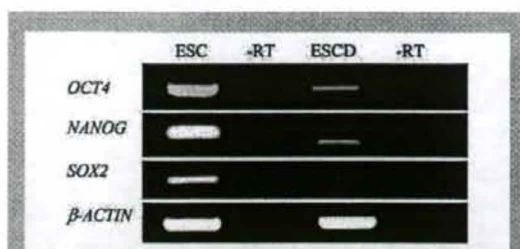

Figure 4 Comparison of the mRNA expression the pluripotency transcription factors *OCT4*, *NANOG* and *SOX2* in undifferentiated cjes001 ESC versus differentiated ESCs (ESCD).

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*,

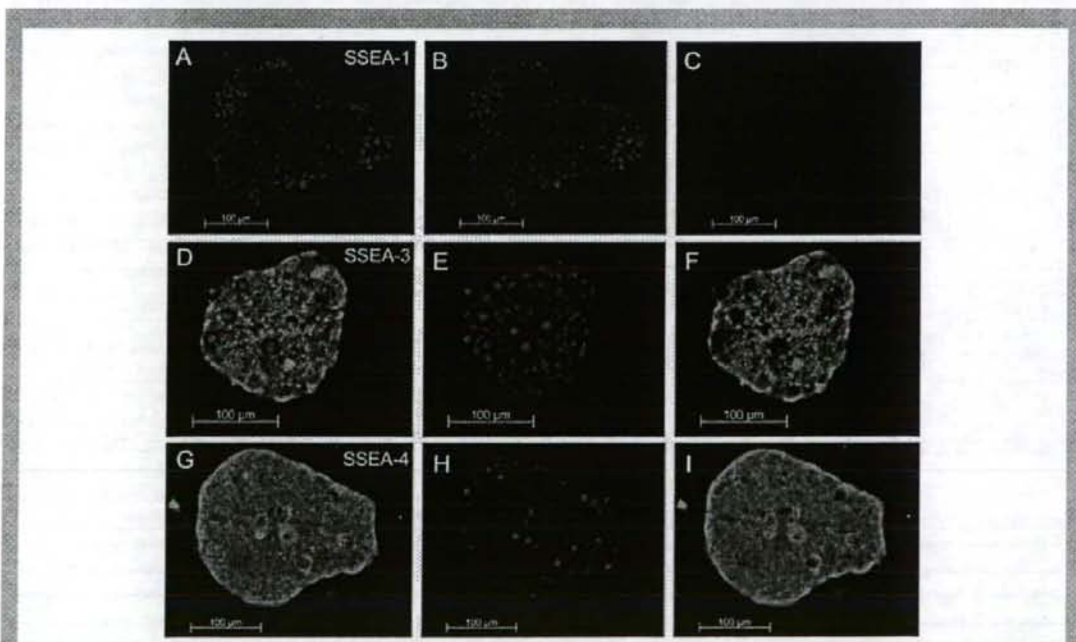

Figure 5 Stage-specific embryonic antigens.

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.

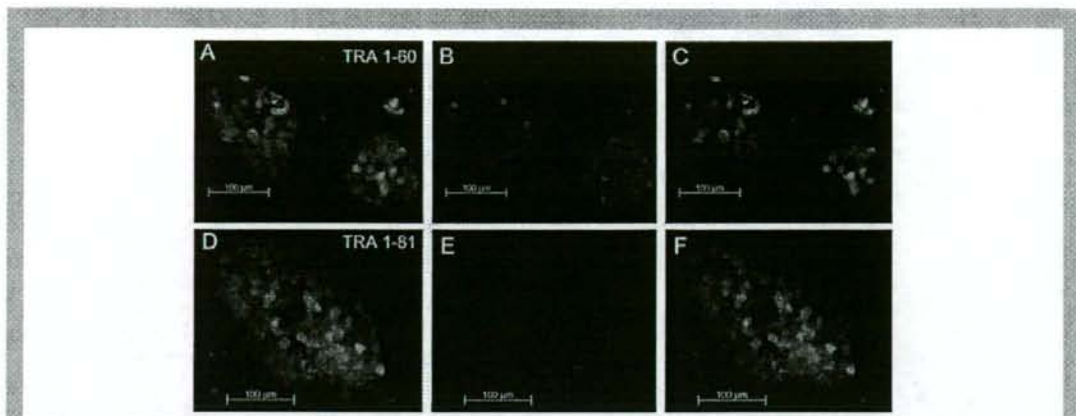

Figure 6 Positive staining for TRA-1-60 (**A–C**) and TRA-1-81 (**D–F**).
B and E show counterstaining by PI; C, F and I shows Alexa 488 staining. A and D the merged pictures.

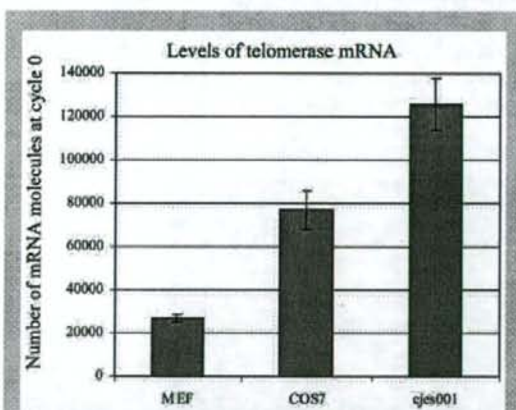

Figure 7 Telomerase activity quantified by real-time PCR.
To determine a normalized arbitrary value for the mRNA, every data point was normalized to the reference of an artificial molecule 'TSR9' from the kit. Data (mean ± SEM) are from triplicates. MEF, mouse feeder cells; COS7, immortalized green monkey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

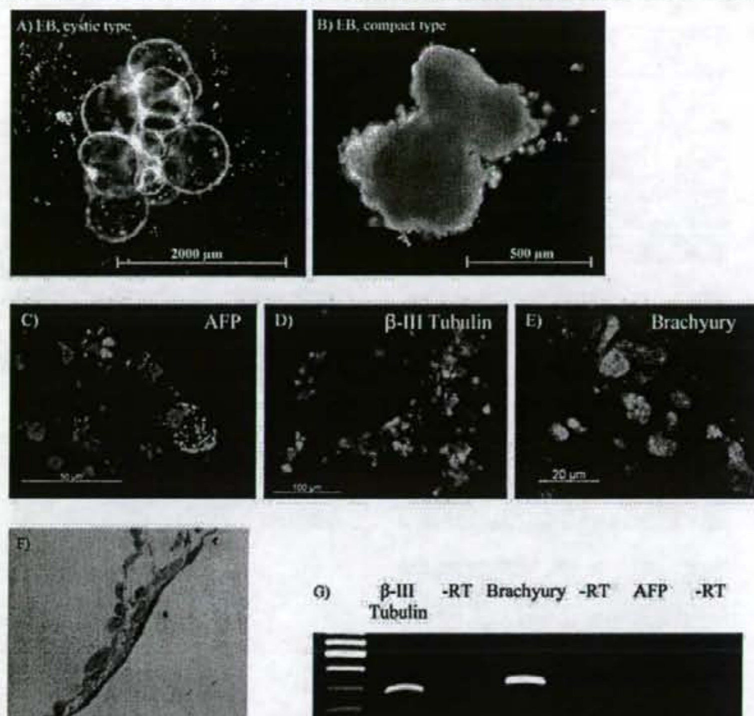

Figure 8 cjes001 cells can form EB with a (**A**) cystic or (**B**) compact (solid) phenotype.

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

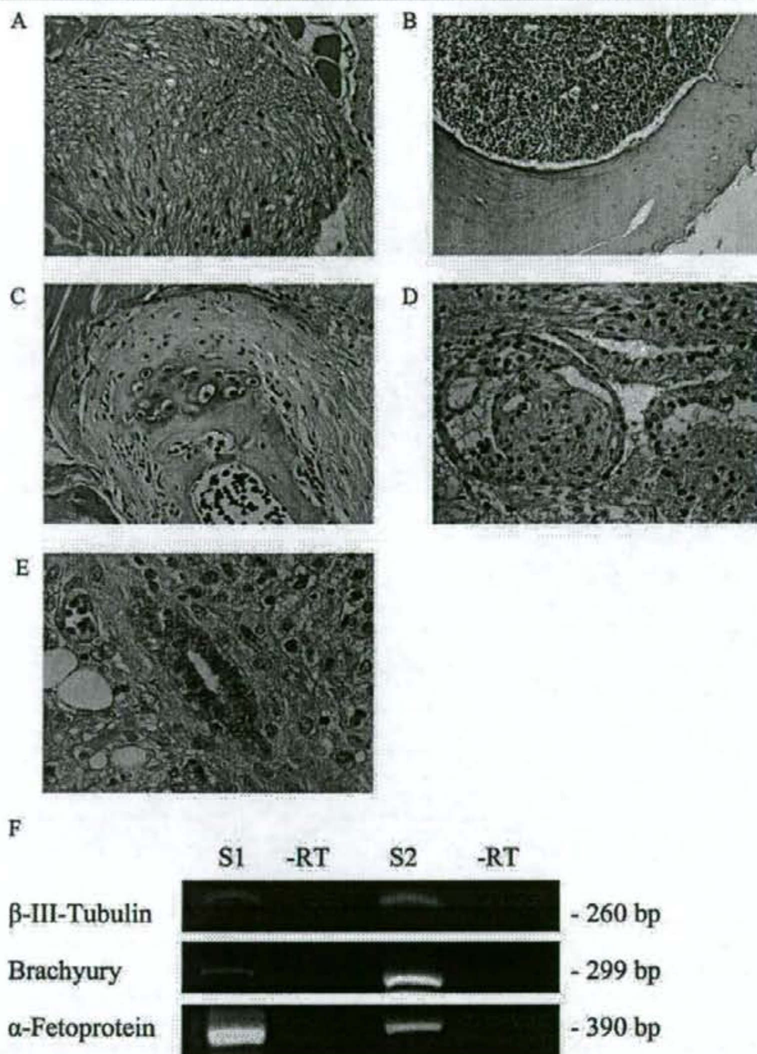

Figure 9 cjes001 cells form tumors containing derivatives of all three embryonic germ layers. (**A**) Neural structures within the tumor represent ectoderm. (**B**) Osteogenic and hematopoietic differentiation show mesoderm formation. (**C**) Chondrocytes and osteogenesis with adnate muscles tissue also 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

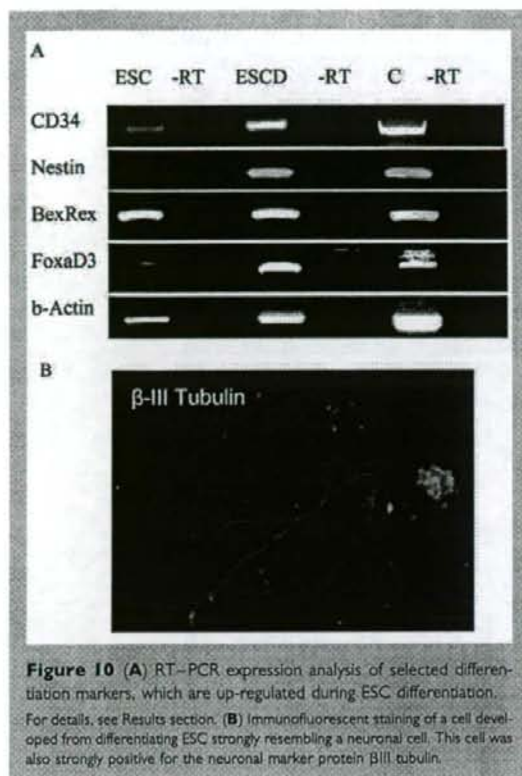

Figure 10 (**A**) RT–PCR expression analysis of selected differentiation markers, which are up-regulated during ESC differentiation. For details, see Results section. (**B**) Immunofluorescent staining of a cell developed from differentiating ESC strongly resembling a neuronal cell. This cell was also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

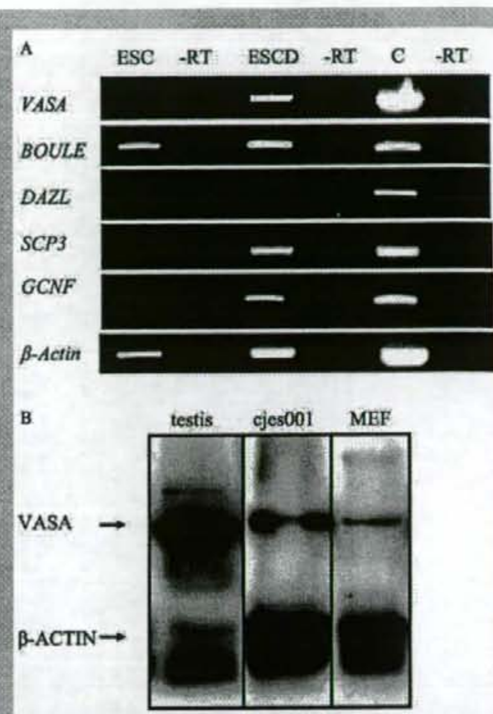

Figure 11 Analysis of germ cell-specific gene expression by RT–PCR (**A**) and western blot (**B**).
(A) The germ cell markers *VASA* and synaptonemal complex protein 3 (*SCP3*) are not detectable in undifferentiated ESC (ESC). In contrast, in differential ESC (ESCD), these germ cell markers are expressed 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

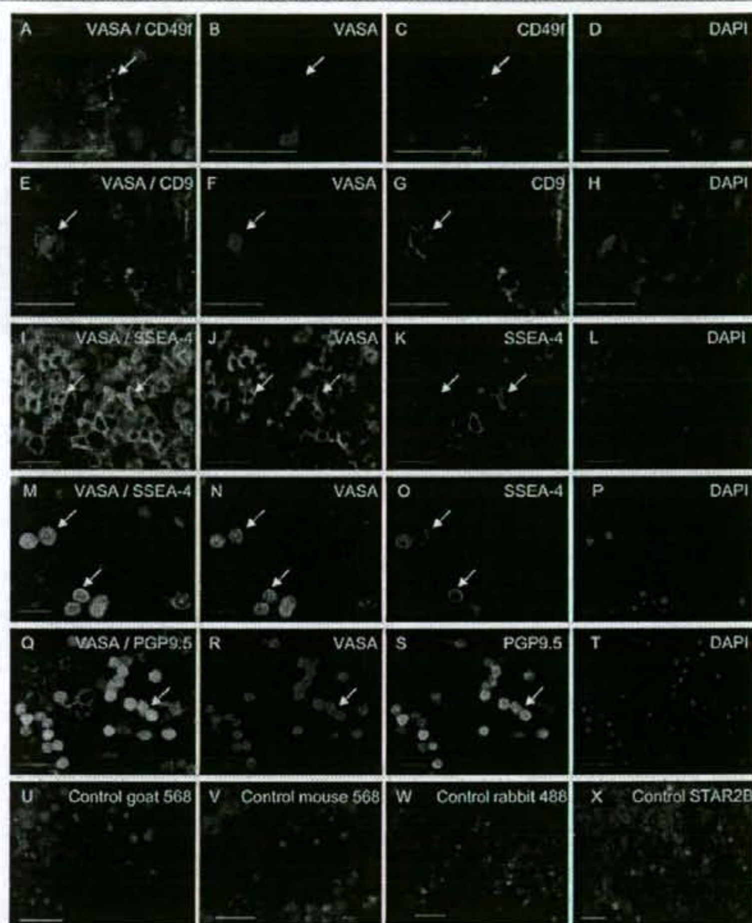

Figure 12 Co-localization of VASA together with different established germ cell markers in cells developing from differentiating ESC.

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

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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