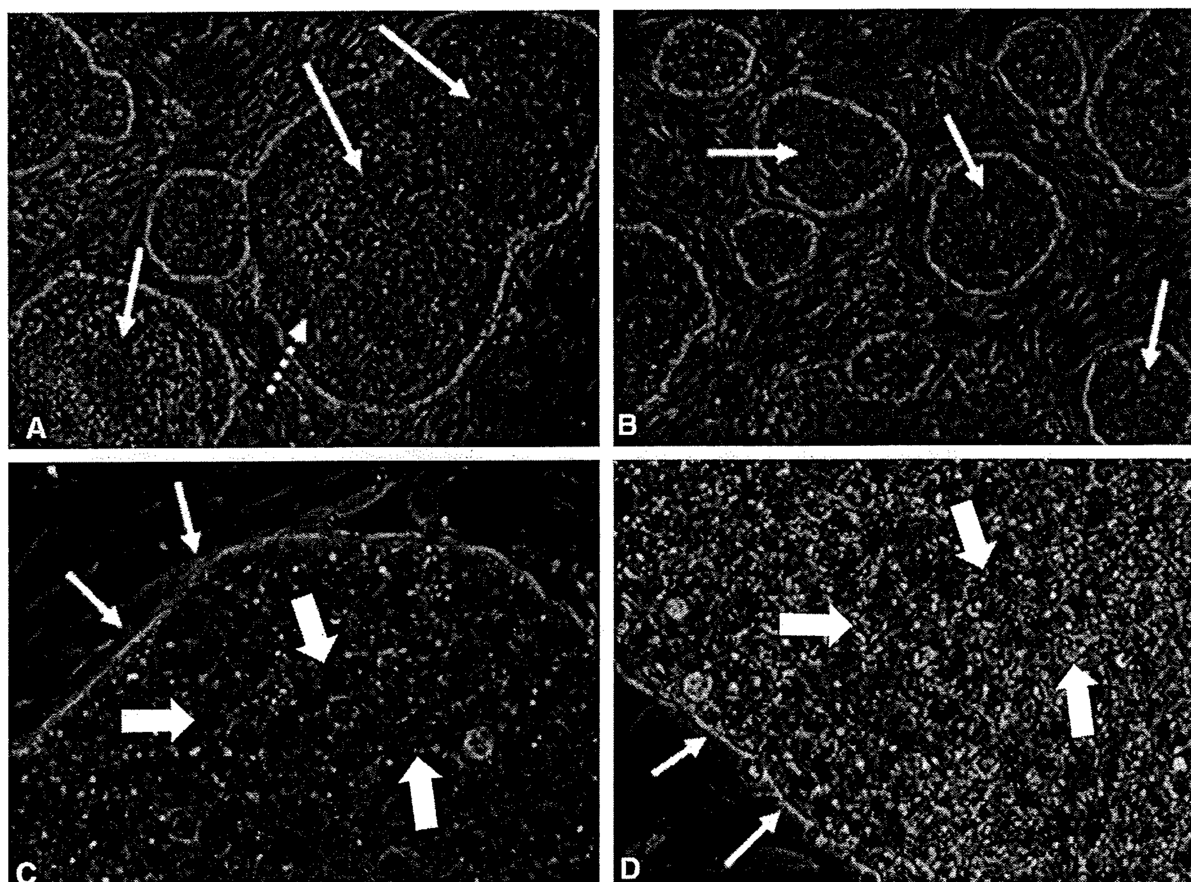

FIG. 6. (A–D) Larger ESC colonies with blur borders on Mitomycin C-treated MEF (A,C). The thin arrows in **A** show the beginning of differentiation in the middle of the colony. The dashed arrow at the border shows the undifferentiated morphology of cells in this colony. In comparison, **B** shows "shiny"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

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

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

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

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

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

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

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

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

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

Acknowledgments

These studies were supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) for the Cluster of Excellence ReBirth (from Regenerative Biology to Reconstructive Therapy). We furthermore would like to thank Kirsten Elger and Marion Niebeling for excellent technical assistance and the working group of LEBAO, especially Prof. Dr. Ulrich Martin and Dr. rer. nat. Kristin Schwanke for excellent cooperation.

Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

References

Amit, M., and Itskovitz-Eldor, J. (2006a). Feeder-free culture of human embryonic stem cells. Methods Enzymol 420, 37–49.

Amit, M., and Itskovitz-Eldor, J. (2006b). Maintenance of human embryonic stem cells in animal serum- and feeder layer-free culture conditions. Methods Mol. Biol. 331, 105–113.

Beattie, G. M., Lopez, A. D., Bucay, N., A. et al. (2005). Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers. Stem Cells 23, 489–495.

Bigdeli, N., Andersson, M., Strehl, R., et al. (2008). Adaptation of human embryonic stem cells to feeder-free and matrix-free culture conditions directly on plastic surfaces. J. Biotechnol. 133, 146–153.

Dvorak, P., and Hampl, A. (2005). Basic fibroblast growth factor and its receptors in human embryonic stem cells. Folia Histochem. Cytobiol. 43, 203–208.

Ezashi, T., Das, P., and Roberts, R.M. (2005). Low O2 tensions and the prevention of differentiation of hES cells. Proc. Natl. Acad. Sci. USA 102, 4783–4788.

Fischbach, G.D., and Fischbach, R.L. (2004). Stem cells: science, policy, and ethics. J. Clin. Invest. 114, 1364–1370.

Fleischmann, G. (2007). Symposium in stem cell repair and regeneration. Cloning Stem Cells 9, 141–143.

Fry, R.C. (1992). The effect of leukaemia inhibitory factor (LIF) on embryogenesis. Reprod. Fertil. Dev. 4, 449–458.

Hehlgans, T., and Pfeffer, K. (2005). The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. Immunology 115, 1–20.

Hong-mei, P., and Gui-an, C. (2006). Serum-free medium cultivation to improve efficacy in establishment of human embryonic stem cell lines. Hum. Reprod. 21, 217–222.

Horn, P.A., Tani, K., Martin, U., et al. (2006). Nonhuman primates: embryonic stem cells and transgenesis. Cloning Stem Cells 8, 124–129.

Humphrey, R.K., Beattie, G.M., Lopez, A.D., et al. (2004). Maintenance of pluripotency in human embryonic stem cells is STAT3 independent. Stem Cells 22, 522–530.

Kurosawa, H., Kimura, M., Noda, T., et al. (2006). Effect of oxygen on in vitro differentiation of mouse embryonic stem cells. J. Biosci. Bioeng. 101, 26–30.

Lavine, K.J., Yu, K., White, A.C., et al. (2005). Endocardial and epicardial derived FGF signals regulate myocardial proliferation and differentiation in vivo. Dev. Cell 8, 85–95.

Li, F., Liu, Y., Chen, D., et al. (2007). Leukemia inhibitory factor-expressing human embryonic lung fibroblasts as feeder cells for human embryonic germ cells. Cells Tissues Organs 186, 221–228.

Marie, P.J. (2003). Fibroblast growth factor signaling controlling osteoblast differentiation. Gene 316, 23–32.

Metcalf, D. (1991). The leukemia inhibitory factor (LIF). Int. J. Cell Cloning 9, 95–108.

Mountford, J.C. (2008). Human embryonic stem cells: origins, characteristics and potential for regenerative therapy. Transfus Med. 18, 1–12.

Nakatsuji, N., and Suemori, H. (2002). Embryonic stem cell lines of nonhuman primates. Sci. World J. 2, 1762–1773.

Ponchio, L., Duma, L., Oliviero, B., et al. (2000). Mitomycin C as an alternative to irradiation to inhibit the feeder layer growth in long-term culture assays. Cytotherapy 2, 281–286.

Prowse, A.B., McQuade, L.R., Bryant, K.J., et al. (2007). Identification of potential pluripotency determinants for human embryonic stem cells following proteomic analysis of human and mouse fibroblast conditioned media. J. Proteome Res. 6, 3796–3807.

Rose-John, S. (2002). GP130 stimulation and the maintenance of stem cells. Trends Biotechnol. 20, 417–419.

Roy, A., Krzykwa, E., Lemieux, R., et al. (2001). Increased efficiency of gamma-irradiated versus mitomycin C-treated feeder cells for the expansion of normal human cells in long-term cultures. J. Hematother. Stem Cell Res. 10, 873–880.

Sone, M., Itoh, H., Yamahara, K., et al. (2007). Pathway for differentiation of human embryonic stem cells to vascular cell components and their potential for vascular regeneration. Arterioscler. Thromb. Vasc. Biol. 27, 2127–2134.

Suemori H., and Nakatsuji, N. (2006). Generation and characterization of monkey embryonic stem cells. Methods Mol. Biol. 329, 81–89.

Wobus, A.M. (2001). Potential of embryonic stem cells. Mol. Aspects Med. 22, 149–164.

Xu, C., Inokuma, M.S., Denham, J., et al. (2001). Feeder-free growth of undifferentiated human embryonic stem cells. Nat. Biotechnol. 19, 971–974.

Xu, C., Rosler, E., Jiang, J., et al. (2005). Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. Stem Cells 23, 315–323.

Yu, K., and Ornitz, D.M. (2008). FGF signaling regulates mesenchymal differentiation and skeletal patterning along the limb bud proximodistal axis. Development 135, 483–491.

Address reprint requests to:
Peter A. Horn, M.D.
Institute for Transfusion Medicine
University Hospital Essen
45147 Essen
Germany

E-mail: Horn.Peter@uk-essen.de

CLONING AND STEM CELLS
Volume 11, Number 4, 2009
© Mary Ann Liebert, Inc.
DOI: 10.1089/clo.2009.0005

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

Yusuke Sotomaru,[1] Reiko Hirakawa,[1,2,3] Akiko Shimada,[2,4] Seiji Shiozawa,[2,5]
Ayako Sugawara,[2] Ryo Oiwa,[4] Asako Nobukiyo,[1] Hideyuki Okano,[5] Norikazu Tamaoki,[2]
Tatsuji Nomura,[2] Eiso Hiyama,[1] and Erika Sasaki [2,3]

Abstract

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

Introduction

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

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

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

[1]Natural Science Center for Basic Research and Development, Hiroshima University, Hiroshima, Hiroshima, Japan.
[2]Central Institute for Experimental Animals, Kawasaki, Kanagawa, Japan.
[3]Center of Integrated Medical Research, Keio University, Shinjuku-ku, Tokyo, Japan.
[4]JAC Inc., Meguro-ku, Tokyo, Japan.
[5]Department of Physiology, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan.

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

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

Materials and methods

Animals

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

Preparation of matured oocytes

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

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

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

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