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Misleading and reliable markers to differentiate between primate testis-derived multipotent stromal cells and spermatogonia in culture

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BACKGROUND: Several studies have reported the generation of spermatogonia-derived pluripotent stem cells from human testes. The initial aim of the present study was the derivation of equivalent stem cells from an established and experimentally accessible non-human primate model, the common marmoset monkey (*Callithrix jacchus*). However, an essential prerequisite in the absence of transgenic reporters in primates and man is the availability of validated endogenous markers for the identification of specific cell types *in vitro*.

METHODS AND RESULTS: We cultured marmoset testicular cells in a similar way to that described for human testis-derived pluripotent cells and set out to characterize these cultures under different conditions and in differentiation assays applying established marker panels. Importantly, the cells emerged as testicular multipotent stromal cells (TMSCs) instead of (pluripotent) germ cell-derived cells. TMSCs expressed many markers such as GFR-α, GPR125, THY-1 (CD90), ITGA6, SSEA4 and TRA-1-81, which were considered as spermatogonia specific and were previously used for the enrichment or characterization of spermatogonia. Proliferation of TMSCs was highly dependent on basic fibroblast growth factor, a growth factor routinely present in germ cell culture media. As reliable markers for the distinction between spermatogonia and TMSCs, we established VASA, in combination with the spermatogonia-expressed factors, MAGEA4, PLZF and SALL4.

CONCLUSIONS: Marmoset monkey TMSCs and spermatogonia exhibit an overlap of markers, which may cause erroneous interpretations of experiments with testis-derived stem cells *in vitro*. We provide a marker panel for the unequivocal identification of spermatogonia providing a better basis for future studies on primate, including human, testis-derived stem cells.

Key words: testis / germ line stem cell / spermatogonia / multipotent stromal cell / non-human primate

Introduction

Pluripotent stem cells are of great interest for regenerative medicine as they are able to generate all cell types of the adult body. The classic source of pluripotent cells is the inner cell mass of blastocysts. For many years now researchers have tried to derive pluripotent cells also from the adult body. Besides the generation of genetically modified induced pluripotent stem cells (Takahashi *et al.*, 2007), the derivation of pluripotent stem cells from unipotent adult human spermatogonia seems to be very promising. For the mouse, this has been demonstrated convincingly (Guan *et al.*, 2006; Seandel *et al.*, 2007; Kanatsu-Shinohara *et al.*, 2008; Ko *et al.*, 2009, 2010a). In recent years, several research groups have also published data suggesting the derivation of pluripotent (Conrad *et al.*, 2008; Golestaneh *et al.*, 2009; Kossack *et al.*, 2009) or multipotent stem cells (Mizrak *et al.*, 2010) from adult human spermatogonia. However, these cells, recently termed htES-like cells (Chikhovskaya *et al.*, 2012), for human testis-derived embryonic stem cell-like cells, are under debate since their developmental potential as well as their origin from spermatogonial stem cells (SSCs) remains unclear (Ko *et al.*, 2010b, 2011; Tapia *et al.*, 2011; Chikhovskaya *et al.*, 2012).

Another adult stem cell type, the multipotent stromal cells (MSCs), is very prevalent in mammals. MSCs are located within the connective tissue of many adult organs and also in the human testis (Gonzalez *et al.*, 2009). Both, MSCs and pluripotent stem cells are known for their great capacity to self-renew. While pluripotent stem cells can differentiate into all cell types of an organism, MSCs display differentiation restricted to cell types of the connective/myoid tissue such as

osteoblasts, adipocytes, chondrocytes and myocytes (Phinney and Prockop, 2007). In contrast to pluripotent cells, MSCs do not express the transcription factors octamer-binding transcription factor 4 (OCT4, also POU5F1) or sex-determining region Y-box 2 (SOX2) and they are not able to generate teratomas when injected into immuno-deficient nude mice. In cell culture, MSCs minimally have to meet the following criteria: (i) adherence to plastic, (ii) the expression of specific cell surface markers, including CD90 (THY-1), CD105 and CD166 and (iii) potential to differentiate into osteoblasts, adipocytes and chondroblasts (Dominici *et al.*, 2006).

While MSCs are multipotent and located within connective tissue, SSCs are a potential source for pluripotent cells. When mouse SSCs are injected into a germ cell-depleted recipient mouse testis, they re-colonize the testis and re-establish spermatogenesis (Brinster and Zimmermann, 1994). This transplantation assay is the gold standard for demonstrating the presence of SSCs. However, when human SSCs are injected into mouse testis they re-colonize the stem cell niche but no SSC proliferation and thus no spermatogenesis occurs (Nagano *et al.*, 2002; Lim *et al.*, 2010; Izadyar *et al.*, 2011). Yet, during both cell culture experiments and heterologous transplantation assays, researchers rely on a set of markers that is endogenously expressed by the primate or human cells of interest. Published markers for spermatogonia include the cell surface markers G protein-coupled receptor 125 (GPR125), GDNF family receptor alpha-1 (GFR-α), THY-1 (CD90), integrin alpha 6 (ITGA6 or CD49f), stage-specific embryonic antigen 4 (SSEA-4) and tumor rejection antigen 1-81 (TRA-1-81) as well as the intracellular markers Sal-like 4 (SALL4), promyelocytic leukemia zinc finger (PLZF) and melanoma-associated antigen 4 (MAGEA4) (Conrad *et al.*, 2008; Golestaneh *et al.*, 2009; Sadri-Ardekani *et al.*, 2009; He *et al.*, 2010; Izadyar *et al.*, 2011).

The histological and spatial organization of the male germinal epithelium of the non-human primate *Callithrix jacchus* (common marmoset monkey) is very similar to the human. Therefore, the common marmoset is a suitable and attractive model for the study of aspects of human germ cell development and spermatogenesis (Millar *et al.*, 2000; Mitchell *et al.*, 2008; Mueller *et al.*, 2008; Albert *et al.*, 2010; Eildermann *et al.*, in press). Thus, we use this new-world primate species to investigate primate-specific aspects of testicular function and organization. However, studies aiming at the isolation and characterization of testicular stem cells rely on the specificity of endogenous markers. The recent literature suggests THY-1 (CD90) and ITGA6 (CD49f) as specific markers for spermatogonia (Lee *et al.*, 2006; He *et al.*, 2010), but, importantly, they are also expressed by MSCs (Dominici *et al.*, 2006; Semon *et al.*, 2010). This fact in combination with the discovery that MSCs can also be derived from adult human testis (Gonzalez *et al.*, 2009; Chikhovskaya *et al.*, 2012) suggests that at least 2-cell types within the human testis express these markers. Hence, the question arises of which proteins are really unequivocal markers for different testicular cell types. In the present study, we demonstrate the culture and characterization of marmoset monkey testicular multipotent stromal cells (TMSCs) and spermatogonia and investigate the expression of frequently used spermatogonia markers in both cell types. We show that VASA, in combination with PLZF or SALL4 or MAGEA4, respectively, allows specific labeling of cultured spermatogonia. In contrast, other 'spermatogonial markers' such as SSEA-4, TRA1-81, GPR125 or GFR-α were also expressed by TMSCs and thus may cause serious misinterpretations of testis cell culture data.

Materials and Methods

Marmoset monkey testis tissue samples

All experiments were performed according to the German Animal Protection Law. Animals were housed according to the standard German Primate Centre practice for common marmoset monkeys. Thirteen adult marmoset monkey (older than 2 years) testes were used for the experiments (Licence numbers AZ10/0063 and AZ11/0395). All data on TMSCs were obtained from three independent primary cultures derived from three individual animals.

Cell culture

Derivation and culture of TMSCs

Testes were kept on ice in Dulbecco's modified Eagle's medium (DMEM)/F-12 immediately after recovery and further processed in a sterile environment. After removing capsule and epididymis, the tubules were digested using DMEM/F-12 containing 1 mg/ml hyaluronidase (Sigma), 1 mg/ml collagenase (Sigma) and 15 U/ml DNase (Roche) for 35–40 min at 37°C under constant mild rotation. Resulting single cells were seeded at a cell density of 2×10^5 cells/cm^2 to plastic culture dishes. TMSC-culture medium was based on a 1:1 mixture of DMEM (GIBCO) and F-12 nutrient mixture (GIBCO) which was supplemented with 10% fetal bovine serum (GIBCO), 100 U/l Penicillin–Streptomycin (GIBCO), 0.25 μg/ml Fungizone® Antimycotic (GIBCO) and 5 ng/ml recombinant human basic fibroblast growth factor (bFGF) (ProSpecTany). Following overnight incubation any cells that did not adhere to the plastic (suspension P0) were re-seeded to a new dish and cultured for an additional 4 days. Adherent cells from the initial cell suspension (termed 'adherent 1 passage 0') and from overnight suspension cells that adhered after an additional 6 days (termed 'adherent 2 passage 0') were passaged upon confluency with 0.05% trypsin in phosphate-buffered saline (PBS) (GIBCO). Adherent cells from the first passage onwards were termed TMSCs. Passaging was performed every 3–5 days at a 1:3 or 1:5 dilution. Generally TMSCs were cultured at 37°C under 5% CO_2 and the medium was exchanged every 2–3 days. A scheme of the different enrichment steps is provided in Supplementary data, Fig. S1.

To analyze the effect of bFGF on proliferation and cell morphology, TMSCs were cultured in medium containing from 0 to 50 ng/ml bFGF. Cells were seeded in 6-well culture plates at a cell density of 1×10^4 cells/cm^2. After TMSCs had been allowed to adhere overnight without bFGF, the growth factor concentration was changed to 0, 1, 5, 10 or 50 ng/ml, respectively. Each condition was analyzed in three independent biological replicates. Cell morphology was documented using a ZEISS cell observer microscope. After 7 days of culture, all samples were trypsinized and counted using a hemocytometer.

Differentiation of TMSCs

The differentiation capacity of TMSCs was assayed using the human mesenchymal stem cell differentiation kit from StemPro® (Invitrogen Life Technologies, Darmstadt, Germany). The differentiation into chondrocytes and osteoblasts was analyzed after 21 days while adipocyte differentiation was performed for 7 days.

Derivation and short-term culture of spermatogonia

Spermatogonia were enriched by differential plating and cultured for 3 days on γ-irradiated TMSCs at 34°C. The spermatogonia culture medium (Supplementary data, Table SII) was a slightly modified version of the

StemPro(R)-34 serum-free medium (Life Technologies, Darmstadt, Germany) based medium that has previously been described to support the growth of mouse spermatogonia (Ko et al., 2009). Two days prior to the isolation of spermatogonia, 5×10^6 3000-rad irradiated TMSCs were seeded into 48-well culture plates in TMSC-medium without bFGF. Following overnight incubation at 37°C, the medium was replaced with spermatogonia medium and cultured at 34°C. For the derivation of spermatogonia, a testicular single cell suspension was seeded in spermatogonia medium onto plastic dishes (2×10^5 cells/cm^2). After 4 h (or alternatively overnight) of differential plating at 34°C, the non-adhering cells were removed and transferred onto the irradiated TMSCs. Spermatogonia were allowed to attach to the TMSC feeder cell layer and cultured under these conditions for 3 and up to 14 days at 34°C.

Characterization of cells

Immunofluorescence staining

Cells were grown in 48-well plastic plates and fixed for 30 min in 4% paraformaldehyde (PFA). For the detection of intracellular antigens, the cells were permeabilized with 0.04% Triton X-100. Primary antibodies (Supplementary data, Table SII) were diluted in PBS/5% bovine serum albumin (BSA) and incubated for 30 min at 37°C. The appropriate Alexa fluor (AF) 488-linked secondary antibody (Supplementary data, Table SIII) was diluted in PBS/5% BSA and applied for 30 min at room temperature in the dark. For double staining, both primary antibodies and subsequently both secondary antibodies, linked to AF488 and AF594, respectively, were applied at the same time. Controls were performed by omitting the primary antibody and by using the corresponding non-specific IgG fraction. Counter staining was performed with 4′,6-diamidin-2-phenylindol and images were taken on a Axio Observer Z1 fluorescence microscope from Zeiss (Germany).

Fluorescence-activated cell sorting

The staining procedure for fluorescence-activated cell sorting (FACS) analysis of cell surface antigens was performed according to the manufacturer's recommendations (Human Multipotent Mesenchymal Stromal Cell Marker Antibody Panel, R&D Systems). Each staining was performed three times using cells from three different animals. FACS analysis was performed with a BD LSR II device and the FACS DIVA software. Data were evaluated with the flowjo software.

Immunohistochemistry

Immunohistochemistry (IHC) on Bouin's solution fixed, paraffin-embedded common marmoset testes tissue sections was performed as previously described (Eildermann et al., in press) using the LSAB Plus-Kit from DakoCytomation (CA, USA). All pictures were taken using a Zeiss microscope and the Nuance™ multispectral camera.

Von Kossa silver staining: detection of osteoblasts

Calcium deposits indicating osteogenesis were detected by von Kossa silver staining, which was performed as described (Romeis, 2010), with slight modifications. In brief, cultured cells were fixed in 4% PFA, washed three times and then incubated for 1 h in 5% aqueous silver nitrate solution in the dark. After rinsing the cells in distilled water, they were incubated in formaldehyde–sodium carbonate solution [5 g sodium carbonate, 25 ml formaldehyde (35–40%), 75 ml distilled water] for 2 min. Following an additional washing step, the staining was fixed in 5% sodium thiosulfate solution for 5 min. Nuclei were stained with 0.1% nuclear fast red solution for 5 min. Sites of calcification appear dark brown and nuclei appear red.

Oil Red O staining: detection of adipocytes

Fatty acid incorporations can be detected with the Oil Red O staining procedure. The cells were fixed with 4% PFA. After rinsing with water, the cells were incubated with 0.3% Oil red solution (0.3% Oil red in 60% isopropyl alcohol) for 1 h. Then, the cells were rinsed once with 60% isopropyl alcohol and several times with distilled water. Fatty acids appear bright pink.

Alcian blue staining: detection of chondrocytes

Chondrocytes are characterized by the production of proteoglycans, which can be detected with alcian blue. The staining was performed as described in the manual of the StemPro Chondrogenesis Differentiation Kit (StemPro #A10582) with slight modifications. In brief, cell accumulations were fixed in 4% PFA, embedded in Paraffin and sectioned at 5 μm. Following deparaffinization and rehydration, sections were incubated in 1% alcian blue (in 0.1 M HCL) for 1 h and washed three times with 0.1 M HCL. After rinsing with water and 2 min of incubation in isopropyl alcohol, slides were transferred to xylol and covered in Eukitt® (Sigma). Proteoglycans appear blue.

Reverse transcription-polymerase chain reaction

Specific marmoset mRNA sequences were derived from http://www.ensembl.org/Callithrix_jacchus/Info/Index and confirmed using the marmoset genomic database available at the UCSC homepage (http://genome.ucsc.edu/cgi-bin/hgGateway?db=calJac1). Sequence-specific primers (if possible intron-spanning) were designed using the CLC DNA workbench and the Primer3 software (Supplementary data, Table SIV). RNA was isolated with the RNeasy mini kit from Qiagen (Hilden, Germany, #74106) and reverse transcription was performed using 1 μg of the total RNA and the Omniscript reverse transcriptase from Qiagen (Hilden, Germany). For negative control reverse transcription (RT) samples, no reverse transcriptase was added to the reaction. All polymerase chain reactions (PCRs) were performed on cDNA and the according negative control RT sample using Chrimson Taq polymerase (New England Biolabs). ACTB was analyzed with 25 PCR cycles; all other genes were tested using 32 cycles. PCR products were separated and visualized using the QIAxcel multicapillary electrophoresis device (Qiagen, Hilden, Germany). The optical output of this analyzer is a digitalized graph based on the detected fluorescence in the microcapillaries over time instead of conventional agarose gels. The method of presentation used in this study can be interpreted with regard to signal intensity in the same way as that used for conventional images of electrophoresed DNA within agarose gels. The identity of all PCR products has been confirmed by sequencing (LCG-genomics, Berlin, Germany).

Quantitative RT–PCR

Relative quantitative (q)RT–PCR was performed on common marmoset testicular cells and testis material with at least three samples. Design and testing of all qRT–PCR primers (Supplementary data, Table SV) as well as the qRT–PCR procedure was performed as previously described (Eildermann et al., in press). In brief, 2 μg testicular RNA were reverse transcribed, using random hexamers and Superscript II (Invitrogen, Karlsruhe) to obtain cDNA. For each 20 μl PCR reaction, 2 μl of 1:2 diluted cDNA was used with Power SYBR Green Mastermix (Applied Biosystems), and different primer concentrations ranging between 50 and 900 nM. The extent of fluorescence of the Power SYBR green dye was detected and analyzed using the $2^{-(\Delta\Delta C(t))}$ method and the ABI Prism® 7000 SDS software (Applied Biosystems). Each sample was assayed in triplicate und normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

Results

Multipotent cells from testis emerge as stromal cells

We initially set out to isolate and culture marmoset spermatogonia. However, our culture mainly contained a cell type morphologically resembling mesenchymal cells. Therefore, we tested these Marmoset testis-derived cells (hereafter called TMSCs) according to the minimum criteria for adult MSCs (Dominici *et al*., 2006): (i) adherence to plastic, (ii) expression of specific cell surface antigens, (iii) differentiation into osteogenic-, adipogenic- and chondrogenic cells. The first criterion was fulfilled by deriving TMSCs through their adherence to plastic. To test the second criterion, i.e. marker expression, we performed FACS and immunofluorescence (IF) using a human MSC-marker antibody panel (R&D Systems, Minneapolis). Conventional IF revealed the presence of the MSC-positive markers CD105, CD90, CD166 and CD44 in the majority of TMSCs, while only a sub-fraction of cells expressed, yet very intensely, STRO-1 (Fig. 1A). The MSC-negative markers CD19 and CD45 did not display signals in IF. FACS revealed that CD105 and CD90 were expressed in over 95% of the cells (Fig. 1B). In contrast to these robust markers, the percentage of CD44-positive cells varied from 64 to 92% and STRO-1 was present in only 8–26% of the cells (Fig. 1B). The MSC-absent markers CD19 and CD45 were detected in 4–54% of the cells by FACS (Fig. 1B). Additionally, RT–PCR indicated the expression of the MSC markers, VCAM1 and CD166, in TMSCs (Fig. 1C, illustrated as QIAcube software gel picture). To test the third criterion, i.e. differentiation capacity, we applied the protocols of the human MSC differentiation kits from StemPro® (Fig. 1D). The differentiation into adipocytes was shown by Oil red O staining. When applying the osteoblast differentiation protocol, the cells generated long, multi-layered and bulging network-like structures. Von Kossa silver staining indicated calcification providing evidence for the differentiation into the osteogenic lineage. The ability to differentiate into the chondrogenic lineage was verified by Alcian blue staining of cell aggregates that had formed after transfer of the cells to the respective medium. Moreover, upon confluency TMSCs formed clusters morphologically slightly resembling primate ES cells colonies (Fig. 1E) and continued cultivation without passaging led to the formation of large contracting myotubes (Fig. 1F). Thus the third criterion, i.e. multipotency of the stromal cells, was met.

bFGF affects proliferation and morphology of TMSCs

The bFGF concentration strongly influenced proliferation and TMSC morphology (Fig. 2). Absence of bFGF led to a fibroblast-like appearance and very low proliferation rates. This morphology was maintained at 1 ng/ml bFGF, while proliferation strongly increased. When cultured with 5 and 10 ng/ml bFGF, TMSCs formed a cohesive monolayer and the cells became more spindle-shaped. The proliferation rates increased up to a bFGF concentration of 10 ng/ml. At 50 ng/ml proliferation declined and large spaces separated individual TMSCs. All cells appeared vital and no signs of apoptosis were detected.

TMSCs originate in testicular connective tissue

Mammalian testis function relies on the presence of characteristic cell types, including germ cells, Sertoli cells, Leydig cells and peritubular cells. To define the cellular origin and identity of marmoset TMSCs, we screened them for markers that are specifically expressed in different testicular cell types (Fig. 3). Vimentin, *in situ* expressed in Sertoli-, peritubular-, endothelial- and interstitial cells (Fig. 3A), was detected at different intensities in TMSCs by IF (Fig. 3B) and qRT–PCR (Fig. 3C). Both techniques also demonstrated the presence of alpha smooth muscle actin (ACTA2), a marker for peritubular, some interstitial and vascular cells, but not for Sertoli- or Leydig cells. The presence of Vimentin and ACTA2 in TMSCs has also been confirmed by western blot (data not shown). Androgen receptor (AR), a marker for Sertoli-, Leydig- and peritubular cells (Fig. 3A) was not detected in TMSCs, neither using IF (Fig. 3B) nor RT–PCR (Fig. 3D). qRT–PCR revealed the absence of the Sertoli cell markers, *SOX9*, follicle-stimulating hormone receptor (*FSHR*) and the Leydig cell marker, luteinizing hormone receptor (*LHR*) and conventional RT–PCR demonstrated the absence of the Leydig cell marker, insulin like 3 (*INSL3*). Importantly, a germ cell identity of TMSCs was excluded through the lack of VASA expression at the protein and mRNA levels (see below).

TMSCs express the cell surface markers SSEA-4, TRA-1-81, GFR-α, GPR125, THY-1 (CD90) and ITGA6

We tested the expression of SSEA-4, TRA-1-81, GFR-α, GPR125, THY-1 (CD90), ITGA6 (CD49f) and the cytoplasmic protein PGP9.5 on the protein and/or the mRNA level in the adult testis and TMSCs. SSEA-4, TRA-1-81, GPR125 and PGP9.5 labeled spermatogonia in testis sections (Fig. 4A). While the GPR125 and the SSEA-4 antibody additionally detected another intracellular structure in later germ cell stages, there was apparently no other cell type stained using the TRA-1-81 antibody. IHC for PGP9.5 localized the protein to spermatogonia and to a few interstitial cells (Fig. 4A, inset). Importantly, IF demonstrated the presence of SSEA4, TRA-1-81, GPR125, PGP9.5 and GFR-α also in TMSCs (Fig. 4B). qRT–PCR confirmed the expression of GFR-α and GPR125 (Fig. 4C) and conventional RT–PCR confirmed the expression of GFR-α, ITGA6, PGP9.5 and THY-1 (CD90) in TMSCs (Fig. 4D).

PLZF, SALL4 and MAGEA4 are expressed by spermatogonia but not by TMSCs

VASA protein is present within all germ cells except elongating spermatids (Fig. 3A). SALL4 and PLZF specifically labeled the nuclei of a sub-fraction of spermatogonia, namely spermatogonial stem and progenitor cells (Phillips *et al*., 2010; Eildermann *et al*., in press), while the MAGEA4 antibody labeled all spermatogonial cells (Fig. 5A) and also early spermatocytes with lower intensities. This demonstrates the *in situ* specificity of the proteins for germ cells and spermatogonia (and in case of MAGEA4 also for spermatocytes), respectively. IF on TMSCs with the same antibodies resulted in no staining for VASA (Fig. 3) and very weak, probably non-specific signals for PLZF and SALL4. VASA, SALL4 and PLZF were also undetectable in TMSCs by western blot, while these proteins were detected in the positive

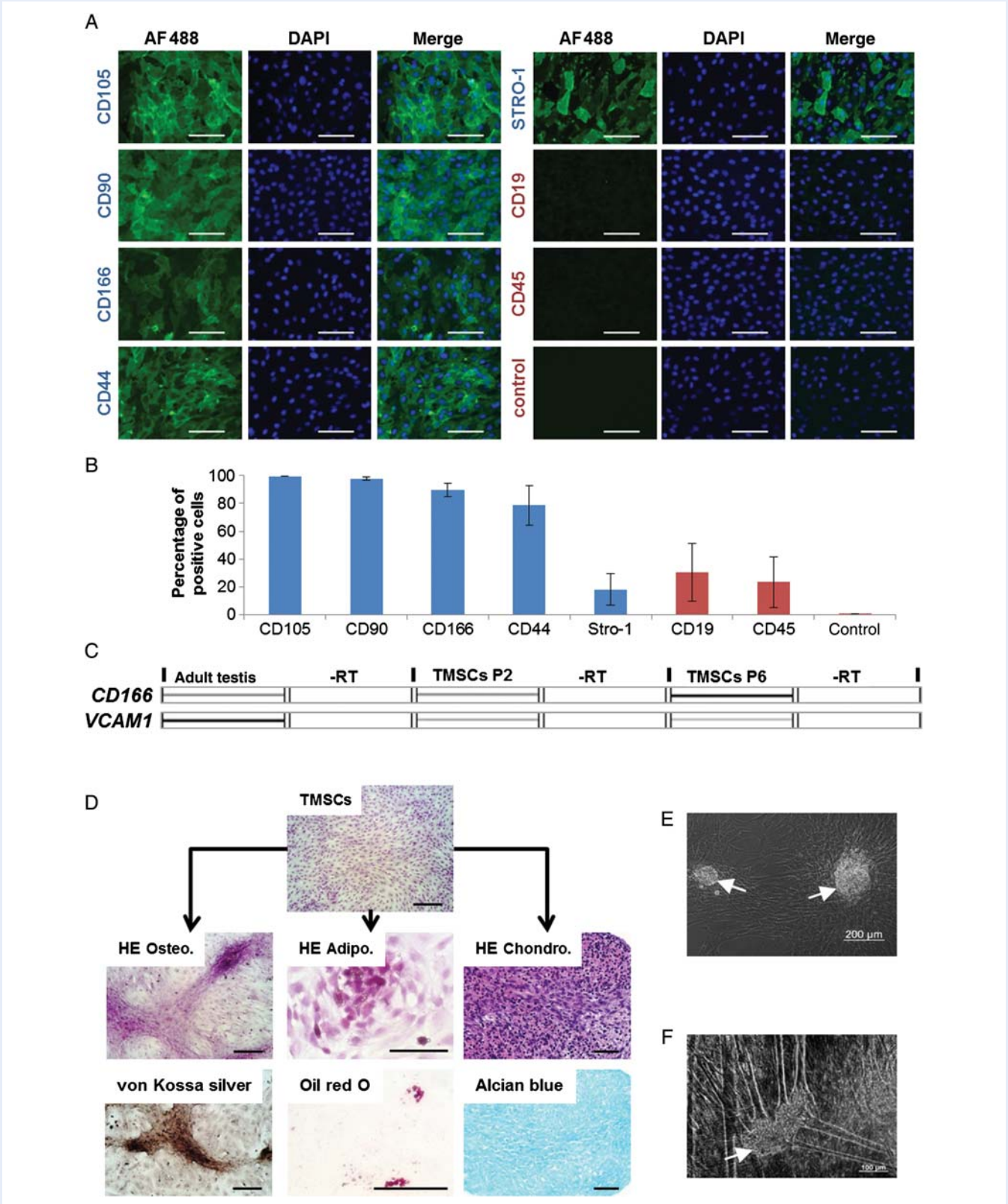

Figure 1 Characterization of TMSCs. (**A**) Immunofluorescence analysis of the cell surface markers of MSCs. Scale bar: 100 µm. (**B**) FACS analysis of MSC markers, $n = 3$. (**C**) RT–PCR on testis and two TMSC samples for MSC markers CD166 and VCAM1. (**D**) Hematoxylin–eosin staining of differentiated TMSCs. Von Kossa silver staining demonstrates osteoblasts, oil red-o staining demonstrates adipocytes and alcian blue staining demonstrates chondrocytes. Scale bar: 250 µm. (**E**) Morphology of a TMSC colony (arrow). (**F**) Contractile myoid structures within differentiated TMSCs.

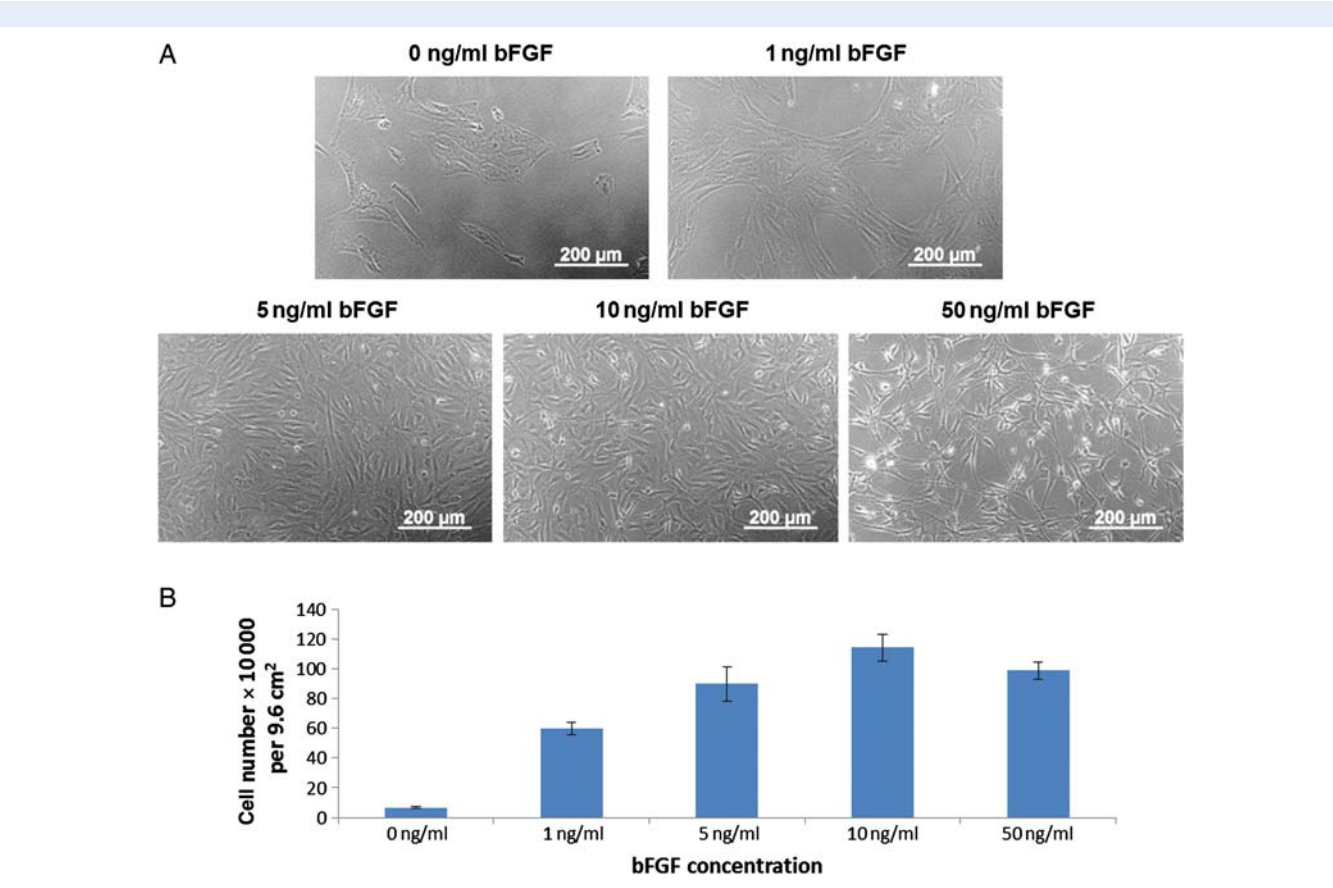

Figure 2 The effect of bFGF on TMSC morphology and proliferation. (**A**) TMSCs cultured with 0, 1, 5, 10 and 50 ng/ml bFGF, respectively. (**B**) The effect of bFGF on cell proliferation. Cell numbers determined after ~7 days of culture. Seeding density was 1×10^4 cells/cm^2. Mean value and standard variation of $n = 3$ different TMSC preparations.

controls, i.e. testis or embryonic stem cell (ESCs) (Mueller *et al.*, 2009), respectively (data not shown). IF staining of TMSCs with MAGEA4 was completely blank (Fig. 5B). However, it is important to note that the PLZF, SALL4 and MAGEA4 antibodies robustly stained cultured cells in other experimental settings (see below). These protein data were verified at the mRNA level by qRT–PCR (Fig. 5C) and by conventional RT–PCR. As TMSCs express neither the germ cell marker VASA nor the spermatogonial markers PLZF, SALL4, nor MAGEA4, which is expressed in spermatogonia and early spermatocytes, these genes represent a marker panel which, in combination, is useful for the identification of spermatogonial stem and progenitor cells in culture.

Detection of spermatogonia and somatic cell types within adherent and suspension fractions

We used the markers *SALL4*, *PLZF*, *MAGEA4* and *VASA* to screen different plastic-adhering and non-adhering cell factions for the presence of spermatogonia (Fig. 6A; see also Supplementary data, Fig. S1). All markers were present in both suspension cell fractions, indicating the enrichment of germ cells in the suspension fraction. While SALL4 and MAGEA4 were never detected in the adhering cells, weak signals for PLZF and VASA suggested the presence of some germ cells including spermatogonia also in the adhering cell fraction. However, cells from the second adherence fraction completely lacked germ cell markers. We screened the same fractions for the presence of somatic cell types and found that AR, labeling Sertoli-, Leydig- and peritubular but not vascular cells, was present mainly in adherent cells. Insl3, expressed by Leydig cells, was detected in P0 adherent cells and the suspension cell fractions, indicating that Leydig cells have only a limited affinity to plastic.

VASA in combination with spermatogonial markers clearly distinguishes spermatogonia from TMSCs

The presence of *VASA* and *PLZF* within the first adherent cell fraction (Fig. 6A) led to the idea that TMSCs might support spermatogonia. To test this, suspension cells (exhibiting spermatogonia markers) were seeded onto irradiated, i.e. proliferation-arrested TMSCs. Using the germ cell-specific marker VASA, we detected single and paired germ cells that were attached to the feeder (Fig. 6B). The VASA-positive cells were morphologically indistinguishable from the feeder cells (Fig. 6B). To analyze whether those VASA-positive germ cells were spermatogonia, we performed double staining using several spermatogonia-expressed proteins. As demonstrated above, GFR-α, GPR125, PGP9.5, SSEA4 and TRA-1-81 were expressed also by the feeder layer, while PLZF, SALL4 and MAGEA4 were not. Importantly,

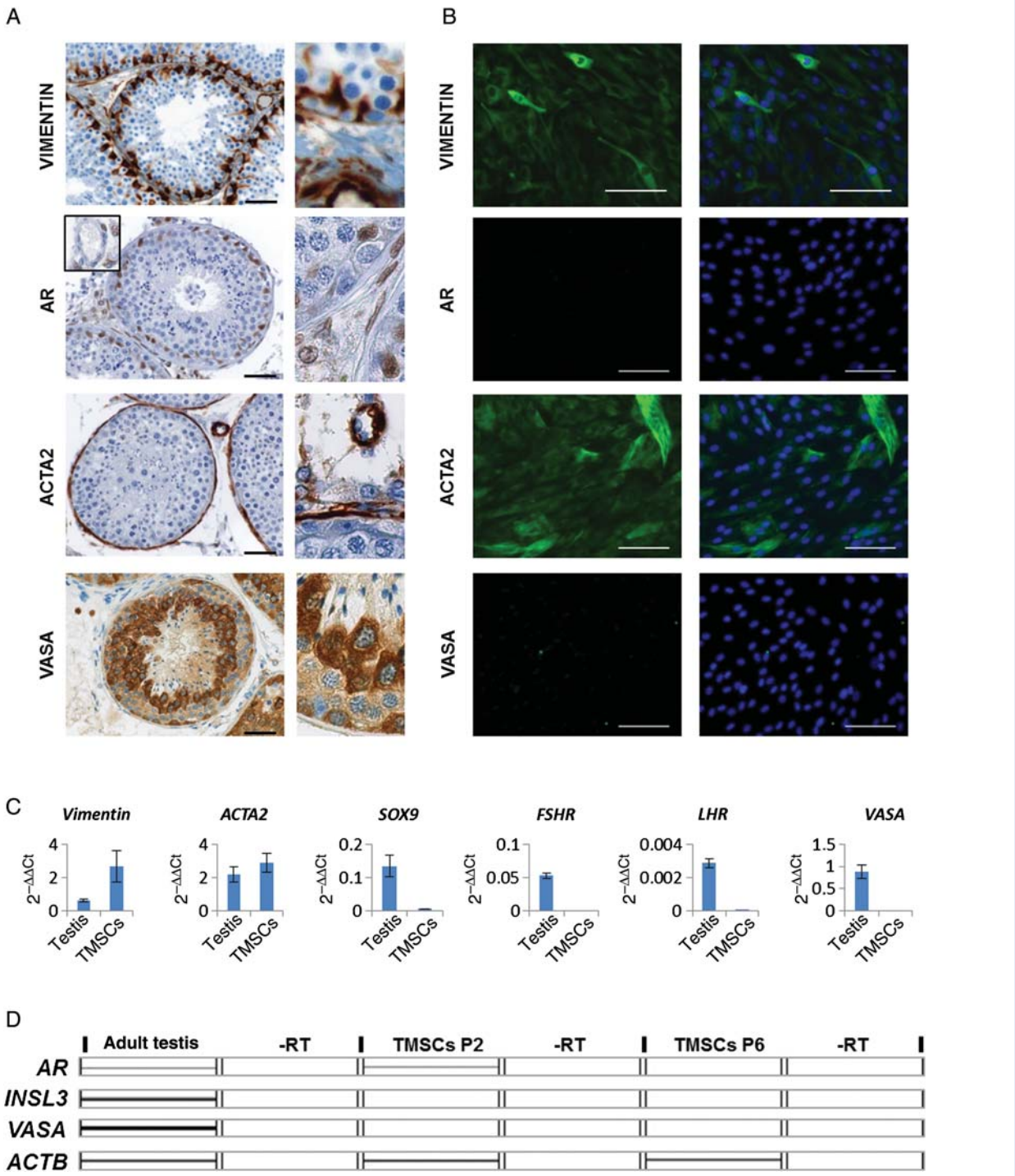

Figure 3 Analysis of the cellular origin of TMSCs. (**A**) Immunohistochemical detection of Vimentin, AR, ACTA2 and VASA in marmoset testis. Scale bar: 50 μm (**B**) Immunofluorescence detection of Vimentin, ACTA2, AR and VASA in TMSCs. (**C**) qRT–PCR for Vimentin, *ACTA2*, *SOX9*, *FSHR*, *LHR* and *VASA* using marmoset adult testis and TMSC cDNA. Mean value of $n = 2$ samples. (**D**) RT–PCR detecting *AR*, *INSL3* and *VASA* in marmoset testis and TMSCs.

the expression of all markers in spermatogonia was robust and the distribution within their respective cellular compartments was steady (Fig. 7). This was in contrast to the distribution of the respective molecules within TMSCs, where they appeared rather heterogeneous and spotty (compare spermatogonia and TMSCs in Fig. 7D–H). All SALL4-positive cells and all MAGEA4-positive cells were also robustly VASA positive, clearly indicating a germ cell identity of SALL4-stained and MAGEA4-stained cells (Fig. 7B and C). The strongest signals for PLZF were seen in VASA^{low} cells (Fig. 7A, orange arrow). Only a subfraction of robustly VASA-positive cells displayed clear PLZF

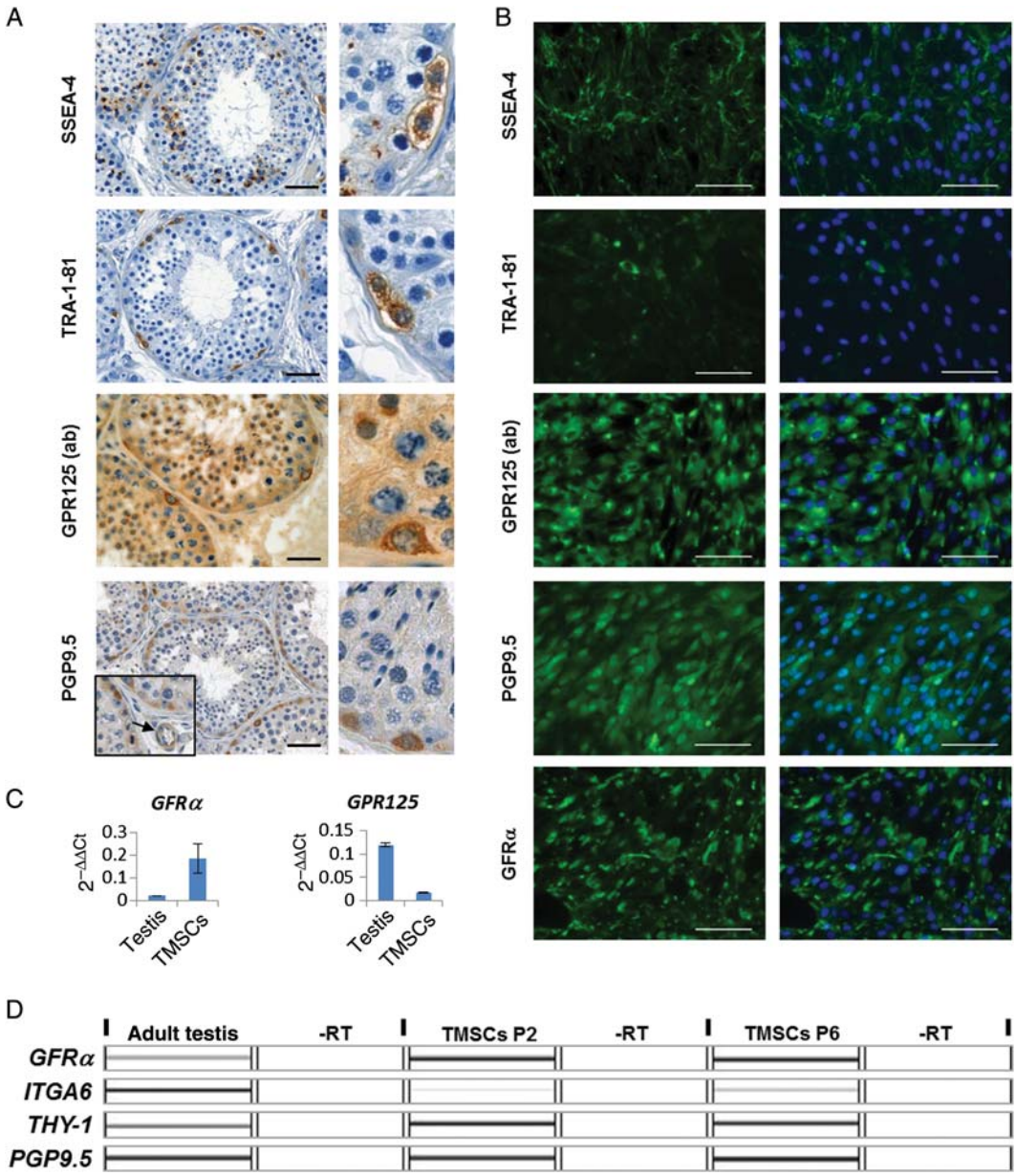

Figure 4 Several spermatogonial markers are also expressed by TMSCs. (**A**) Immunohistochemical detection of SSEA-4, TRA-1-81, GPR125 and PGP9.5 in marmoset testis. Scale bar: 50 μm (**B**) Immunofluorescence detection of SSEA-4, TRA-1-81, GPR125, PGP9.5 and GFR-α in TMSCs. (**C**) qRT–PCR for *GPR125* and *GFR-α* expression in marmoset testis and TMSCs, $n = 2$ samples. (**D**) RT–PCR analysis of *GFR-Aα*, *ITGA6*, *THY-1* and *PGP9.5* in adult testis and TMSCs. The internal control (*ACTB*) for all samples is shown in Fig. 3.

expression (Fig. 7A, white arrows). Spermatogonia can be maintained on irradiated TMSCs for at least 14 days (Fig. 7I).

Discussion

Testicular multipotent stromal cells

To culture non-human primate SSCs and eventually derive pluripotent cells, we set out using culture systems that were published previously for human testis-derived pluripotent stem cells (e.g. Conrad *et al.*, 2008). Interestingly, we propagated fibroblast-like cells that upon confluency formed colonies (Fig. 1E) resembling those shown previously (Conrad *et al.*, 2008; Golestaneh *et al.*, 2009; Kossack *et al.*, 2009) and further resembling pluripotent embryonic stem cell colonies. We identified these cells as MSCs (Dominici *et al.*, 2006). MSCs have already been derived from the human testis and were termed mesenchymal stem cells (Chikhovskaya *et al.*, 2012) or gonadal stem cells (GSCs; Gonzalez *et al.*, 2009). However, as the mesenchyme is an embryonic tissue but the cultured cells were derived from adult tissue, the term mesenchymal stem cell seems inappropriate. The abbreviation GSCs has previously also been used for germline stem cells and would be confusing. Therefore, we propose the term TMSCs.

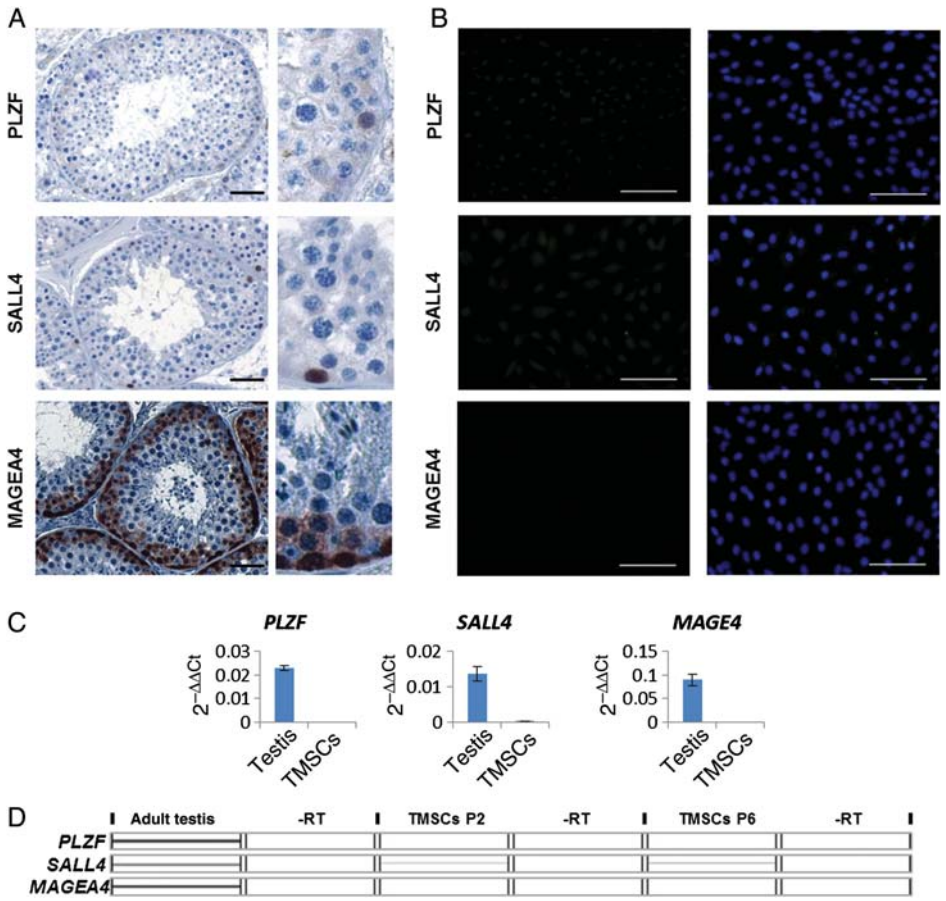

Figure 5 Spermatogonia-specific markers not expressed by TMSCs. (**A**) Immunohistochemical detection of PLZF, SALL4 and MAGEA4 in marmoset testis. Scale bar: 50 μm (**B**) Immunofluorescence detection of PLZF, SALL4 and MAGEA4 in TMSCs. (**C**) qRT–PCR analysis of *SALL4*, *MAGEA4* and *PLZF* in marmoset testis and TMSCs. Mean value of $n = 2$ samples. (**D**) RT–PCR analysis of *SALL4*, *PLZF* and *MAGE4* in marmoset testis and TMSCs.

Similar to the equivalent human cells (Gonzalez *et al.*, 2009; Chikhovskaya *et al.*, 2012), the marmoset testicular fibroblast-like cells expressed the MSC-characteristic cell surface antigens CD105, CD166, CD44 and CD90. In contrast to the human cells, where the MSC-negative markers CD45 and CD19 showed no or only low labeling, marmoset TMSCs showed quantitatively moderate expression in FACS analysis (Fig. 1B) while IF demonstrated only weak signals. These inconsistencies in marker expression might be explained by the presence of other not yet fully characterized cell types within our primary culture or by species differences. Nevertheless, the marmoset TMSCs adhered to plastic and differentiated into adipocytes, chondrocytes and osteoblasts. Additionally, they display a great self-renewing capacity as they could be maintained as a primary culture for 15 passages without loss of proliferation. Altogether, these experiments demonstrated MSC identity.

To analyze whether TMSCs were multipotent or pluripotent, we performed a teratoma formation assay (Phillips *et al.*, 2010). However, three attempts to generate teratomas by subcutaneous injection of TMSCs into immuno-deficient mice failed. We also failed to demonstrate the expression of the pluripotency-related transcription factors OCT4 and SOX2 in TMSCs under different culture conditions including ESC medium. We therefore assume that TMSCs are not pluripotent.

Effect of bFGF on testicular MSCs

There is a strong effect of the bFGF concentration on the proliferation of TMSCs. This is important with regard to the culture of spermatogonia, where the addition of bFGF to the medium may lead to an overgrowth of TMSCs over spermatogonia. Similar observations regarding bFGF were made by Chikhovskaya *et al.* (2012). In that study an expansion of human htES-like cells, likely equivalent to the cells described in this study for the marmoset monkey, was only possible in medium containing bFGF. Importantly, in previous studies bFGF was used at concentrations that effectively promoted TMSC proliferation in our experiments [e.g. 25 ng/ml (Mizrak *et al.*, 2010), 10 ng/ml (Kossack *et al.*, 2009; He *et al.*, 2010; Lim *et al.*, 2010) and 4 ng/ml (Conrad *et al.*, 2008)].

The origin of TMSCs

The testis contains several functionally important organ-specific cell types. These are the germ- and Sertoli cells within the seminiferous

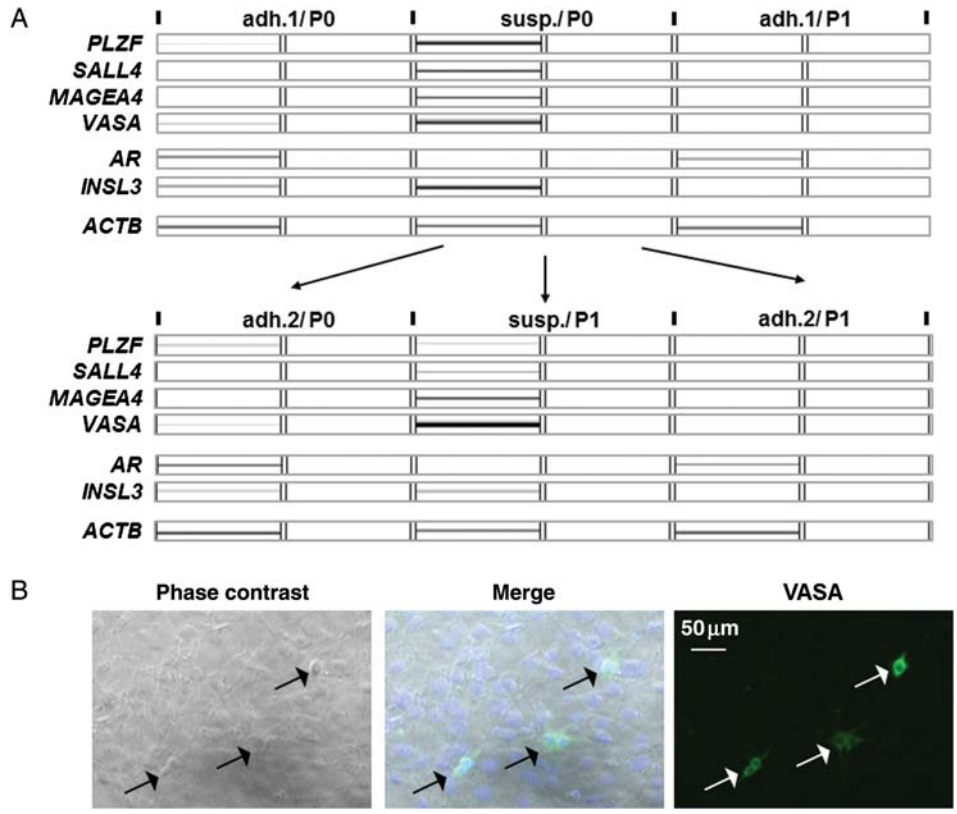

Figure 6 Identification of spermatogonia within heterogenous testicular cell populations. (**A**) RT–PCR for PLZF, SALL4, MAGEA4, VASA, AR, INSL3 and ACTB. For retrieval of the different samples, see Supplementary Fig. S1. (**B**) Immunofluorescence staining for VASA visualized all germ cells that adhered to γ-irradiated TMSCs. Phase contrast images display the morphology of the cells.

tubules, the Leydig cells that are located within the interstitial compartment and the peritubular cells that surround and border the seminiferous tubules. TMSCs lack the expression of the germ cell gene *VASA*, as well as *AR* (expressed in Sertoli-, Leydig and myoid cells, respectively), *FSHR* and *SOX9* (both specific for Sertoli cells) and *LHR* and *INSL3* (specific for Leydig cells). We therefore conclude that TMSCs do not arise from any of these testis-specific cell types. TMSCs express Vimentin and ACTA2, both markers that are expressed not only by peritubular cells but also within the interstitial compartment of the testes and within vascular cells. When we analyzed the expression of the MSC markers CD105, CD44 and STRO-1 on marmoset testis tissue sections, we found CD105 and CD44 exclusively within the interstitial compartment and STRO-1 in the interstitial compartment as well as in some meiotic germ cells (Supplementary data, Fig. S2). Our cells therefore most likely do not represent a testis-specific cell type, but rather derive from the interstitial stroma. Indeed, marmoset adipose tissue-derived MSCs exhibited a similar marker expression pattern (K.E. and R.B., unpublished data).

Markers expressed in spermatogonia and TMSCs

Several markers were published which should allow the identification and the enrichment of spermatogonia (Nagano, 2001; Conrad *et al.*, 2008; Mueller *et al.*, 2008; Maki *et al.*, 2009; Gassei *et al.*, 2010; He *et al.*, 2010; Lim *et al.*, 2010; Reding *et al.*, 2010; Izadyar *et al.*, 2011). We have analyzed marmoset TMSCs for the expression of the cell surface antigens ITGA6, THY-1 (CD90), GFR-α, GPR125, TRA-1-81 and SSEA-4 and found all of them to be expressed. We therefore suggest that none of those markers is suited for the specific isolation of spermatogonia as they are indeed expressed by at least two different testicular cell types, consequently resulting in the enrichment of at least 2-cell populations, TMSCs and spermatogonia. The expression of ITGA6, THY-1 (CD90) and SSEA-4 has been described for MSCs (Dominici *et al.*, 2006; Phinney and Prockop, 2007; Gonzalez *et al.*, 2009; Semon *et al.*, 2010; Chikhovskaya *et al.*, 2012). THY-1 is even considered as an essential characteristic of MSCs (Dominici *et al.*, 2006). However, to our knowledge the present study demonstrates for the first time the expression TRA-1-81, GPR125 and GFR-α by TMSCs. In addition to these cell surface markers, PGP9.5 (UCHL1), a cytoplasmic protein that is widely used to identify spermatogonia (e.g. He *et al.*, 2010), has also only limited use as a spermatogonial marker since it is also present in TMSCs. Using FACS analysis, Chikhovskaya *et al.* demonstrated that human htES-like cells lack TRA-1-81, while IF on the same cells displayed the marker (Mizrak *et al.*, 2010). In our hands, TRA-1-81 appears to be rather cytoplasmic and limited to a small population of cells in IF. This might explain the discrepancy using these two different methods.

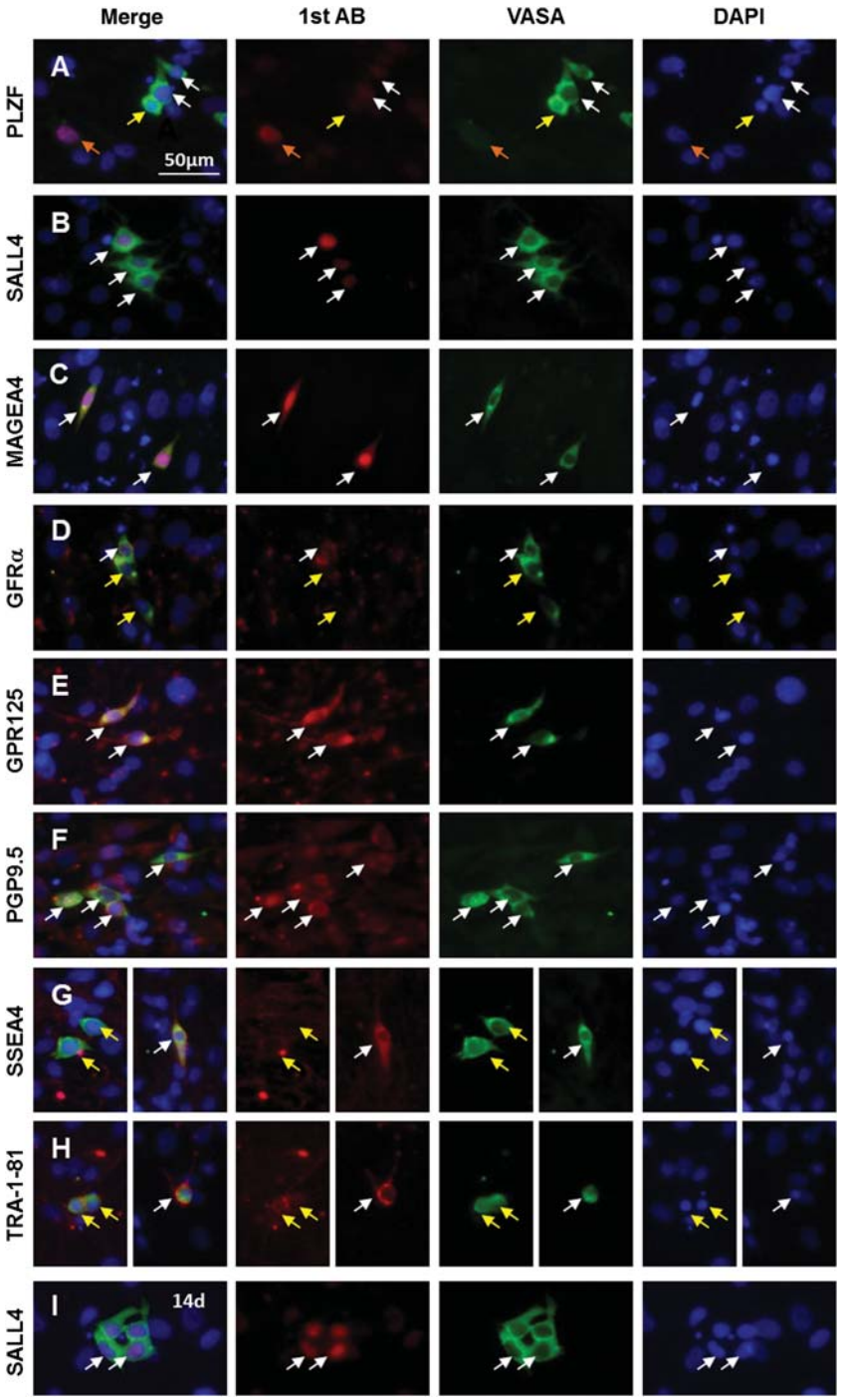

Figure 7 Characterization of marmoset monkey germ cells cultured on irradiated TMSCs. Suspension cells were seeded onto proliferation-inactivated TMSCs and cultured for 3 (**A**–**H**) or 14 (**I**) days. (**A**–**H**) Double staining of VASA and the respective spermatogonia markers. (**I**) Spermatogonia after 14 days of culture expressing SALL4 and VASA. White arrows highlight double-positive cells, yellow arrows cells that were only VASA positive and orange arrows cells that were VASA negative but displayed the second marker.

Characterization of marmoset spermatogonia

The spermatogonia markers SALL4, PLZF, MAGEA4 and the germ cell marker VASA were not expressed by TMSCs. Thus, they represent, when used in specific combinations, good candidates to reliably detect spermatogonia within a mixture of testicular cells. Our experiments indicated that spermatogonia do not adhere to plastic surfaces. However, they do adhere to TMSCs. In order to further characterize spermatogonia on a feeder cell layer consisting of TMSCs, we transferred the plastic non-adherent testicular cell fraction to irradiated

TMSCs. A similar approach has been performed previously with mouse spermatogonia (Kim *et al*., 2008). As TMSCs completely lack the expression of VASA, we have chosen this protein to identify germ cells. To identify spermatogonia within this germ cell population, we performed double staining with spermatogonia markers (Fig. 7). The combination of the respective markers with VASA enabled the identification and characterization of spermatogonia on the TMSC feeder layer. Surprisingly, double staining of VASA and PLZF revealed the strongest expression of PLZF in VASA-negative cells. A possible explanation might be the presence of a PLZF positive, VASA negative or $VASA^{low}$ spermatogonial subfraction (compare Fig. 3A). In summary, our IF-data show that spermatogonia can be detected in cell culture using several markers in combination with VASA. The combination of VASA and SALL4 might be the most appropriate since double staining for VASA and MAGEA4 may also detect early spermatoctyes in addition to spermatogonia, as both latter proteins are also present in some spermatocytes (Fig. 5).

Human testis-derived pluripotent stem cells

In the recent literature, different human testis-derived stem cell types and cultures have been described (e.g. Conrad *et al*., 2008; Golestaneh *et al*., 2009; Kossack *et al*., 2009; Mizrak *et al*., 2010), and there is a considerable degree of confusion regarding the identity of these cells. Besides the challenges that were raised by the Schöler group (Ko *et al*., 2010b, 2011; Tapia *et al*., 2011), novel data published recently by Chikhovskaya *et al.* (2012) further strengthen the view that those human testis-derived cells that were initially considered as pluripotent germ cell-derivatives capable of producing teratoma-like structures (Conrad *et al*., 2008; Golestaneh *et al*., 2009; Kossack *et al*., 2009) are indeed rather MSCs. Those tissues considered as teratomas in the above-mentioned studies may either derive from MSCs (which have a remarkable differentiation capability) or from invaded tissue of the host. Indeed, the origin (host or graft) of the cells in the teratoma (-like structures) has so far not been analyzed. Altogether, data available to date including that presented in this study may suggest the following scenario for the previous reports on pluripotent germ line-derived human cells. The very initial single cell suspension contained all cells of the human testis. Subsequent sorting of this suspension using either ITGA6 (CD49f) (Conrad *et al*., 2008) or GFRalpha (Kossack *et al*., 2009) may have led to the enrichment of spermatogonia and htES-like cells/TMSCs. Assuming that the great marker-overlap between spermatogonia and TMSCs, which we observed in the non-human primate, is also present in humans, the characterization of the sorted population might indeed have led to the correct conclusion of a spermatogonia (but also htES-like cell/TMSC)-enriched cell suspension. However, co-culturing of these 2-cell types in the presence of bFGF may have led to the fast proliferation of htES-like cells/TMSCs. Over time most other testicular cells, including spermatogonia, were diluted out or even completely lost under these conditions. The similarities between TMSCs and spermatogonia with regard to marker expression and the remarkable differentiation capacity of TMSCs may eventually have led to erroneous conclusions.

Importantly, we also experienced severe non-specific staining of some OCT4 and SOX2 antibodies during their characterization (unpublished data). Therefore, we speculate that some immunostainings which were performed during the characterization of testicular cells may also be non-specific. Unfortunately, in most cases there are no specific details provided on the antibodies used in the previous studies thus preventing a direct comparison. However, it is striking that there is a surprising discrepancy in one report (Conrad *et al*., 2008) between the very intense VASA IF signals and the very low level of VASA mRNA. Moreover, their immunohistochemical localization of VASA clearly deviates from the published pattern for human testis (Castrillon *et al*., 2000) and all VASA staining patterns we obtained in human and different monkey testes. Inconsistencies like these make it even more important to analyze primate (and also human) testis-derived cell cultures in more detail to better understand what cell types are present in these cultures and how the cells behave *in vitro* after removal from their original tissue.

In summary, we established an appropriate marker panel to distinguish marmoset TMSCs and spermatogonia. We show that frequently used markers such as SSEA-4, TRA1-81, GPR125 or GFR-α that were considered as spermatogonia-specific, are expressed in both cell types. However, VASA in combination with PLZF or SALL4 or MAGEA4, respectively, allowed specific labeling of cultured marmoset monkey spermatogonia. We conclude that (i) some studies on primate spermatogonia probably have to be re-evaluated to prevent misinterpretation of data and (ii) future studies should employ unequivocal combinations of technically and biologically well-established markers to avoid unjustified conclusions from cell culture experiments with the rare primate (and human) testis tissue.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

K.E.: study design, collection and assembly of data; data analysis and interpretation, manuscript writing, final approval of manuscript. J.G.: collection and assembly of data; data analysis and interpretation, final approval of manuscript. R.B.: study design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

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