POTENTIALLY PLURIPOTENT CELLS IN THE COMMON MARMOSET MONKEY (CALLITHRIX JACCHUS) TESTIS

BY

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ABSTRACT

Pluripotent stem cells are of outstanding interest for regenerative medicine due to their remarkable ability to generate all cell types of the body. Although pluripotent stem cells are classically derived from the inner cell mass of blastocysts, recent findings indicate that also spermatogonia can be a source for such cells. The initial aim of this project was to derive pluripotent stem cells from common marmoset monkey (*Callithrix jacchus*) spermatogonia.

At the outset, the expression of the pluripotency factor SALL4 was investigated in marmoset embryonic stem cells and preimplantation embryos in mammalian germ line stem cells at different developmental stages and in adult mammalian spermatogonia. These experiments established SALL4 as an appropriate marker for pluripotent stem cells and undifferentiated premeiotic germ cells, including a subset of adult spermatogonia. Furthermore, these findings supported the hypothesis that SALL4-positive germ cells are a potential source of pluripotent stem cells. The experimental approach comprises the isolation and culture of spermatogonia including their eventual reprogramming and subsequent culture of spermatogonia-derived pluripotent stem cells. A prerequisite for this protocol is clear identification of the desired cells and their distinction from other cell types. Therefore, a detailed *in situ* analysis of marker expression in the adult primate testis was performed and an appropriate marker panel was established. All attempts to propagate spermatogonia or to derive pluripotent stem cells from testicular cells resulted in the enrichment of a fibroblast-like cell type. Initially these cells were thought to be pluripotent as two antibodies that detect the key pluripotency factor OCT-4 gave positive signals. Further analysis revealed that these signals were false-positive and a third antibody was required to display un-falsified staining. Using a standardized marker-panel and established differentiation assays, these cells were identified as testicular multipotent stromal cells (TMSCs). Importantly, TMSCs displayed the expression of many markers that are considered spermatogonia-specific within the testis, such as SSEA4, TRA-1-81, GFR-α, GPR125, THY-1 (CD90), and ITGA6. However, the germ cell-specific marker VASA and the spermatogonia-expressed factors MAGEA4, PLZF and SALL4 allowed clear distinction between marmoset spermatogonia and TMSCs. Furthermore, a culture-system for marmoset spermatogonia on irradiated TMSCs as a feeder layer was established.

Although the propagation and reprogramming of marmoset spermatogonia to a pluripotent state was not achieved, this study provides for the first time a culture system for marmoset spermatogonia. Furthermore, a severe overlap in marker expression between spermatogonia and TMSCs was found. However, a panel of unequivocal markers for the identification of germ cells in culture was established, which provide a meaningful and reliable basis for future studies on cultured premeiotic (human and non-human primate) germ cells. Altogether, this work provides data which suggest that previous studies in the derivation of pluripotent stem cells from the human testes should be re-evaluated with regard to the suitability of the markers used for isolation and characterization of the cells.

LIST OF PUBLICATIONS

I. K. Eildermann, N. Aeckerle, K. Debowski, M. Godmann, H. Christiansen, M. Heistermann, S. Schweyer, M. Bergmann, S. Kliesch, J. Gromoll, J. Ehmcke, S. Schlatt, and R. Behr. Developmental expression of the pluripotency factor SALL4 in the monkey, human, and mouse testis: restriction to premeiotic germ cells. Accepted for publication in Cells Tissues Organs. DOI: 10.1159/000335031; S. Karger AG, Basel.

II. R. Warthemann*, K. Eildermann*, K. Debowski, J. Gromoll, and R. Behr. False-positive antibody signals for the pluripotency factor OCT4A (POU5F1) may lead to erroneous data and misinterpretations. Submitted to Molecular Human Reproduction. *Indicates equal contribution to this study.

III. K. Eildermann, J. Gromoll, and R. Behr. Misleading and reliable markers to differentiate between primate testis-derived multipotent stromal cells and spermatogonial stem cells in culture. Human Reproduction des091 first published online March 22, 2012. http://humrep.oxfordjournals.org/content/early/2012/03/22/humrep.des091.full.pdf+html

LIST OF ABBREVIATIONS

ACTA2 (ASMA)	Alpha-actin-2 (alpha smooth muscle actin)
AR	androgen receptor
CD	cluster of differentiation
CD90 (THY-1)	cluster of differentiation 90
ESC	embryonic stem cell
FSHR	FSH (follicle-stimulating hormone)- receptor
GFR-α	GDNF (glial cell-derived neurothrophic factor) family receptor alpha
GPR125	G protein-coupled receptor 125
htES-like	human testis-derived embryonic stem cell-like cell
ICM	inner cell mass
INSL3	Insulin-like 3
ITGA6 (CD49f)	Integrin alpha-6
LHR	LH (*luteinizing hormone)- receptor*
MAGEA4	Melanoma-associated antigen 4
MSC	multipotent stromal cells (if not otherwise stated)
NANOG	Tír na nÓg - “land of the ever young”
ND	not determined
OCT4 (POU5F1)	Octamer-binding transcription factor 4)
PGC	primordial germ cells
PGP9.5 (UCHL-1)	protein gene product 9.5 (ubiquitin-C-terminal hydrolase 1)
PLZF (ZBTB16)	Promyelocytic leukaemia zinc finger protein
POU5F1 (OCT4)	POU domain, class 5, transcription Factor 1
SALL4	Sal-like protein 4
SOX2	SRY (sex determining region Y)-box 2
SOX9	Transcription factor SOX-9
SSC	spermatogoinal stem cells
SSEA4	stage specific antigen 4
THY-1 (CD90)	cluster of differentiation 90
TMSC	testicular multipotent stromal cell
TRA-1-81	tumor rejection antigen-1-81
VASA (DDX4)	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4

1 GENERAL INTRODUCTION

The initial aim of this thesis was to derive pluripotent stem cells from common marmoset spermatogonia. This introduction therefore provides information about (1) the key cell types (e.g. pluripotent stem cells, spermatogonia and adult multipotent stromal cells), (2) current knowledge in the field of this study and (3) the common marmoset monkey (*Callithrix jacchus*) as the model organism.

1.1 PLURIPOTENCY

The zygote and its very early descendants are totipotent. Totipotent cells are able to generate a complete organism *and* all extraembryonic tissues, such as the placenta, amnionic sac and chorion. The cells of the inner cell mass (ICM) of blastocysts are pluripotent; they can generate a complete organism, but in contrast to totipotent cells they have lost the ability to generate the extraembryonic cell types. As development proceeds, the developmental potential of individual cells decreases until their fate is determined. Most cells of the adult organism are determined, with the exception of adult multipotent stem cells that enable tissue renewal and regeneration by generating the cell types of their host-tissue. Stem cells are defined by their ability for (theoretically) unlimited self-renewal on the one hand and generation of differentiating progeny on the other. Interestingly, there seems to be a great discrepancy between a cell's differentiation capacity and stem cell characteristics when comparing the *in vivo* and *in vitro* situations. For example, the pluripotent cells of the ICM exist for only a short period of time *in vivo*, whereas these cells *in vitro* (now called embryonic stem cells (ESCs)) gain the ability for unlimited self-renewal without losing their differentiation capacity. The advantage of such cultured pluripotent stem cells is their ability to provide a renewable source of healthy cells and possibly also tissues for the potential treatment of diseases through cell replacement therapy and/or tissue engineering (e.g. for treating heart disease, diabetes, burn victims or Parkinson's disease) (Stem cell Basics, 2009).

As each isolation and creation of a new embryonic stem cell line requires the use and destruction of an embryo, which is at least in humans ethically controversy and illegal in many countries including Germany, scientists search for alternative sources of pluripotent cells. In 2007 the Yamanaka group discovered such a source when they managed to induce pluripotency in adult human fibroblast by introducing defined factors, forcing them to actively reprogram into a pluripotent state (Takahashi *et al.*, 2007). Another promising cell type is the adult spermatogonial stem cell (SSC), which will be discussed below in more detail.

There are several experimental approaches used to determine whether a cell is pluripotent or not. The most conclusive is the chimera assay, whereby labeled test cells are injected into a non-labeled blastocyst. If the labeled test cells are pluripotent, they will contribute to all embryonic germ layers of the resulting organism, resulting in a chimera. This test is not applicable to humans for obvious ethical reasons. However, another functional pluripotency test, the teratoma formation assay, can be performed. In this test the potentially pluripotent stem cells are injected into the subcutis of an immunodeficient mouse. Pluripotent donor stem cells will generate an encapsulated tumor that contains cell types or even organ-like structures of all three embryonic germ layers. Such a tumor is called a teratoma. In addition to these two *in vivo* assays, pluripotency can be tested *in vitro* by directly differentiating the cells into the specialized cell types of the three embryonic germ layers using defined media and protocols (Stem cell Basics, 2010).

Aside from testing differentiation capacity functionally, pluripotency can be demonstrated by the detection of characteristic pluripotency-indicating genes. These are e.g. NANOG, OCT4, SOX2, TRA-1-81, TRA-1-60, SSEA4 (Sasaki *et al.* 2005; Stem cell Basics, 2010) SALL4 (Wu *et al.*, 2006) and LIN28 (Qiu *et al.*, 2010).

1.2 SPERMATOGONIAL STEM CELLS AS A POTENTIAL SOURCE OF PLURIPOTENT CELLS

Spermatogonial stem cells (SSCs) are the stem cells of the germ line. They are unipotent *in vivo* as their differentiating progeny exclusively develops into sperm and they are located at the basal lamina within the seminiferous tubules of the adult testis. As mentioned above, SSCs provide a potential source of pluripotent stem cells from the adult organism. This hypothesis first arose when it was found that primordial germ cells (PGCs), the ancestors of SSCs can generate pluripotent stem cell lines in mice (Resnick *et al.,* 1992) and humans (Shamblott *et al.*, 1998; Turnpenny *et al.*, 2003). Although unipotent *in vivo*, these findings indicated a highly undifferentiated state of PGCs where the loss of surrounding cells resulted in pluripotency. Similar but not identical to PGCs, SSCs retain the expression of several pluripotency related genes indicating that they might also be rather undifferentiated (for review see Zwaka and Thomson, 2005; Rajpert-De Meyts, 2006).

To probe a cell suspension for the presence of SSCs it is first injected into germ cell-depleted mouse-testis. Mouse SSCs will re-colonize the germinal epithelium and re-establish spermatogenesis (Brinster *et al.*, 1994). This transplantation assay is the golden standard to prove the SSC-identity of a cell. However, when human SSCs are injected into mouse testis they re-colonize the stem cell niche but no spermatogonial stem cell proliferation and thus no spermatogenesis occurs (Nagano et al, 2002; Izadyar *et al*, 2011). Due to the unsuitability of the

transplantation assay for the identification of functional human SSCs, researchers rely on the specificity of markers that are endogenously expressed by these cells. 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 (also named 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; Izadyar *et al.*, 2011; He *et al.*, 2010; Sadri-Ardekani *et al.*, 2009).

The generation of pluripotent stem cells from the adult testis has already been shown for the mouse (Guan *et al.*, 2006; Kanatsu-Shinohara *et al.*, 2008; Seandel *et al.*, 2007). The same has been claimed for the human (Conrad *et al.*, 2008; Kossack *et al.*, 2009, Golestaneh *et al.* 2009; Mizrak *et al.*, 2010). Nevertheless, it was only convincingly demonstrated for the mouse that the resulting pluripotent stem cells originated in SSCs (Ko *et al.*, 2009). The pluripotency as well as SSC origin have recently been challenged for the human htES (human testis-derived embryonic stem cell) -like cells (Ko *et al.,* 2010; Tapia *et al.,* 2011; Chikhovskaya *et al.,* 2012). "Gene expression microarray analysis revealed that htES-like cells possess a transcriptome distinct from human ESCs and fibroblasts, but closely resembling the transcriptome of mesenchymal stem cells (MSCs). The similarity to MSCs was confirmed by detection of SSEA4/CD146 expressing cells within htES-like colonies and efficient *in vitro* differentiation toward three mesodermal lineages (adipogenic, osteogenic, chondrogenic)" (Chikhovskaya *et al.*, 2012). Thus, besides undifferentiated germ cells, MSCs also came into focus for the project.

1.3 MULTIPOTENT STROMAL CELLS (MSC)

Firstly, testicular MSCs were termed *mesenchymal* stem cells (MSC) (Chikhovskaya *et al.*, 2012) or gonadal stem cells (GSCs) (Gonzalez *et al.*, 2009) in previous studies. However, as the mesenchyme is an embryonic tissue but the cultured cells are derived from adult tissue, the term mesenchymal stem cell seems inappropriate. In addition, the abbreviation GSC has also previously been used for germline stem cells and could create confusion. Therefore, we proposed the term testicular multipotent stromal cells (TMSCs).

MSCs are located within the connective tissue of many adult organs and as previously described also in the human testis (Gonzalez *et al.*, 2009, Chikhovskaya *et al.*, 2012). In cell culture, MSCs meet the following criteria: (1) adherence to plastic, (2) expression of specific cell surface markers, including CD90 (THY-1), CD105 and CD166 and (3) potential to differentiate into osteoblasts, adipocytes and chondroblasts (Dominici *et al.*, 2006).

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 that is restricted to the cell types of the connective/myoid tissue such as osteoblasts, adipocytes, chondrocytes and myocytes (Dominici *et al.*, 2006; Phinney and Prockop, 2007). Both cell types obviously share several characteristics. But in contrast to pluripotent stem 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 are not able to generate a teratoma when injected into immunodeficient nude mice. Although the markers THY-1 (CD90) and ITGA6 (CD49f) are considered spermatogonia-specific by some researches (He *et al.*, 2010; Lee *et al.*, 2006), they were already reported to also be expressed in MSCs (Dominici *et al.*, 2006; Semon *et al.*, 2010). Therefore, the question arose as to which proteins/epitopes are really unequivocal markers for different testicular cell types.

1.4 THE COMMON MARMOSET MONKEY (*Callithrix jacchus*)

The common marmoset monkey (*Callithrix jacchus*) is a New World monkey native to Brazil. This non-human primate is a very valuable model species due to the advantages of short birth intervals (about 5 month), high reproductive rate (up to 3 neonates per litter), constant menstrual cycle, low body weight (300-450g) and small size (25cm long) and the resulting relatively low maintenance costs. Presently the common marmoset is used in numerous areas of biomedical research (König, 2011).

Figure 1: *Callithrix jacchus* (DPZ archive)

One major aim when working with pluripotent stem cells is to potentially use them in cell replacement therapy and regenerative medicine. Therapy approaches must be tested in appropriate animal models. Such models must either naturally develop a similar disease to that afflicting humans or the symptoms must be induced to provide a useful disease model. Regarding reproductive biology, it is a tremendous advantage that the histological and spatial organization of the marmoset male germinal epithelium resembles that of the human, making the marmoset a good candidate 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). Taken together the common marmoset monkey provides a very advantageous non-human primate model for the project of this PhD-thesis.

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2 AIMS OF THE PROJECT

The initial aims of this PhD thesis were:

1. The establishment of an *in situ* characterization of the different testicular cell types.
2. The establishment of a feasible marker-panel that enables a clear identification of the different testicular cell types in culture.
3. The establishment of a culture system for common marmoset spermatogonia.

The principal objective of this PhD thesis was:

4. The derivation of pluripotent stem cells from common marmoset spermatogonia.

With the current debate on the origin and the differentiation-capacity of the published htES-like cells in mind - where it was stated that htES-like were truly TMSCs - additional aims were:

5. The establishment of a culture-system for common marmoset TMSCs.
6. A detailed characterization of marmoset TMSCs.

3 DEVELOPMENTAL EXPRESSION OF THE PLURIPOTENCY FACTOR SALL4 IN THE MONKEY, HUMAN, AND MOUSE TESTIS: RESTRICTION TO PREMEIOTIC GERM CELLS

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KEY WORDS: Testis - Germ cell - Stem cell - Embryo - Primate

ABSTRACT SALL4 (sal-like protein 4) is a pluripotency transcription factor, that is highly expressed in embryonic stem cells (ES) and that is essential for mouse preimplantational development. In adult mouse organs, *Sall4* mRNA is highly expressed in the testis and ovary, while there is only little or no expression in other organs. There is high expression of SALL4 in human testicular germ cell tumors. However, as there is no detailed analysis of SALL4 expression during mammalian testicular development yet, we analyzed SALL4 expression in ES cells, preimplantation embryos, and the developing and adult testis of a non-human primate (NHP) species, the common marmoset monkey (*Callithrix jacchus*). Immunofluorescence revealed SALL4 in the nuclei of marmoset ES cells and preimplantation embryos. Marmoset SALL4 isoform analysis in ES cells, newborn and adult testis by RT-PCR and Western blotting showed two different isoforms, SALL4-A and SALL4-B. Immunhistochemistry localized this transcription factor to the nuclei of primordial germ cells and most gonocytes in the prenatal and early postnatal marmoset testis. In the pubertal and adult testis SALL4 was present in undifferentiated spermatogonia. In the developing and adult human and mouse testis SALL4 expression mimicked the pattern in the marmoset. Adult testes from additional NHP species, the treeshrew, the cat, and the dog exhibited SALL4 also in undifferentiated spermatogonia, indicating a conserved expression in the mammalian testis. Taking into account the importance of SALL4 for mouse development, we conclude that SALL4 may play an important role during mammalian germ cell development and is involved in the regulation of spermatogonial proliferation in the adult testis.

LIST OF ABBREVIATIONS:

ES	embryonic stem
HRP	horseradish peroxidase
MEF	mouse embryonic fibroblast
NHP	nonhuman primate
ORF	open reading frame
PGC	primordial germ cell
PND	postnatal day
SALL4	Sal-like protein 4

INTRODUCTION

SALL4 (sal-like 4) is a zinc finger transcription factor, which belongs to the spalt protein family (Sweetman and Munsterberg, 2006). The spalt proteins are conserved in diverse phyla of the animal kingdom such as mammals, fish, amphibians and birds, as well as in insects and worms. In mammals, the spalt-like proteins are encoded by a family of 4 genes, spalt-like 1-4 (*SALL1-4*). Evolutionary conservation of proteins suggests their involvement in fundamental biological processes. Indeed, the spalt and SALL proteins, respectively, play elementary roles during *Drosophila* and mouse development (Jurgens, 1988; Elling *et al.*, 2006; Sakaki-Yumoto *et al.*, 2006). Functional impairment of SALL4 in humans causes the Okihiro syndrome (Kohlhase *et al.*, 2002b), which is primarily characterized by forearm malformations in combination with Duane syndrome of eye retraction. Further symptoms of impaired SALL4 activity in humans can be heart and kidney malformations. At least parts of this human syndrome have been modeled using SALL4

heterozygous mice (Koshiba-Takeuchi *et al.*, 2006; Sakaki-Yumoto *et al.*, 2006) indicating haploinsufficiency of *SALL4*. Complete lack of SALL4 in mice leads to peri-implantational death of the embryos (Elling *et al.*, 2006; Sakaki-Yumoto *et al.*, 2006; Tsubooka *et al.*, 2009). These embryos failed to develop the epiblast and the primitive endoderm and no or only very limited numbers of atypical embryonic stem cell-like cell lines could be established from *Sall4*-deficient mouse embryos proving an important role of SALL4 in the pluripotent cells of the embryoblast (also called inner cell mass) (Elling *et al.*, 2006; Tsubooka *et al.*, 2009). Indeed, several studies recently showed that SALL4 belongs to the so-called group of *pluripotency genes*, which are highly expressed in embryonic stem cells and functional studies identified SALL4 as part of the transcriptional network regulating pluripotency (Zhang *et al.*, 2006; Zhou *et al.*, 2007; Yang *et al.*, 2008a; Rao *et al.*, 2011). Notably, SALL4 appears to be at the top of the transcriptional hierarchy since it has been shown to modulate OCT4 expression (Zhang *et al.*, 2006) and to physically interact with NANOG (Rao *et al.*, 2011), which are considered as *the* key factors for pluripotency. Cauffman and colleagues (Cauffman *et al.*, 2009) recently demonstrated that SALL4 is also expressed in the early human embryo in the embryoblast as well as in the trophoblast. Supporting a role of SALL4 in pluripotency, this factor enhanced reprogramming of human and mouse somatic cells to induced pluripotent stem (iPS) cells (Tsubooka *et al.*, 2009). In another experimental setup, where somatic cells were fused with embryonic stem cells to regain pluripotency, SALL4 also enhanced reprogramming (Wong *et al.*, 2008). Interestingly, besides pluripotent stem cells, SALL4 has been implicated in multipotent hematopoietic stem cell and fetal hepatic progenitor cell function (Ma *et al.*, 2006; Yang *et al.*, 2007; Yang *et al.*, 2008b; Oikawa *et al.*, 2009). Altogether, there is strong evidence that SALL4 is essentially involved in the regulation of pluripotent and multipotent stem and progenitor cell development.

Previously, Kohlhase and colleagues (Kohlhase *et al.*, 2002a) have shown by northern blot analysis that *Sall4* is expressed in adult mouse testis and ovary, while it was undetectable in all other organs tested. The expression of *Sall4* in adult mouse gonads was confirmed by RT-PCR (Tsubooka *et al.*, 2009) and strongest signals were obtained – besides in ES cells – in the adult testis and ovary. Together, these data suggest that in the adult mouse *Sall4* appears to be expressed at a substantial level specifically in the gonads. However, a detailed study of SALL4 expression in the developing and adult mammalian and specifically the primate gonad is still missing.

Here we demonstrate SALL4 expression in the non-human primate pre-implantation embryo and embryonic stem cells. We also provide the first comparative immunohistochemical analysis of SALL4 expression in the developing and adult monkey, human, and mouse gonad and investigate *SALL4* mRNA and protein isoform expression in the marmoset monkey. Published data and the data presented in this study suggest an important role of SALL4 in male mammalian germ cell development.

MATERIALS AND METHODS

Human testis tissues

Fetal human gonads and pediatric testis

The human fetal gonadal tissue employed in this study was made available through the Department of Pathology, University of Göttingen, Germany. The use of the tissues was guaranteed by the parental approval for each individual fetus to perform autopsy. The male fetal gonads (n=14) from gestational weeks 18 - 35 (corresponding to second and third trimester) were obtained after spontaneous miscarriages. The gonads were collected within 24h after death and post-mortem examinations were carried out in the Department of Pathology, University of Göttingen, by approved and experienced pathologists. Testes were dissected, fixed in 10% formalin and embedded in paraffin, and processed routinely for histological examination. Cases with obvious conditions potentially interfering with gonadal development such as chromosomal aberrations, malformations or growth retardation were excluded from the study. Gestational ages were calculated clinically in

relation to the mother's last menstrual cycle and correlated to the foot length and the crown-heel length at autopsy. The biopsy from the pediatric testis from the one year old boy exhibited an age-appropriate developmental stage and was obtained or diagnostic purposes. The use of the tissue was granted by parental approval.

Human adult testes samples

All patients had given written informed consent (Az. 2006-588-fs of Ethikkommission the Medical Faculty of the University of Münster) for these investigations to be performed. In 4 patients (5 testes samples) with obstructive azoospermia after vasectomy biopsies were carried out for diagnostic purposes during vasectomy reversal. Testicular tissue was fixed by immersion in Bouin's fixative and embedded in paraffin using standard techniques. For histological evaluation, 5 µm paraffin sections were stained with hematoxylin. Histological evaluation revealed normal spermatogenesis in these testes.

Marmoset monkey material and tissues

Pre-implantation embryo collection

Common marmoset monkey embryo collection was basically carried out as described earlier (Sasaki *et al.*, 2005; Muller *et al.*, 2009). All experiments were performed according to German Animal Protection Law. Animals were housed according to standard German Primate Centre practice for the common marmoset. Briefly, marmoset pre-implantation embryos were recovered from naturally cycling female adult marmosets 5-8 days after putative ovulation (determined by plasma progesterone measurement twice weekly) by uterus-flush. Progesterone was determined by a direct, non-extraction enzyme-immunoassay using an antiserum raised in sheep against progesterone-11α-hemisuccinate-BSA. The assay has been previously described for determining ovulation in the marmoset monkey by Heistermann and colleagues (Heistermann *et al.*, 1993) except that progesterone-3-CMO-peroxidase was used as enzyme conjugate.

Embryonic stem cell culture

Common marmoset monkey embryonic stem cells (line cjes001) were cultured on 3500-rad γ-irradiated mouse embryonic fibroblasts (MEFs) in medium consisting of 80% Knockout DMEM (GIBCO) and 20% Knockout Serum Replacement (GIBCO) supplemented with 2mM GlutaMAX™ (GIBCO), 0.1mM MEM non-essential amino acids (GIBCO), 0.1mM β-mercaptoethanol (GIBCO), 100 IU/ml penicillin and 100 mg/ml streptomycin as described previously by Müller and collegues (Muller *et al.* 2009).

Testis tissue samples

Testes of 25 adult, 13 pubertal (between postnatal weeks 20 and 42), and 10 newborn common marmoset monkeys were analyzed. Five fetal testes (between gestational days 75 and 117, normal duration of gestation 145 days) were obtained after spontaneous miscarriage or after surgical retrieval of the fetuses.

Mouse tissues

Mouse (strain CD1) tissues were retrieved immediately after killing by cervical dislocation (adult animals) or decapitation (postnatal animals). At least two animals per developmental stage were analysed. The tissues were fixed in Bouin's solution and paraffin-embedded according to routine procedures. Pregnancies were timed by checking the vaginal plugs and the collected embryos were staged according to The Atlas of Mouse Development (Kaufmann, 1992). At least two embryos/animals were analyzed per developmental stage.

Detection of proteins

Immunofluorescence staining

Pre-implantation embryos were fixed for 30 minutes in 2% paraformaldehyde (PFA) containing 0.02% Triton X-100. Embryonic stem cells were grown on *x*-irradiated mouse embryonic fibroblasts (MEFs) in foil-bottom 24-well plates (Lumox™, Greiner Bio-One, Stuttgart, Germany) for 2-5 days, fixed for 30 minutes in 2% PFA, 0.02% Triton X-100 and then washed twice in PBS. The staining with primary antibodies was done according to the manufacturer's recommendations. Antibodies were diluted in PBS supplemented with 5% BSA. After 16 hours incubation in first antibody (SALL4, abcam #ab57577) dilution (1:200) at 4°C cells were washed twice in PBS, incubated for another 60 minutes with the Anti-mouse secondary antibody covalently linked to Alexa dye A488 or A568. Images were taken on a Zeiss Axio Observer Z1 microscope. Counterstaining reagent was DAPI.

Immunohistochemistry on sectioned tissues

Tissues were fixed in Bouin's solution immediately after recovery for at least 6 hours. After several washes in 70% EtOH for at least two days the tissues were embedded in paraffin and sectioned at 5 µm. Tissue sections were deparaffinized and rehydrated and an antigen retrieval step was performed by microwaving the sections in 10 mM citrate buffer for 10 min. Endogenous peroxidase was inhibited by an incubation with peroxidase blocking reagent (DakoCytomation Carpinteria, CA, USA, LSAB+ system-HRP, K0679). A mouse monoclonal SALL4

antibody (abcam ab57577) generated against a recombinant fragment of human SALL4 (amino acids 954-1054 of human SALL4) was used at a 1:200 to 1:300 dilution in 5% BSA in TBS. Identical staining results were obtained with another SALL4 antibody (abcam ab29112, rabbit polyclonal). All incubation steps were done in a humid chamber and incubations with the primary antibody were performed over night at 4°C. DakoCytomation Universal LSAB Plus-kit including biotinylated second antibody polymer and horseradish peroxidase (HRP) conjugated streptavidin was employed for detection of bound primary antibody. 3,3'-diaminobenzidine (DAB) chromogen was used as substrate for the HRP and Mayer's hematoxylin as counterstain. Control stainings were carried out omitting the primary antibody and by replacing the specific primary antibody by the respective unspecific control immunoglobulin.

Western blot analysis

Protein from ~50mg tissue or cell culture material from up to one 9cm dish was isolated using the RNeasy mini Kit from Qiagen (Appendix F in the Handbook describes the Protein precipitation from buffer RLT lysates). Protein precipitate was dissolved in 200µL RIPA 2 resuspension buffer (0,15M NaCl, 15 NP-40, 1% LDS, 2% SARKOSYL (N-Lauroylsacosin-natriumsalt)). For westernblot analysis 15-20µL of the protein lysate (including 10xDTT and 4x loading buffer) and 5µL Novex sharp prestained protein standard from Invitrogen was loaded onto an NuPAGE Novex 4-12% Bis-Tris gel to separate proteins. Proteins were then transferred to a Nitrocellulose membrane. The membrane was washed in PBS-T (1xPBS with 0,1% Tween-20) and blocked for 30 minutes in 5% skim milk/0.1% normal goat serum/PBS-T. Primary-Antibody incubation was performed for one hour at room temperature or over night at 4°C. All antibodies (mouse-anti-SALL4 (abcam, ab57577; dilution 1:3000), mouse-anti-beta-Actin (Sigma, A1978; dilution 1:1000)) were diluted in 5% skim milk/PBS-T. After washing in PBS-T membranes were incubated with a secondary HRP conjugated antibody (goat-anti-mouse-HRP from RandD #HAF007). Signal-detection was carried out using the ECL-Kit from Amersham (RPN2209) and an Ecomaxx x-ray Film developer.

Detection and analysis of mRNA

RNA isolation and RT-PCR

Total RNA from ~50mg frozen tissue-material or cell culture material from up to one 9cm dish was isolated using the RNeasy mini Kit from Qiagen. Reverse transcription was performed using the Omniscript reverse transcriptase from Qiagen. PCRs (32 cycles) were performed with resulting cDNA using Taq Hot Start polymerase from Novagene. PCR products were analysed using the QIAxcel System (Qiagen, Hilden, Germany) or standard gel electrophoresis. All PCR products have been sequenced by LCG-genomics (Berlin) to prove the identity of the amplicons, Beta-Actin served as internal standard. Primer sequences and respective product sizes are given in Table 1.

Real time quantitative RT- PCR

Relative qRT-PCR for *SALL4* was performed on testicular RNA from four newborn, four 8-week-old and four adult monkeys. Primers were designed on the basis of the whole marmoset genome which is available in the trace archive (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_SPEC=TraceArchive&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch). The gene sequences were annotated by aligning them to the corresponding human and mouse genes; they displayed a nucleotide homology of about 85 % between marmoset and human/mouse. Intron-spanning primers were designed with Primer Express® to yield RNA-specific and marmoset-specific detection. Primers were tested to yield a distinct, single amplicon by 2% agarose gel electrophoresis. Primer sequences for qRT-PCR were: fw 5`-AAGGCAACTTGAAGGTTCACTACA-3' and re 5`-GATGGCCAGCTTCCTTCCA-3`. Identity of the amplicon was confirmed by DNA sequencing. QPCR for each primer pair has been optimized following the protocol for Power SYBR® Green PCR Master Mix from Applied Biosystems. A subsequent dissociation step was performed to confirm the specificity of each primer pair. Briefly, 2 µg testicular RNA was reverse transcribed, using random hexamers, by Superscript II (Invitrogen, Karlsruhe) to obtain cDNA; 2 µL of 1:2 diluted cDNA was used for each 20 µl PCR reaction with Power SYBR Green Mastermix (Applied Biosystems), and different primer concentrations ranging between 50 to 900nM. The PCR programme consisted of an initial step of denaturation (5 min. at 95°C) followed by 40 cycles of denaturation (15 sec. at 95°C) and annealing and elongation (1 min. at 60°C for both, annealing and elongation). The

extent of fluorescence of the Power SYBR green dye was detected and analysed using the ABI Prism ® 7000 SDS software (Applied Biosystems). Each sample was assayed in triplicate und normalized to glyceraldehyde-3-phosphate dehydrogenase expression. Relative Quantification was based on the 2(-ΔΔ C(T)) method (Livak and Schmittgen, 2001).

Primer name	Primer sequence	Fragment size
SALL4 fw	5'-atgtcgaggcgcaagcaggc-3'	marmoset *SALL4A*: 3160bp marmoset *SALL4B*: 1852bp
SALL4 re	5'-cttagctgaccgcaatcttgttttc-3'	
SALL4 qFw	5`-aaggcascttgaaggttcactaca-3'	*marmoset SALL4* both isoforms: 77bp
SALL4 qRe	5`-gatggccagcttccttcca-3`	
mmSALL4 qFw	5'-gcgggcgggccttca -3'	*mouse SALL4* both isoforms: 63bp
mmSALL4 qRe	5'-tgttggccccatgagtcat-3'	
ACTB fw	5'-cactcttccagccttctttcc-3'	*marmoset ACTB*: 175bp
ACTB re	5'-gtgatctccttctgcatcctg-3'	
GAPDH fw	5'-tgctggcgctgagtatgtg-3'	*marmoset GAPDH*: 64bp
GAPDH re	5'-agccccagccttctccat-3'	

Table 1: Primer sequences and sizes of the amplicons generated by the different primer combinations used in this study.

RESULTS

SALL4 protein is highly abundant in marmoset ES cells and in newborn testis

In order to obtain an overview over the general SALL4 protein expression and abundance in newborn and adult non-human primate testes, we performed western blot analysis (Fig.1A). Protein from four different passages of the recently established pluripotent *Callithrix jaccus* ES cell line Cjes001 (Muller *et al.*, 2009) was included as positive control for this pluripotency factor. All four ES cell protein samples exhibited two major bands corresponding to SALL4-A (160 kDa) and SALL4-B (80 kDa) isoforms, respectively (Rao *et al.*, 2011). A third band of medium intensity exhibited an apparent molecular weight of ~ 140 kDa. A fourth band of minor intensity of about 100 kDA was detected only in ES cells. Three bands corresponding to the 160 (SALL4-A), 140, and 80 (SALL4-B) kDa isoforms were clearly visible in the newborn testis samples. Relative to the *ACTB* signal, which was very weak in newborn testis, the SALL4-A signals in the newborn testis were even stronger than the ones in the ES cells. The 100 kDa band showing up in the ES cell samples was not detectable in the testis. In the adult marmoset monkey testis, SALL4 showed two bands (Isoform A and the 140 kDa band). However, the intensities of the bands were significantly lower than those obtained with newborn gonads. Comparable findings were obtained with mouse postnatal day (PND) 1, PND9 and adult testis. Newborn and PND9 testis exhibited clear SALL4-A and –B signals, while these bands were hardly detectable in the adult testis (data not shown).

SALL4 transcripts are alternatively spliced in marmoset ES cells and testes

Western blot analysis suggested the presence of different *SALL4* isoforms in the testis. Therefore we performed RT-PCR to analyze *SALL4* expression in newborn and adult marmoset testes and ES cells. The *SALL4* gene has four exons (Fig.1B), and all exons contribute to the open reading frame. This gene structure is highly conserved in man, non-human primates, mouse and other mammals (http://www.ensembl.org/index.html). Two SALL4-isoforms were detected in undifferentiated marmoset ES cells (Fig.1C, ESC). Besides the longest ORF (corresponds to isoform A; size of respective PCR product: 3160 bp) there was an alternatively spliced transcript which encodes SALL4 protein isoform B (1852 bp PCR product). The shorter ORF resulted from the use of an alternative splice donor site in the second exon thereby excluding the 1308 nucelotides located at the 3' end of exon 2 (Fig.1B). Performing non-quantitative RT-PCR (32 cycles), both SALL4 transcripts were detected in the newborn and adult marmoset testis (Fig.1C).

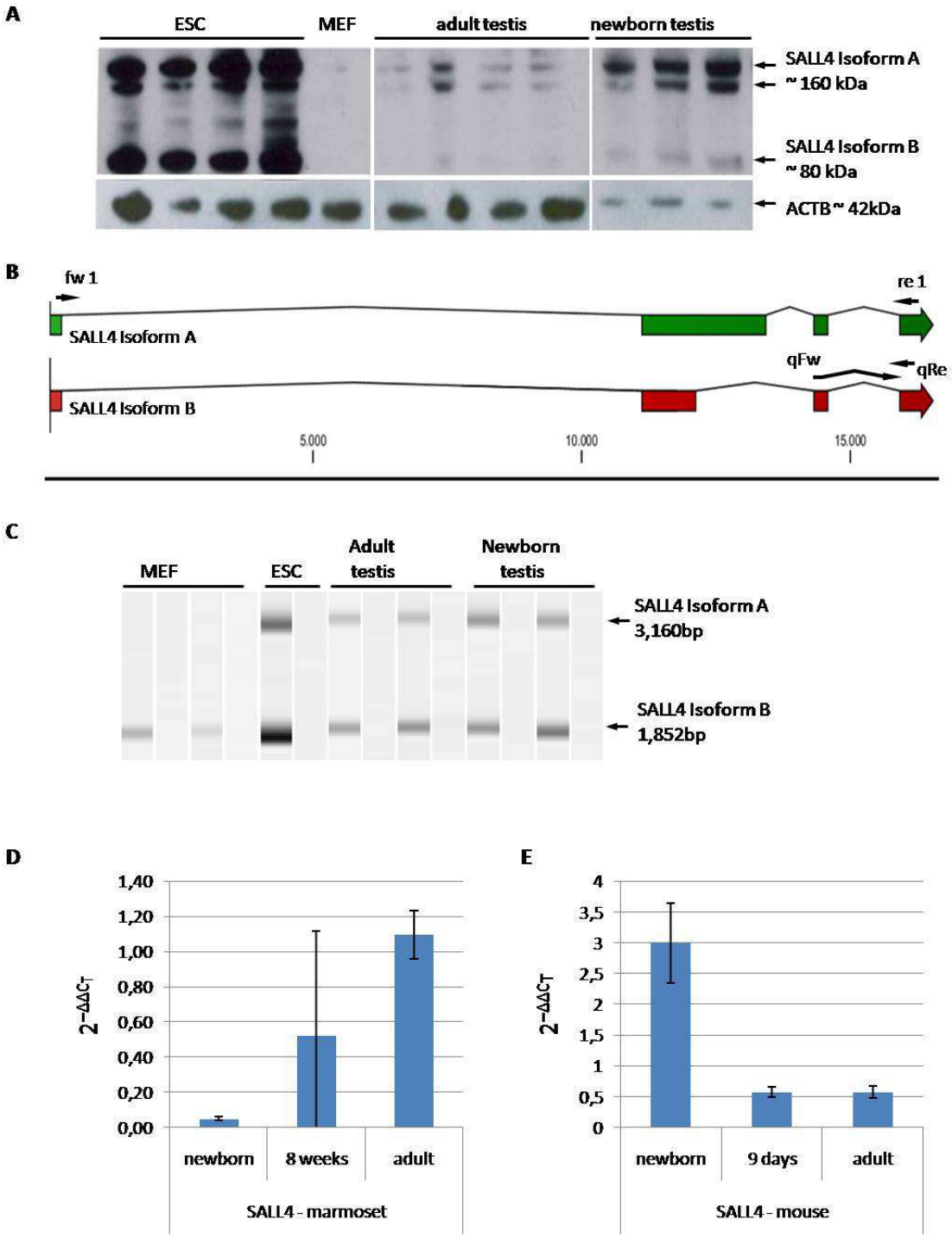


Figure 1: SALL4 protein and transcript expression in neonatal and adult marmoset testes. A) Western blot. In ES cells, four different bands were seen. The strongest bands represented the SALL4 isoforms A and B, respectively. The lower band between isoform A and B was detected in ES cells only. SALL4 was undetectable in mouse embryonic feeder (MEF) cells. Robust SALL4 signals were seen in the newborn testes, while in the adult testis the signals were much weaker. B) Scheme of the exon-intron-structure of *SALL4* transcripts encoding isoforms A and B, respectively. The transcripts differ in the usage of a splice-donor site in exon 2. The primers fw1 and re1 were used to amplify the ORF, primers qFw and qRe were used for qRT-PCR. Primer qFw is intron-spanning to prevent amplification of genomic DNA. The scale provides the size of the gene on the genomic level in base pairs. C) Non-quantitative RT-PCR revealed the presence of the *SALL4*-isoforms A and B in ES cells and in newborn and adult testes. Only isoform B was weakly detectable in MEFs. D) Quantitative real time RT-PCR of marmoset testes. Primers (qFw and qRe, displayed in B). In contrast to the protein, which was low in the adult

gonad, *SALL4* transcripts were up-regulated about twenty-fold in the adult testis compared to the newborn testis. The 8 week-old testes showed an intermediate expression level. E) Quantitative real time RT-PCR of mouse testes displayed a relative decrease of *Sall4* expression during postnatal testis development.

SALL4 is expressed in marmoset monkey preimplantation embryos and in ES cells

In order to demonstrate SALL4 expression on the cellular level in pluripotent cells, we performed immunofluorescence with preimplantation embryos and pluripotent marmoset ES cells. In the marmoset pre-implantation embryo, SALL4 was detected in the nuclei of all cells of the compacting morula stage (Fig.2A). In the blastocyst (Fig.2B), the protein was detected in the nuclei of the embryoblast (inner cell mass) and the trophoblast cells (outer cell layer). Omission of the SALL4 antibody resulted in no staining (Fig.2C). In undifferentiated ES cells, which were derived from the inner cell mass of a blastocyst, SALL4 was detected in the nuclei of almost all cells (Fig.3A). Figure 3B shows the negative control. Summarizing these data, SALL4 expression is pluripotency-associated also in the non-human primate Callithrix jacchus.

SALL4 is expressed in marmoset gonocytes and spermatogonia

In order to analyze SALL4-expression in primate male germ cells from the early fetal stage to adulthood, we performed immunohistochemistry for SALL4 on different developmental stages of the marmoset monkey testis. SALL4 is expressed in late primordial germ cells, gonocytes and (pre-) spermatogonia in the fetal, newborn and pre-pubertal marmoset testis, respectively (Fig.4A-E). During the early phase of testis organogenesis (embryonic day 75; embryonic development is very delayed in the marmoset compared to the human (Li *et al.*, 2005)), there were SALL4-positive germ cells which are included in the forming cords (red dotted line in Fig.4A) as well as germ cells that are clearly surrounded by stromal cells, thus being primordial germ cells (PGC; Fig.4A, B, red arrows). These PGCs have large nuclei and are also characterized by their relatively large soma (Fig.4B). Within the PGCs and the gonocytes the SALL4 signal was restricted to the nucleus. PGCs exhibited stronger SALL4 signals than gonocytes. There were also obvious differences in the staining intensities between individual gonocytes in the fetal and postnatal testes, and a sub-fraction of these germ cells exhibited no SALL4 signal at all (e.g. Fig.4A and D, yellow and blue arrows, respectively).

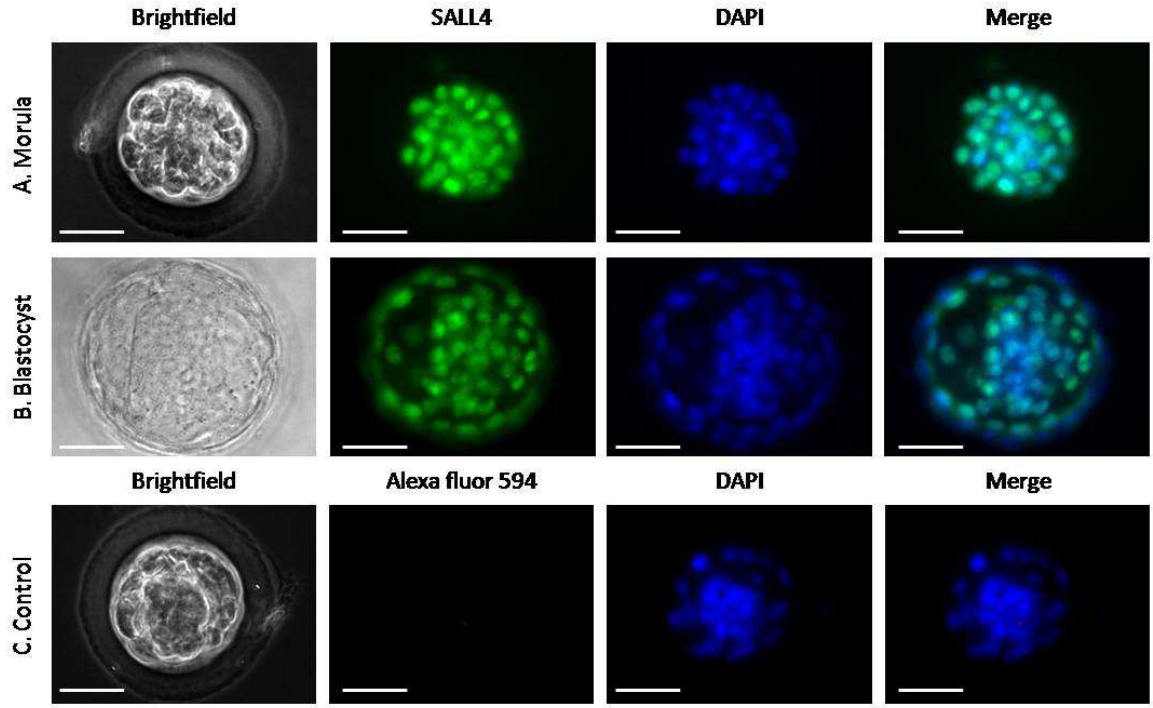
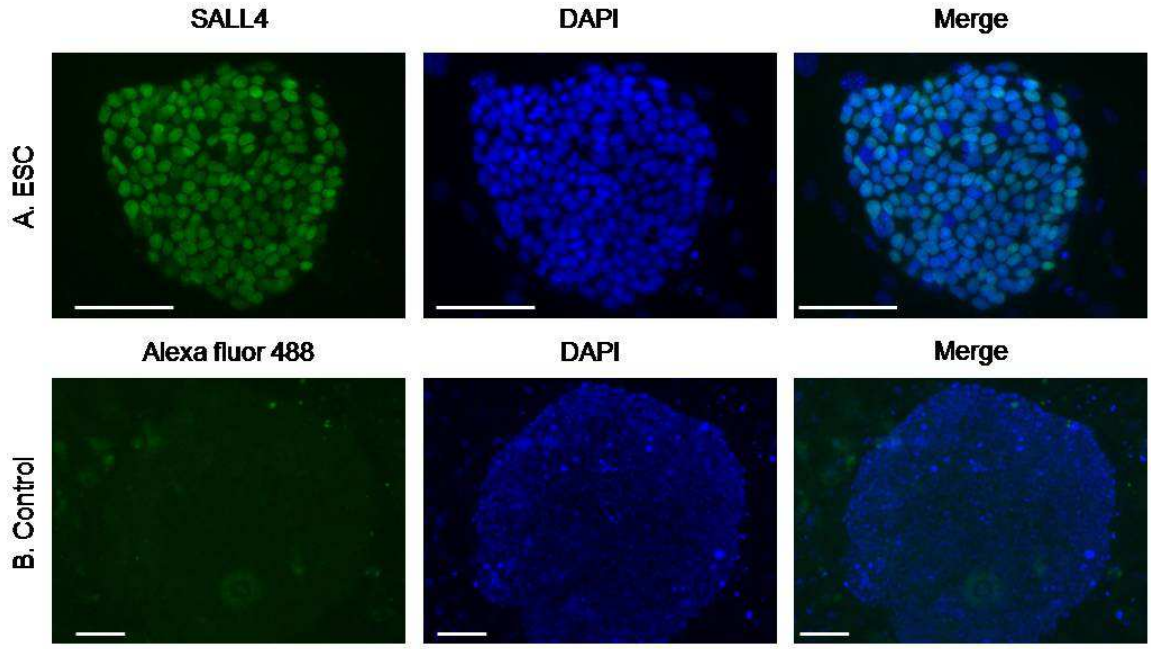


Figure 2: SALL4 in common marmoset pre-implantation embryos. A) SALL4 expression in a morula stage embryo. The protein is detectable in all cells. B) SALL4 expression in a blastocyst stage embryo. SALL 4 can be detected in the cells of the inner cell mass (embryoblast) as well as in most cells of the trophoblast. C) Negative control. The

morula was exposed only to the secondary antibody (anti mouse alexa fluor 594). The scale bar represents 50 µm.



<u>Figure 3:</u> SALL4 expression in marmoset monkey embryonic stem cells. Staining was performed 3-5 days after passaging. (A) Undifferentiated ES cell colony. SALL4 is expressed in the nuclei of the ES cells. (B) Negative control. The ES cells were exposed only to the secondary antibody (anti mouse alexa fluor 488). No staining was observed. The scale bar represents 50 µm.

In pubertal marmoset monkey testes (Fig.4F), the SALL4-positive germ cells became more and more scattered as the testis cords / seminiferous tubules grow (compare (Albert *et al.*, 2010)) and from the onset of puberty the SALL4-positive cells were exclusively found in contact with the basal membrane of the developing germinal epithelium (Fig.4F). Differentiating type B spermatogonia and meiotic cells were negative for SALL4. In the adult marmoset testis, SALL4 is restricted to type A spermatogonia, where SALL4 is present predominantly in the nucleus and with less intensity sometimes also in the cytoplasm (Fig.4G, H). For further details on spermatogonial subtypes see Fig.7.

SALL4 expression in the fetal, postnatal and adult human testis

There were nuclear SALL4 signals in most gonocytes in the fetal human testes (Fig. 5A, red arrows), but some germ cells exhibited, like in the marmoset, no or only faint signals (Fig.5A, green arrows). The pediatric testis from a one year old boy exhibited normal age-appropriate spermatogenesis with central pre-spermatogonia and peripheral type A spermatogonia. The nuclei of most germ cells were strongly SALL4-positive (Fig.5B). However, detailed histological evaluation was difficult due to limitations of the sample size and preservation. In the normal adult testes with complete spermatogenesis, SALL4 was, like in the marmoset, strongly expressed in a subset of spermatogonia with predominant localization in the nucleus (Fig.5C; see also Fig.7B). Faint staining could also be seen in the adult human testis in some later germ cell stages. However, these signals were very weak compared to the signals in spermatogonia.

Spermatogonial SALL4 expression is conserved in additional primate and non-primate species and exhibits no stage-dependency

The expression pattern of SALL4 in marmoset monkey, human, and mouse testis was confirmed in adult testes from *Goeldi's* marmoset monkey (Callimico goeldii, another new world primate Fig.6A), the Rhesus monkey (Macaca mulatta; Fig.6B), the Lion-tailed macaque (Macaca silenus; Fig.6C), the baboon (Papio hamadryas; Fig.6D), and the mandrill (Mandrillus sphinx; Fig.6E), which are all old-world primates, as well as in the treeshrew (Tupaia belangeri; a “linking species” between insectivores and primates; Fig.6F), the cat (Felis

silvestris catus; Fig.6G) and the dog (Canis lupus familiaris, Fig.6H). All show strong SALL4 expression in undifferentiated spermatogonia and no or only faint signals in later germ cell stages. In all species analyzed, we did not observe any stage-dependent expression of SALL4 in spermatogonia.

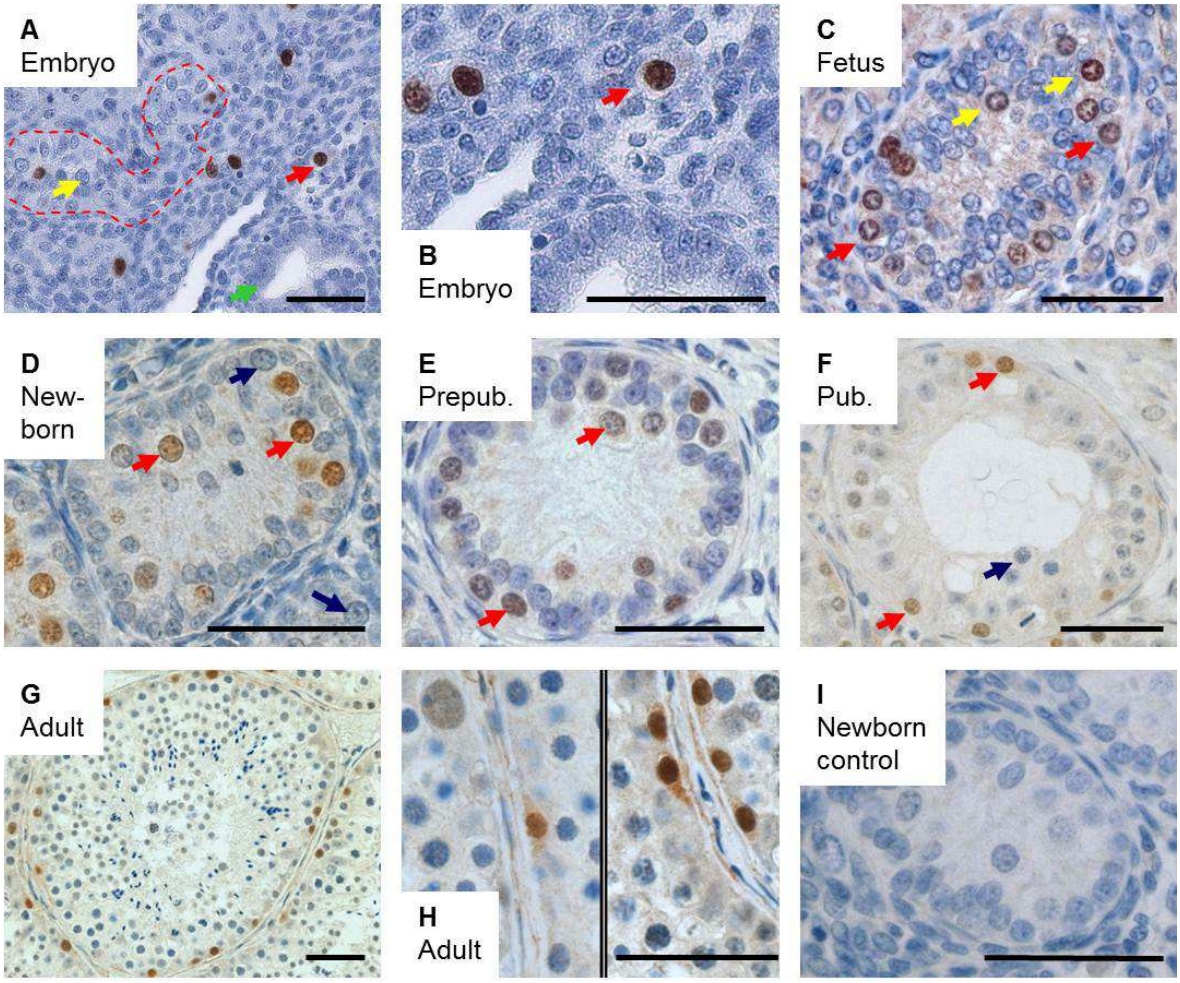


Figure 4: SALL4 expression in pre- and postnatal marmoset testis. A) Early fetal testis, at the transition from embryonic to fetal development (~GD 70; duration of pregnancy in marmosets: 143 days). The first testicular cords have formed (red dashed line), but differentiated peritubular cells were still missing. SALL4-positive and negative (yellow arrow) germ cells were detected. Some SALL4-positive germ cells with large nuclei and a large soma were still surrounded by stromal tissue thus being primordial germ cells (red arrow). The green arrow points to the epithelium of the developing epididymis. B) Higher magnification of A). PGCs display a large and prominent nucleus and a ring-like cytoplasm (red arrow). C) Fetal testis (GD 100). SALL4 was specifically expressed in gonocytes (yellow arrow) and pre-spermatogonia (red arrow), that were located in clearly delimited testicular cords. D) Newborn testis. SALL4 was expressed in gonocytes (red arrows). Pre-spermatogonia were sometimes negative for SALL4 (dark blue arrows). E) Pre-pubertal testis (5 months). Puberty starts at 8-10 months. SALL4 was detected in different intensities in gonocytes and immature spermatogonia (red arrows). F) Early pubertal testis (30 weeks) with first meiotic germ cells (blue arrow). SALL4 was restricted to spermatogonia (red arrows). G) Adult testis. SALL 4 was detected in spermatogonia. H) Adult testis. SALL4-positive spermatogonia. Strongest SALL4-singals were detected in the nuclei of spermatogonia, weaker signals were also seen in the cytoplasm. I) Newborn testis. Negative control. (Scale bar: 50 µm).

SALL4 expression in the developing and adult mouse testis parallels that in the primate testis

To compare these primate data with the mouse, the most utilized mammalian model in developmental biology and research on reproduction, we analysed different developmental stages of the mouse testis. In general, SALL4 expression in the developing and adult mouse testis is similar to the pattern seen in

the marmoset and human testis. All early gonocytes and PGCs outside the cords in the forming mouse E12.5 day testes were strongly SALL4-positive (Fig.5D). However, the signal distribution within the nuclei differed between different gonocytes.

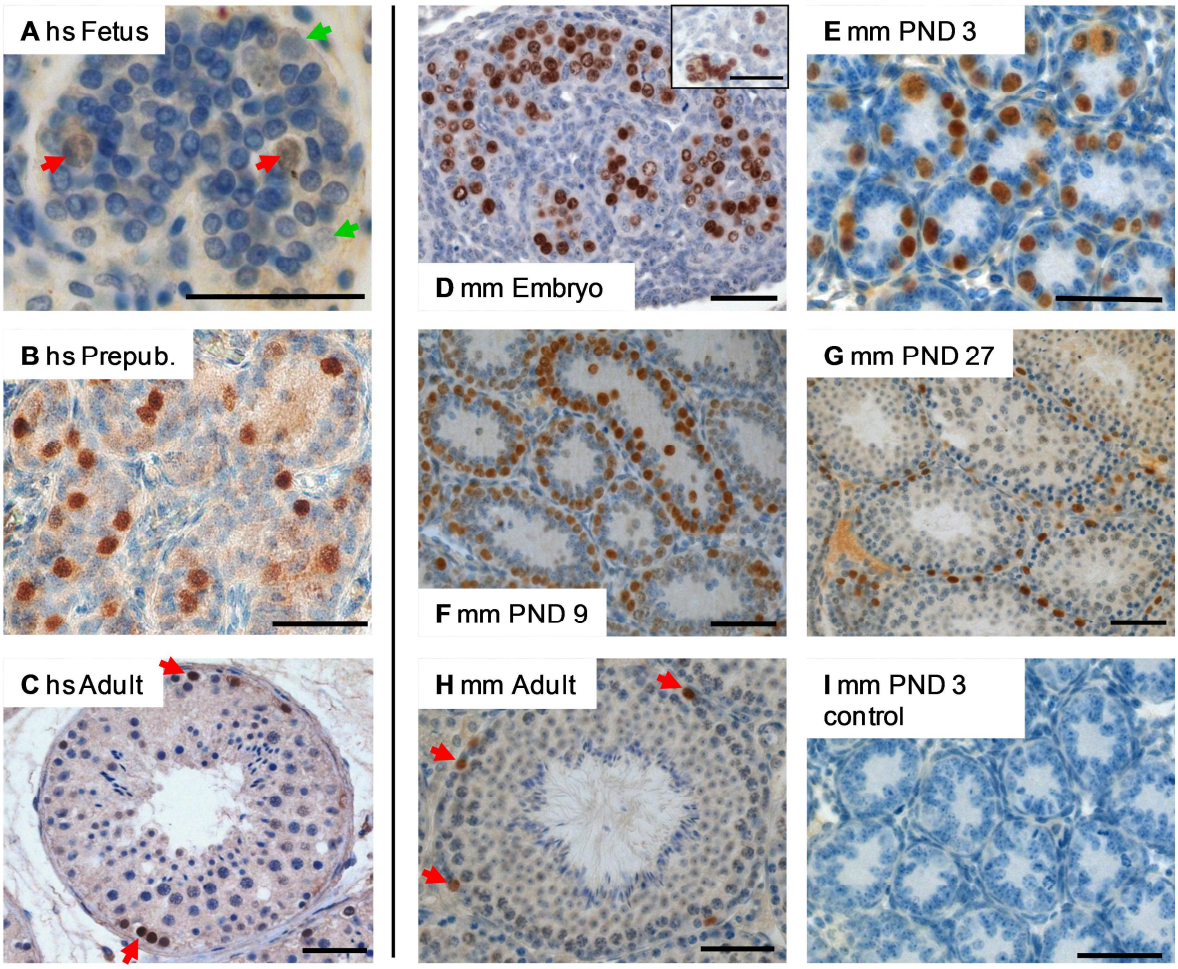


Figure 5: SALL4 expression in human (A-C) and mouse (D-I) testis. A) Fetal human testis (gestational week 21, formalin-fixed). SALL4 was present in gonocytes (red arrows). Pre-spermatogonia were faintly stained (green arrows). B) One year old testis with nuclear stain in all (pre-)spermatogonia. C) Adult testis with complete spermatogenesis. SALL4 was strongly expressed in spermatogonia. D) E12.5d mouse testis. SALL4 exhibited strong and specific expression in intra-gonadal gonocytes and late extra-gonadal primordial germ cells (inset). Some gonocytes displayed a homogenous (green arrows) and others a rather peripheral signal (red arrows) distribution within the nucleus. E) PND 3 testis. Spermatogonia were SALL4-positive. F) PND 9 testis. Spermatogonia showed moderate or strong SALL4 expression. G) PND 27 testis. Spermatogonia strongly express SALL4. Brown stain in the interstitium is unspecific. H) Adult testis. Strong staining was obtained in spermatogonia. I) Negative control, day 3 testis. The scale bar represents 50 µm in all figures.

While in some nuclei the signal was concentrated in the periphery of the nuclei (red arrows), other nuclei showed a rather homogenous nuclear signal (green arrows). Postnatal immature spermatogonia in three days old testes established contact with the basal membrane and were also strongly stained (Fig.5E). At this stage, the signal was always homogenous within the nucleus. In the 9 day old testis (Fig.5F) SALL4 expression was predominant in spermatogonia. At 27 days (Fig.5G) and in the adult testis with a fully developed seminiferous epithelium, SALL4 was expressed again very predominantly in spermatogonia (Fig.5H) during all stages of the spermatogenic cycle. Meiotic spermatocytes and postmeiotic spermatids sometimes showed very faint signals. *SALL4 is expressed in type A spermatogonia in the adult marmoset and human testis*

All pre-meiotic germ cells in the adult mammalian testis are called spermatogonia. In primates including man they are subdivided into type A

spermatogonia and type B spermatogonia. B spermatogonia are committed to differentiation, i.e. entry into meiosis to become a sperm cell. Type A spermatogonia are further subdivided into A_{dark} and A_{pale} spermatogonia. A_{dark} spermatogonia are considered as mitotically rather inactive undifferentiated spermatogonia, which are activated in cases where the germinal epithelium must be repopulated with germ line stem cells. A_{pale} spermatogonia are actively dividing undifferentiated spermatogonia, which support continuous sperm production. B spermatogonia derive from A_{pale} spermatogonia. SALL4 was expressed in A_{dark} and A_{pale} spermatogonia in the marmoset (Fig.7A) and human (Fig.7B) testis, while it was undetectable in marmoset B spermatogonia and only very faintly detectable in human B spermatogonia (Fig.6A, B). However, between individual A_{dark} and A_{pale} spermatogonia there were considerable differences in the SALL4 staining intensities indicating molecular heterogeneity between the cells belonging to one specific histologically identifyable type of spermatogonia.

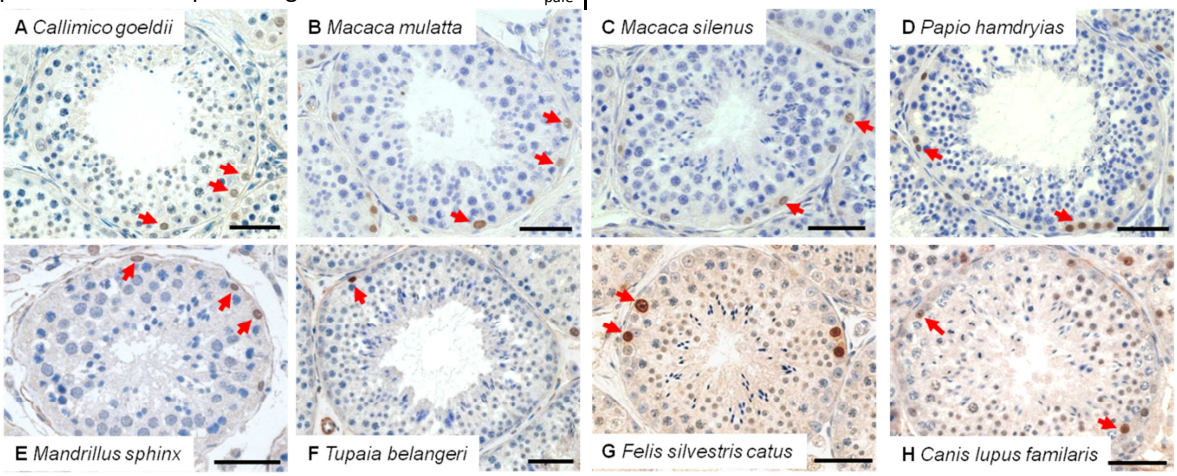


Figure 6: SALL4 expression in additional mammalian testes. A) *Goeldi's* marmoset (*Callimico goeldii;* new world primate species). B-E) Old world primate species. B) Rhesus monkey (*Macaca mulatta*), C) Lion-tailed macaque (*Macaca silenus*), D) Baboon (*Papio hamadryas*), E) Mandrill (*Mandrillus sphinx*). F) Treeshrew (*Tupaia belangeri*). G) Cat (*Felis silvestris catus*) and H) Dog (*Canis lupus familiaris*) are representaives of non-primate mammalian species. All species analysed expressed SALL4 in undifferentiated spermatogonia (highlighted by red arrows). Cat and Dog also show weak signals in some later germ cell stages. The scale bar represents 50 µm in all figures.

DISCUSSION

In the present work we analysed the expression of the pluripotency-associated transcription factor SALL4 during male germ cell development in common marmoset monkey as well as in pre-implantation embryos and ES cells. Recently, Cauffman and colleagues (Cauffman *et al.*, 2009) found SALL4 expressed in the nuclei of human pre-implantation embryos and in undifferentiated human ES cells. We confirmed strong nuclear SALL4 expression in the present study in the non-human primate pre-implantation embryo at the morula and the blastocyst stage. Also, strong SALL4-expression in undifferentiated ES cells is common for human (Cauffman *et al.*, 2009) and marmoset ES cell lines (this study). Using gene targeting studies, *Sall4* has been found to be essential for peri-implantation embryo stages in mice (Elling *et al.*, 2006; Tsubooka *et al.*, 2009). Since experimental studies with human embryos are banned in several countries and ethically very controversial, the experimental prove of the importance of the role of SALL4 in the human embryo has not been made. Due to the very limited number of monkey embryos and the experimental difficulties in this species, functional studies using common marmoset embryos are currently also not possible. Nevertheless, altogether, the published data and those presented in this study suggest that the essential role of *Sall4* in the mouse pre-/peri-implantation embryo might be conserved in the human and non-human primate embryo. Testicular germ line stem cells (spermatogonia) are unipotent *in vivo*. They exclusively produce sperm cells. However, as shown in mice (Kanatsu-Shinohara *et*

al., 2004; Guan *et al.*, 2006; Seandel *et al.*, 2007; Ko *et al.*, 2009), when removed from their stem cell niche in the testis, these cells can spontaneously reprogram their developmental state from unipotency to pluripotency without any genetic modification like the introduction of the set of pluripotency transcription factors used for the generation of iPS cells. This spontaneous reprogramming strongly emphasizes the very special developmental state of germ line stem cells, which may be represented by the expression of pluripotency markers which are also expressed by ES cells (for review see (Zwaka and Thomson, 2005; Rajpert-De Meyts, 2006)). Among these pluripotency markers are OCT4, NANOG, AP2γ, SSEA4, and TRA-1-81, which are also present in premeiotic germ cells of the marmoset testis (Mitchell *et al.*, 2008; Muller *et al.*, 2008; Albert *et al.*, 2010).

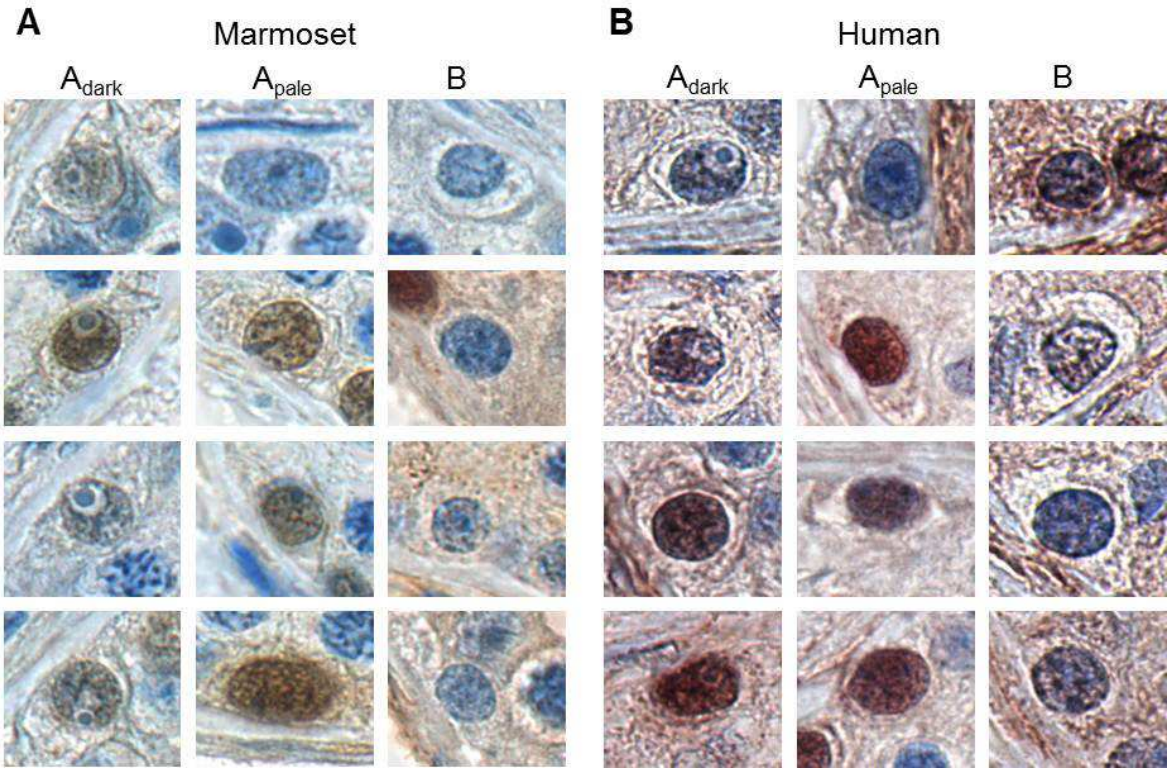


Figure 7: SALL4 expression in spermatogonial subtypes of marmoset monkey and man. A) marmoset. B) human. Four examples per cell type (A_{dark} spermatogonia, A_{pale} spermatogonia, B spermatogonia) are shown. A_{dark} spermatogonia and A_{pale} spermatogonia show highly variable staining intensities ranging from strong staining to complete lack of staining. B spermatogonia were not (marmoset monkey) or only sometimes very faintly (human) stained.

Presenting the data on SALL4, we add an additional pluripotency marker to the list of proteins that are shared by ES cells, the pre-implantation embryo and testicular germ line stem cells. In addition to the expression of SALL4 in ES cells and adult spermatogonia, we show here for the first time that SALL4 is also expressed in fetal human, monkey and mouse gonocytes as well as in monkey and mouse PGCs during the late migratory phase. Interestingly, in the fetal and newborn marmoset testis the gonocytes show, in contrast to the mouse, very differential SALL4 signals which is in agreement with the findings by Gaskell and colleagues, (Gaskell *et al.*, 2004) who showed that the gonocytes in the human fetal testis are not a homogeneous population of cells as it appears to be in the mouse. However, in general, it can be stated that SALL4 is expressed in undifferentiated cell types of the male mammalian germ line: the morula, the inner cell mass, the PGCs, the gonocytes and the undifferentiated spermatogonia. Expression of SALL4 in undifferentiated spermatogonia appears to be a general characteristic of the mammalian testis as all species analyzed in our study showed conserved expression pattern. A_{dark} spermatogonia are undifferentiated reserve spermatogonia. A_{pale}

spermatogonia are the mitotically active undifferentiated spermatogonia, which supportcontinuous sperm production. However, there appears to be certain plasticity in the spermatogonial subpopulations (Ehmcke and Schlatt, 2006; Ehmcke *et al.*, 2006; Nakagawa *et al.*, 2010). It might be, that undifferentiated spermatogonia can switch from one to another type (Adark to Apale, and back). It is also conceivable, like in the mouse, that a clone of spermatogonia breaks apart and that the daughter cells meet different fates: some may differentiate and enter meiosis while others from the same syncytium may re-enter a functional stem cell niche and thus regain full stem cell potential. In general, A_{pale} spermatogonia appear to exhibited stronger SALL4 signals than A_{dark} spermatogonia, although both spermatogonial populations contained SALL4-positive and negative cells. In the light of the emerging data on the plasticity of the spermatogonial stem cell pool it is conceivable that SALL4 expression is rather associated with a specific developmental state or potential of the spermatogonia than with the morphological criteria of A_{dark} and A_{pale} spermatogonia.

We have shown on the mRNA as well as on the protein level that the marmoset testis and ES cells express different SALL4 isoforms. Since this study is restricted to a detailed descriptive expression analysis, functional analysis of the different SALL4 isoforms still awaits. However, a recent paper by (Rao *et al.*, 2011) provided first hints to functional differences of the SALL4-A and –B isoforms in pluripotent mouse ES cells. SALL4-B binds preferentially to promoters of genes showing high expression in undifferentiated ES cells. In contrast, DNA binding of SALL4-A occurred preferentially in promoter regions of genes upregulated during ES cell differentiation. However, both isoforms can also form heterodimers, which were found preferentially responsible for the regulation of pluripotency genes. If this control of gene expression by SALL4 isoforms is also true for primate (germ) cells needs to be shown in future functional studies.

Functional *in vivo*-analysis of SALL4 in germ cells is impossible in the primates. However, keeping the relevance of SALL4 in the early mouse embryo in mind, it is conceivable that SALL4 might also have important functions in male mammalian germ cells as suggested by its strong expression in PGCs, gonocytes and spermatogonia. Future studies on the function of SALL4 in mammals have to be conducted in mice to finally prove the functional importance of SALL4 in the mammalian germ line.

There is an apparent discrepancy between marmoset SALL4 protein (Fig.1A) and *SALL4* mRNA (Fig.1D) data in our present study. A possible explanation for this basically unexpected finding could be that translational efficiency and/or *SALL4* transcript stability are significantly different in newborn and adult testis. Such regulation on the translational level has been shown for other testicular germ cell transcripts like RNF4 (Pero *et al.*, 2003). Another explanation could be the presence of a well-conserved *SALL4* pseudogene, which is located on marmoset chromosome 4 (ENSEMBL database, data not shown). Based on the sequence and on some motifs present in the pseudogene, it is likely that it arose by retroposition of a spliced *SALL4-B* mRNA. There are other genes that arose by retroposition and which are specifically expressed in the testis (Marques *et al.*, 2005) or even in postmeiotic germ cell stages (Hendriksen *et al.*, 1997; Vemuganti *et al.*, 2007). However, to date it is not clear whether the *SALL4* pseudogene is transcribed (but probably not translated) in meiotic or postmeiotic germ cells. The increasing *SALL4* mRNA abundance during postnatal testis development in the marmoset is not only surprising with regard to the marmoset protein data, but also regarding the mouse mRNA data. During mouse testis development *Sall4* mRNA is (relatively) down-regulated as expected from the immunohistochemical stainings. Future experiments will elucidate this problem.

In summary, we provide for the first time a comprehensive SALL4 expression analysis in human, non-human primate and mouse testes as well as in marmoset monkey pre-implantation embryos. Testicular PGCs, gonocytes as well as spermatogonial stem cells of the adult testis also exhibit strong SALL4 signals. In the early embryo, SALL4 is detectable in the blastomeres of the morula and in the blastocyst in the cells of the embryoblast as well as in trophoblast. Regarding the entire germ line of a generation, SALL4 is expressed in the premeiotic phase in those developmental germ cell stages that have pluripotent and/or stem cell

characteristics. The functional relevance of the different SALL4 isoforms during germ cell development remains to be analysed in future studies.

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4 FALSE-POSITIVE ANTIBODY SIGNALS FOR THE PLURIPOTENCY FACTOR OCT4A (POU5F1) IN TESTIS-DERIVED CELLS MAY LEAD TO ERRONEOUS DATA AND MISINTERPRETATIONS

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KEY WORDS OCT4 - Stem cell - Pluripotency - Reprogramming - Testis - Non-human primate

ABSTRACT Octamer-binding protein 4 (OCT4) is a key player in pluripotent ES cells and is essential for the generation of induced pluripotent stem cells. Recently, several reports indicated the spontaneous recovery of pluripotency in cultured adult human testis-derived cells. This was evidenced also by the detection of OCT4 using antibodies. However, the soundness of some data was recently put into question. During our attempts to derive pluripotent cells from the common marmoset monkey testis, we obtained inconsistent data which prompted us to deeper analyze the characteristics of three independent OCT4 antibodies that were used in numerous published studies. All antibodies detected OCT4 by immunofluorescence (IF) in a marmoset monkey ES cells line. Two out of the three OCT4 antibodies gave also robust nuclear signals in testis-derived cells. However, the latter cells did express no *OCT4* mRNA as revealed by quantitative RT-PCR and turned out to be mesenchymal cells. When tested in western blot analyses, all antibodies detected heterologously expressed marmoset monkey OCT4. But, importantly, those antibodies that resulted in non-specific signals in IF also showed additional non-specific bands. In summary, some commercially available OCT4 antibodies result in false-positive signals, which may provoke erroneous conclusions when used in studies aiming at the generation of pluripotent cells in vitro. We conclude that antibodies must be carefully characterized before use to prevent misleading observations.

INTRODUCTION

Octamer-binding protein 4 (OCT4, also POU5F1) is a key player in pluripotent cells (Nichols *et al.*, 1998; Niwa *et al.*, 2000; Boyer *et al.*, 2005). This transcription factor is essential for the reprogramming of differentiated cells into induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006; Okita *et al.*, 2007; Takahashi *et al.*, 2007) and OCT4 alone is sufficient, but also essential to reprogram adult neural stem cells to pluripotency (Kim *et al.*, 2009). Recent publications presented data indicating that OCT4 is quickly and robustly up-regulated in human cultured testis-derived cells even without any genetic modification of the cells (Conrad *et al.*, 2008; Golestaneh *et al.*, 2009; Gonzalez *et al.*, 2009; Kossack *et al.*, 2009; Mizrak *et al.*, 2010). Altogether, these facts indicate the vital importance of specific and reliable detection of *the* key pluripotency marker OCT4 in cell culture experiments aiming at the generation of pluripotent cells from differentiated cells lacking OCT4. This issue is even more critical when studies with human or non-human primate cells are performed, where no transgenic OCT4-GFP-reporter cells are available.

OCT4 exists basically as two isoforms that differ by the alternative use of the leader exons encoding the N-terminal part of the respective OCT4 proteins. Importantly, these different OCT4 proteins have different functional properties (Wang and Dai, 2010). While the OCT4A isoform

is clearly related to stemness and pluripotency, OCT4B seems to be involved in the cellular stress response. The role of the recently discovered OCT4B1 isoform is still unclear. The cellular localization of OCT4A and –B is different. OCT4A is present in the nucleus, while OCT4B is mainly cytoplasmic (Cauffman *et al.*, 2005). Important for functional analyses, the "pluripotency isoform" OCT4A is the only isoform that includes exon 1. Thus, exon 1 is a unique and specific part of OCT4A and hence its presence is the only reliable indicator of pluripotency (Liedtke *et al.*, 2008; Wang and Dai, 2010). All other OCT4 exons (and the corresponding parts of the proteins) are not meaningful with regard to pluripotency.

The common marmoset monkey (*Callithrix jacchus*) is a well-established non-human primate species in preclinical and especially in reproduction research, since its testicular development and organization is very similar to the human (Millar *et al.*, 2000; Weinbauer *et al.*, 2001; Mitchell *et al.*, 2008) . During our attempts to derive pluripotent cells from the common marmoset monkey testis, as it was recently published for the human testis (Conrad *et al.*, 2008), we used three different antibodies to detect OCT4 in cultured cells by immunofluorescence (IF). However, our data were inconsistent prompting us to further characterize the different frequently used polyclonal OCT4 antibodies. The first antibody (Abcam ab19857) was generated against the C-terminal part of human OCT4, which is common to both, OCT4A and OCT4B. The second antibody was from Santa Cruz (Sc-9081) and was generated against the OCT4A-specific part (amino acids 1-134 of OCT4A). The third antibody was also from Santa Cruz (Sc-8628) and directed against the OCT4A-specific 19 N-terminal amino acids of OCT4A. Importantly, two of these antibodies generated signals in IF which can easily be misinterpreted and eventually lead to erroneous conclusions when used in studies aiming at the generation of pluripotent cells.

MATERIALS AND METHODS

Embryonic stem cells (ESCs)

Common marmoset monkey (*Callithrix jacchus*) embryonic stem cells (line cjes001; (Muller *et al.*, 2009)) were cultured on 3500-rad γ-irradiated mouse embryonic fibroblasts (MEFs) in medium consisting of 80% Knockout DMEM (GIBCO) and 20% Knockout Serum Replacement (GIBCO) supplemented with 2mM GlutaMAX™ (GIBCO), 0.1mM MEM non-essential amino acids (GIBCO), 0.1mM β-mercaptoethanol (GIBCO), 100 IU/ml penicillin and 100 mg/ml streptomycin as described previously(Muller *et al.*, 2009).

Derivation and primary culture of testicular multipotent stromal cells (TMSCs)

Common marmoset testis were kept on ice in 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 (GIBCO) containing 1mg/mL hyaluronidase (Sigma), 1mg/mL collagenase (Sigma) and 15U/mL DNase (Roche) for 35-40 minutes at 37°C under constant mild rotation. Resulting single cells were seeded at a cell density of $2x10^5$ cells/cm^2 to plastic culture dishes. TMSC-culture medium was based on a 1:1 mixture of D-MEM (GIBCO) and F-12 nutrient mixture (GIBCO) which was supplemented with 10% FBS (GIBCO), 100U/L Penicillin-Streptomycin (GIBCO), 0,25µg/mL Fungizone® Antimycotic (GIBCO) and 5ng/mL recombinant human basic fibroblast growth factor (ProSpecTany). Following overnight incubation any non-adherent cells were removed from the dish and remaining adherent cells were cultured until they reached confluency. Generally TMSCs were cultured at 37°C under 5% CO_2 and the medium was changed every 2 -3 days. Passaging was performed with 0,05% trypsin in PBS (GIBCO) every three to five days (in general upon confluency) at a dilution of 1:3 - 1:5.

Cloning of marmoset monkey OCT4A and transfection into HEK-293 cells

Marmoset monkey OCT4A cDNA was amplified with primers 5′-GATCGGATCCTTGGGGCGCCTTCCTTC-3′ and 5′-CTGATCTAGACTCCTCTCCCTGTCCCCC-3′ from single strand cDNA of the marmoset ES cell line cjes001 (passage51). The PCR product of 1159 bp was digested with BamHI and XbaI, cloned into the vector pBluecsriptII SK- (Stratagene) and sequenced. Compared to the published sequence (UCSC Genome Bioinformatics, http://genome.ucsc.edu/, the Mar. 2009 *Callithrix jacchus* draft assembly (WUGSC 3.2 (GCA_000004665.1)) the amplified ORF had two silent mutations: C363T and T1014A. The complete sequence of the amplified OCT4 ORF has been deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/), accession number JQ627833. Subsequently the *OCT4* ORF was amplified with primers 5′-CGGGATCCCCACCATGGCGGGACACCTGGCTTCG and 3′-GCTCTAGATCAGTTGGAATGCATGGGAGAGC including additional restriction sites (BamHI, XbaI) for cloning into the expression vector pcDNA3.1$^+$ (Invitrogen). For a more robust expression of the OCT4 protein the CMV promoter was replaced by the CAG promoter, which was synthesized by GenScript (www.genscript.com) and cloned into the puc57 vector by digestion with BamHI and EcoRI. Subsequent digestion of both vectors pcDNA3.1$^+$-OCT4 and pUC57-CAG with BglII and NheI allowed the promoter exchange resulting in the final expression vector pcDNA3.1$^+$-CAG-OCT4, which was sequenced and used in this study.

Twenty-four hours prior transfection 4.5 x 10^6 HEK-293 cells were seeded to a 9 cm culture dish and maintained in MEM (GlutaMAX, Invitrogen) containing 10 % FBS (Gibco/BRL), 1 % penicillin/streptomycin (Gibco/BRL) and 1 % non-essential-aminoacids (Gibco/BRL) at 37 °C under 5 % CO_2.The transfection was performed using 0.02 µg/µl pcDNA3.1$^+$-CAG-OCT4 expression vector and the FuGENE HD Reagent (Promega) according to the manufacturer's manual. The cells were harvested for protein isolation 48 hours post transfection.

Immunofluorescence (IF)

For IF, cells were grown in 48-well-plastic plates and fixed for 30 minutes in 4% PFA. The cells were permeabilized with 0.04 % Triton X-100 for 10 minutes. After rinsing with PBS, the primary antibodies (see table I), diluted in PBS/5% BSA, were applied for 1 hour at 37°C. Following two PBS washing steps the appropriate Alexa fluor (AF) 488-linked secondary antibodies (Table II), diluted in PBS/5% BSA, were applied for 30 minutes at room temperature in the dark. Controls were performed omitting the primary antibody and with the corresponding IgG fraction. Cells were counter-stained with DAPI, covered with citifluor (Citifluor Ltd) and images were taken on a Axio Observer Z1 fluorescence microscope from Zeiss (Germany).

Table I: primary antibodies used in this study

Immunogen / Isoform specificity	company	cat. No.	Species / Isotype	Times cited according to supplier's webpage
aa 300-360 (OCT4A and OCT4B	abcam	#ab19857	rabbit IgG	41
aa 1-19 (OCT4A)	santa cruz	#sc-8628	goat IgG	171
aa 1-134 (OCT4A)	santa cruz	#sc-9081	rabbit IgG	102

Table II: secondary antibodies used in this study

Antigen	linked fluorophore	host species	company	cat. No.
anti-goat IgG	Alexa fluor 488	donkey	Invitrogen	#A11055
anti-rabbit IgG	Alexa fluor 488	donkey	Invitrogen	#A21206
anti-rbt IgG	Alexa fluor 594	donkey	Invitrogen	#A21207

Western blot analysis

Western blots were basically performed as previously described (Eildermann *et al.*). Cytoplasmic and nuclear protein from wild-type and *cj*OCT4 expressing HEK-293 cells was isolated with the Qproteom nuclear protein kit from Qiagen (Hilden, Germany) according to the manufacturer's instructions. Protein concentrations were measured using the Bradford-assay and 20 µg of protein per sample was separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane (Immobilon-P, Millipore). Membranes were incubated with one of the three different OCT4 primary antibodies (see table I), followed by the incubation with the according HRP-coupled secondary antibody (table III). Signals were detected with ECL Western blot detection reagents (GE Healthcare) and the ChemoCam-System (INTAS, Goettingen, Germany).

Table III: secondary antibodies for Western Blots

Antigen	host species	company	cat. No.
anti-rabbit IgG-HRP	goat	R&D	#HAF008
anti-goat IgG-HRP	rabbit	R&D	#HAF017

Quantitative (q) RT-PCR

Relative qRT-PCR was performed on common marmoset testicular multipotent stromal cells (TMSCs), ESCs and testis material. Design and testing of the qRT-PCR Primers (*OCT4* fw 5`AAACCCACACTTCAGCAGATCA 3`, re 5`CACACGGACCACATCCTTCTC 3`; *SOX2* fw 5` GAGAACCCCAAGATGCACAAC 3`, re 5`TCTCGGACAGCAGCTTCCA 3`) as well as the qRT-PCR procedure was performed as previously described (Eildermann *et al.*) . In brief, 2µg testicular RNA were reverse transcribed, using random hexamers, by Superscript II (Invitrogen, Karlsruhe, Germany) to obtain cDNA. 2µL of 1:2 diluted cDNA was used for each 20 µl PCR reaction with Power SYBR Green Mastermix (Applied Biosystems), and different primer concentrations ranging between 50 to 900nM. The extent of fluorescence of the Power SYBR green dye was detected and analyzed using the $2^{-(\text{Delta Delta C(T)})}$ 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.

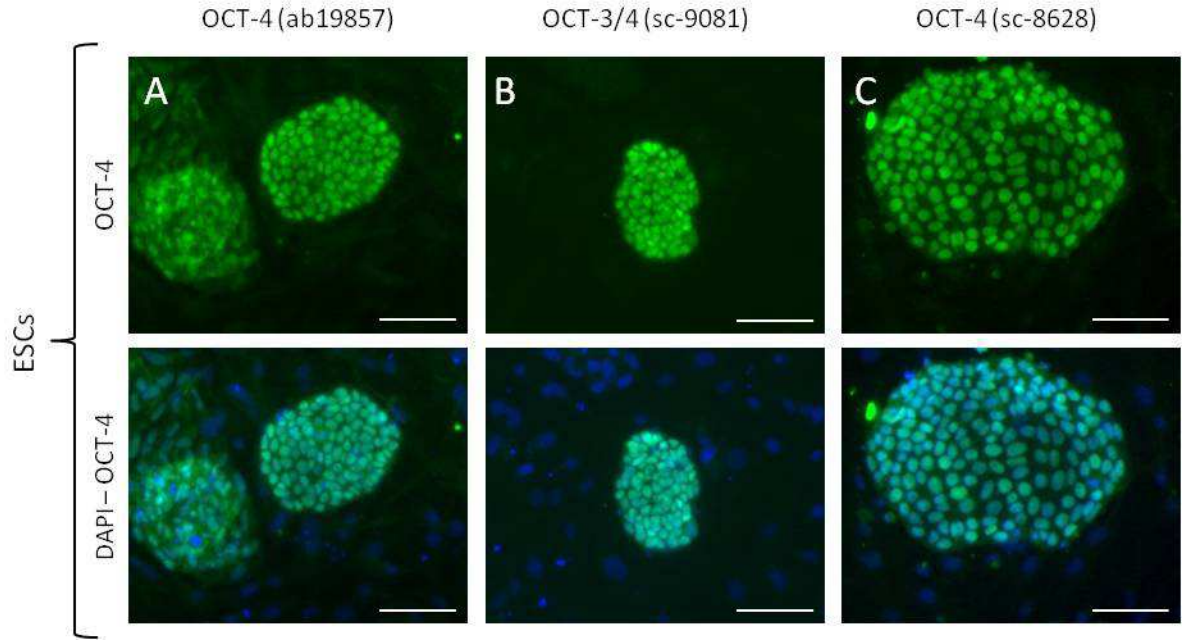

Figure 1: **OCT4-signals obtained in marmoset monkey ESCs with three different polyclonal antibodies. A) Abcam ab19857 OCT4 antibody directed against the C-terminus recognizing OCT4A and OCT4B (upper panel). Nuclei were stained with DAPI (respective lower panels). Scale bar: 100 µm in all panels. B) Santa Cruz Sc-9081 OCT4 antibody directed against the N-terminus recognizing only OCT4A. C) Santa Cruz Sc-8626 OCT4 antibody directed against the N-terminus recognizing** only OCT4A.

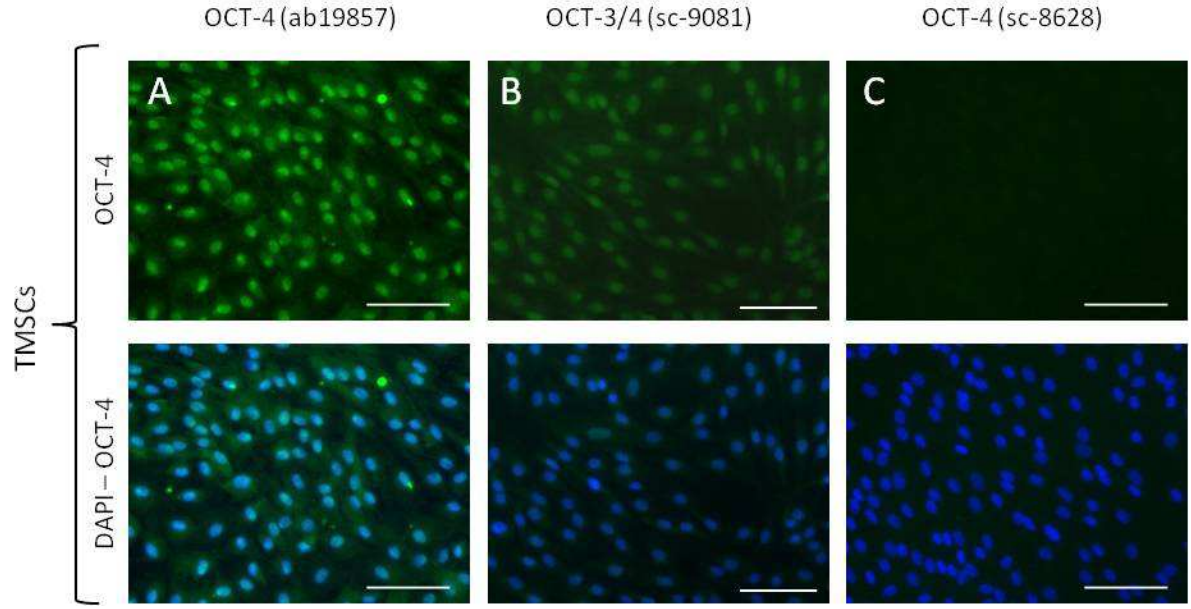

Figure 2: **OCT4-signals obtained in marmoset monkey testicular multipotent stromal cells (TMSCs) with three different polyclonal antibodies. A) Abcam ab19857 OCT4 antibody (upper panel). This antibody showed strong signals in testis-derived stromal cells. Nuclei were stained with DAPI (respective lower panels). Scale bar: 100 µm in all panels. B) Santa Cruz Sc-9081 OCT4 antibody showing signals of intermediate intensity. C) Santa Cruz Sc-8626 OCT4 antibody showing no signals.**

RESULTS

All three tested antibodies detect OCT4 in ES cells

We used marmoset monkey ES cells as a positive control for OCT4-expressing cells (Muller *et al.*, 2009). All three antibodies irrespective of the epitope used for immunization of the animals, detected OCT4 in the nuclei of the marmoset monkey ES cells (Fig.1). Antibody ab19857 also produced a slight cytoplasmic signal (Fig.1, left panel).

Two out of three OCT4 antibodies strongly stain primary testicular multipotent stromal cells (TMSC)

Importantly, two of the OCT4 antibodies showed also robust nuclear staining in marmoset monkey TMSCs, which are non-pluripotent cells from connective tissue (Fig 2; Eildermann et al. under revision). The ab19857 OCT4 antibody displayed a strong nuclear signal in TMSCs which was comparable to the one seen in pluripotent ES cells. Additionally, weak cytoplasmic staining was visible (Fig. 2 left panels). The sc-9081 OCT3/4 antibody displayed moderate nuclear and also faint cytoplasmic signals. Only the sc-8628 OCT4 antibody displayed no signal in TMSCs. A comparable result was obtained when HEK-293 cells of human origin were stained: very intense staining with the ab19857 OCT4 antibody, moderate staining with the sc-9081 OCT3/4 antibody and only faint signals with the sc-8628 OCT4 antibody (data not shown) indicating a staining pattern of the different OCT4 antibodies which is neither species- nor cell type-specific. qRT-PCR reveals that TMSCs do not express the pluripotency markers OCT4A and SOX2. In order to either confirm or disprove the unexpected IF OCT4 stainings in marmoset testicular cells, we performed qRT-PCR for OCT4 (Fig.3). The primers bind to exons 4 and 5 so that both, OCT4A and OCT4B would be detected. Importantly, the TMSCs did not express any OCT4 mRNA. In contrast, pluripotent ES cells, which were included as positive controls, expressed OCT4 mRNA, while OCT4 was also not detectable in the adult marmoset testis. To further verify that the pluripotency-determining transcriptional network is not activated in the TMSCs, we also tested the expression of SOX2, another key player in pluripotency (Boyer et al., 2005). Confirming the OCT4 mRNA data, SOX2 was also absent from the TMSCs, while it was detectable at low levels in whole testis mRNA and at high levels in ES cell RNA.

Western blot analysis

Following the contradictory results from IF and qRT-PCR, we performed western blot analysis on nuclear and cytoplasmic protein of wild type HEK-293 cells and with HEK-293 cells transfected with marmoset *OCT4A* (Fig 4). The western blot signals at an apparent molecular weight of ~ 45kDa (black arrows) corresponded to OCT4A since it appeared only in the *OCT4A*-transfected cells. This positive control signal was obtained with all three different antibodies and was, as expected for a transcription factor with a distinct nuclear localization signal, more intense in the nuclear fraction than in the cytoplasmic fraction. However, importantly, the sc-9081 and the ab19857 OCT4 antibodies displayed additional. bands of variant size and intensity in both, wild type and OCT4A-transfected HEK-293 cells (Fig. 4, red arrows). Furthermore, the unspecific bands were much stronger in the nuclear protein fraction than in the cytoplasmic fraction. Only the sc-8628 OCT4 antibody resulted in no unspecific signals.

DISCUSSION

The human and the marmoset *OCT4* genes are very well conserved with regard to the gene structure and the encoded amino acid sequence. Both, the human and the marmoset *OCT4A* isoform encode a protein consisting of 360 amino acids (aa). The marmoset *OCT4* gene displays 97% nucleotide homology to the human gene. The protein products derived from the human *OCT4B* mRNA variant range between 164 and 265 aa depending on the translational start site and

the use of an internal ribosomal entry site (Wang and Dai, 2010). Crucially, the pluripotency-associated OCT4A isoform has a higher molecular weight than all other OCT4 isoforms that are not associated with pluripotency (Liedtke *et al.*, 2008; Wang and Dai, 2010). This indicates that the signals that were obtained in western blots above the OCT4A positive controls are not related to OCT4 and are thus unspecific. The appearance of the unspecific band at ~ 50 kDa in the nuclear fraction in western blot analysis using the isoform-nondistinctive ab19857 OCT4 antibody correlates very well with the immunofluorescence signals, which also show strong nuclear staining. Notably, this unspecific band was already observed earlier ((Bhartiya *et al.*, 2010), see also ttp://www.abcam.com/index.html?pageconfig=reviews&intAbreviewID=22268&intAbID=19857).Similar false-positive results were obtained with the OCT4A-directed sc-9081 antibody, although the additional bands appeared at an apparent molecular weight below 30 kDa. Only the OCT4A-specific antibody sc-8628 detected exclusively the positive control in western blot analyses. These western blot results correlate very well with the IF signals in ESCs and TMSCs. In summary, all three tested antibodies detect OCT4 in ESCs and in western blots. However, if they are used to demonstrate the pluripotency-indicating OCT4A isoform, two of the tested antibodies are inappropriate since they also may detect OCT4B or an even completely OCT4-unrelated epitope. This may result in false-positive signals, which

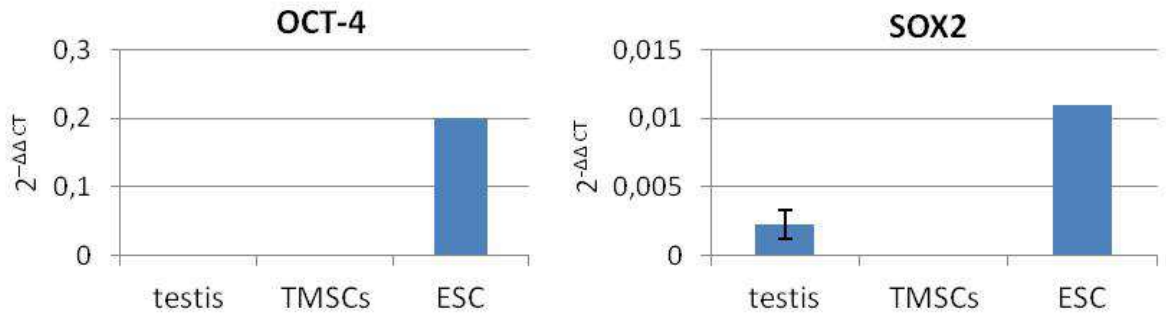

Figure 3: Quantitative real-time RT-PCR for OCT4 and SOX2 on marmoset monkey testis-, TMSC- and ESC RNA. A) OCT4 was detected only in ES cell RNA, while it was undetectable in testis and testicular stromal cell RNA. B) SOX2 was detected at high levels in ES cell RNA, at lower levels in testis RNA and was not detected in testicular stromal cell RNA.

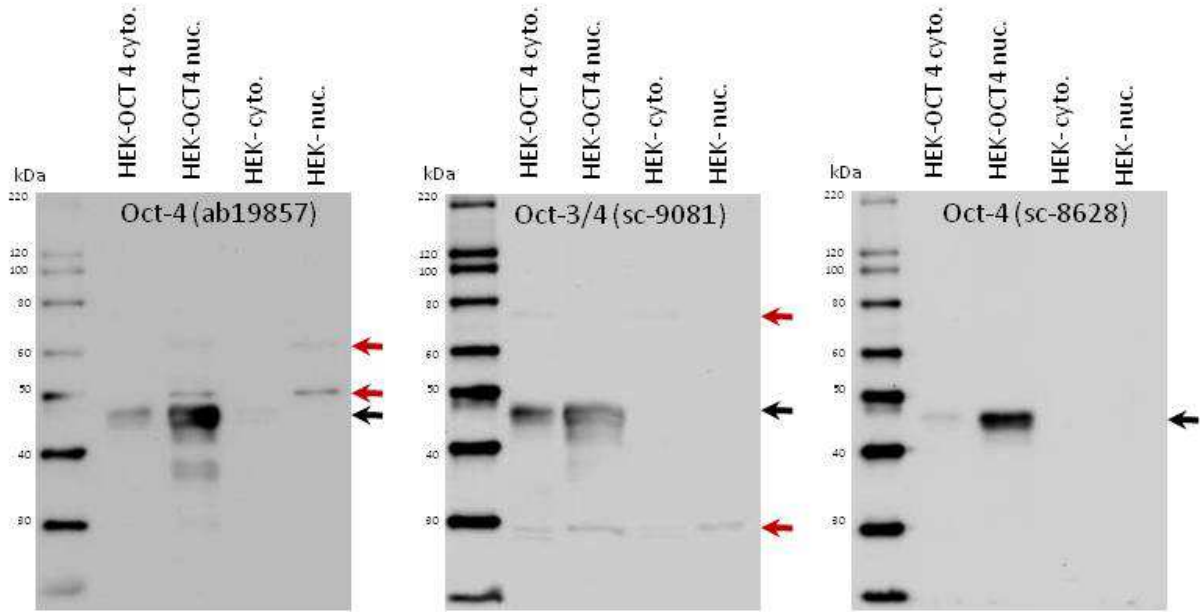

Figure 4: **Western blot analysis with three different OCT4 antibodies on cytoplasmic and nuclear protein of OCT4 transfected and non-transfected human embryonic kidney (HEK-293) cells. A) Abcam ab19857 OCT4 antibody detected the OCT4-positive control in transfected cells (black arrow). Additional unspecific bands were also detected (red arrows) in the non-transfected controls. B) Santa Cruz Sc-9081 OCT4 antibody detected the OCT4-positive control in transfected cells (black arrow). Additional unspecific bands were also detected (red arrows) in the non-transfected controls. C) Santa Cruz Sc-8626 OCT4 antibody detected exclusively the positive control. With non-transfected cells no unspecific bands were obtained.**

probably provoke erroneous interpretations when they are used in studies on pluripotent cells.Several research groups have demonstrated OCT4 either within spermatogonia-derived multi- or pluripotent stem cells {Conrad, 2008 #11; Golestaneh, 2009 #12; Kossack, 2009 #13; Mizrak, 2010 #14} or in putative testicular mesenchymal stem cells {Gonzalez, 2009 #15}. However, at least some of these results have been questioned as - according to {Ko, 2011 #21} - primers were used that did not distinguish between *OCT4A* and *OCT4B* mRNA isoform (Conrad *et al.*, 2008; Golestaneh *et al.*, 2009; Mizrak *et al.*, 2010). Furthermore, the detection of the *OCT4A* mRNA becomes problematic due to the fact that at least six (at least partially transcribed) *OCT4* pseudogenes are present in the human as well as in the marmoset genome which may be detected by RT-PCR (Liedtke *et al.*, 2008).

For the OCT4 analysis on protein level, antibodies with possibly insufficient specificity were used in the studies on testis-derived OCT4-positive cells. Conrad and colleagues (Conrad *et al.*, 2008) used a rabbit polyclonal OCT4 antibody from abcam, which was not further specified in this report. However, it is likely that this antibody was the same as in the present study. Importantly, this antibody does not discriminate between OCT4A and OCT4B and detects an additional epitope unrelated to OCT4 (this study Fig 2 and 3 and (Bhartiya *et al.*, 2010)). Mizrak and colleagues (Mizrak *et al.*, 2010) used an antibody (Santa Cruz Sc-8629) that was directed against the C-terminal epitope of human OCT4. Hence, this antibody was also unable to differentiate between OCT4A and OCT4B. However, the nuclear localization of the OCT4 signals in the cultured cells may indicate the OCT4A variant. Nevertheless, it cannot be excluded that this antibody also binds non-specifically to a nuclear epitope like the ab19857 or the sc-9081 antibody in the present study. Further controls would be necessary to confirm the validity of these data. Gonzalez and colleagues (Gonzalez *et al.*, 2009) used an "OCT3/4 H-134" antibody from Santa Cruz, which is probably the same (sc-9081) we used in the present study. In our study this antibody produced false-positive signals delusively suggesting the presence of OCT4A. Golestaneh and colleagues (Golestaneh *et al.*, 2009) also used an OCT3/4 antibody from Santa Cruz, which was also not further specified. This lack of information prevents an evaluation of the validity of the signals. The same is true for Kossack and colleagues (Kossack *et al.*, 2009). Altogether, the expression analysis of the pluripotency factor OCT4A in some recent publications on testis-derived multi- or pluripotent cells should be further deepened.

To prevent misleading studies on OCT4 in pluripotent cells researchers should consider (i) appropriate positive and negative controls including western blot analyses, (ii) the use the appropriate OCT4A-specific antibody and (iii) to confirm their results with an independent and meaningful method on the RNA level circumventing different pitfalls like the detection of pseudogenes (Liedtke *et al.*, 2008; Tapia *et al.*, 2011).

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human reproduction ORIGINAL ARTICLE *Reproductive biology*

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 subfraction 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

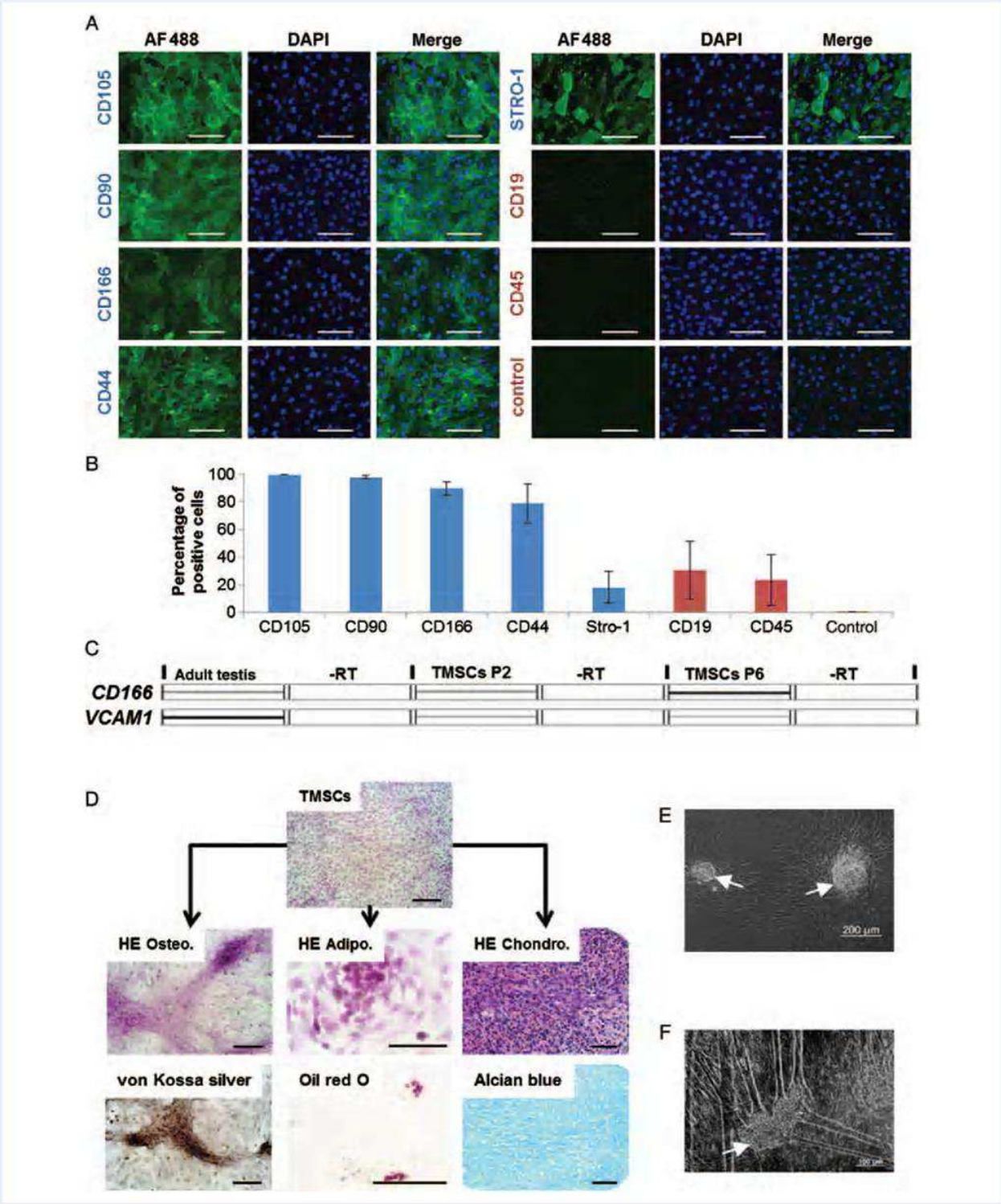

Figure 1 Characterization of TMSCs. (**A**) Immunofluorescence analysis of the cell surface markers of MSCs. Scale bar: 100 µm. (**B**) FACS analysis of MSC markers, $n = 3$. (**C**) RT–PCR on testis and two TMSC samples for MSC markers CD166 and VCAM1. (**D**) Hematoxylin–eosin staining of differentiated 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.

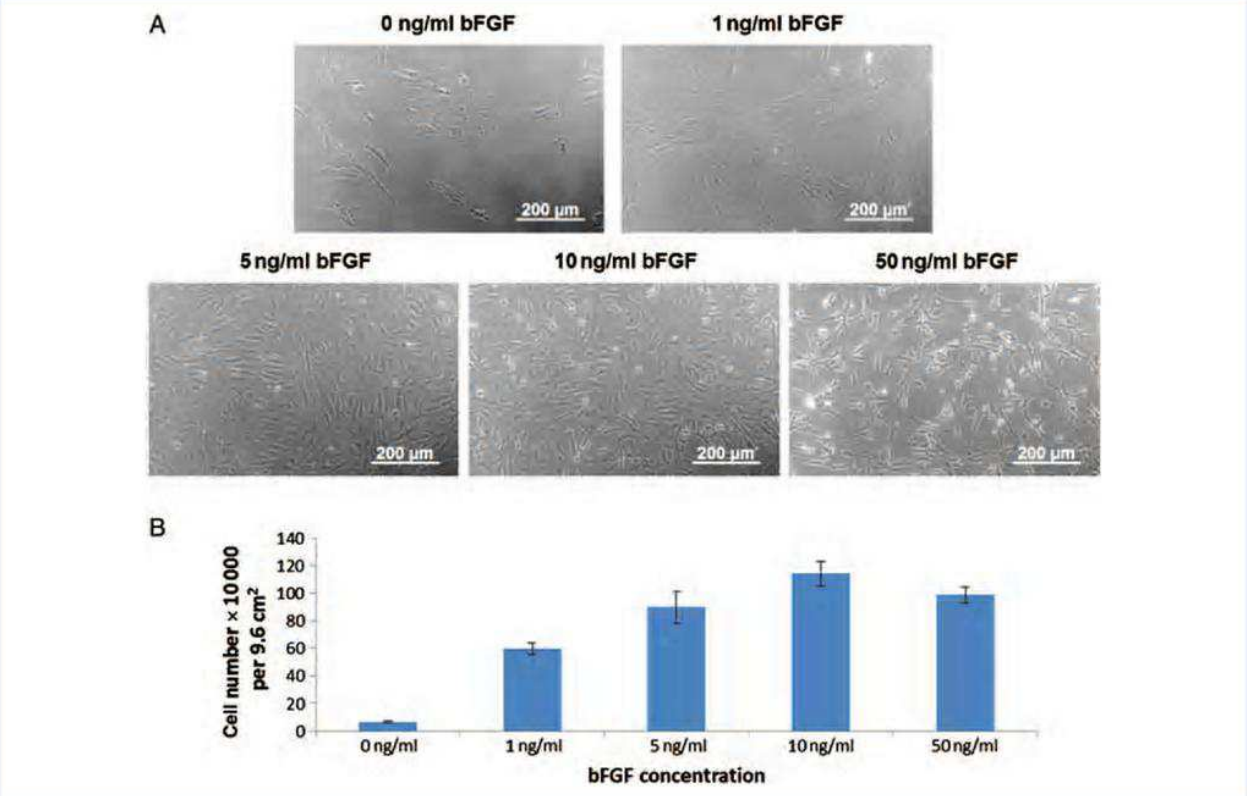

Figure 2 The effect of bFGF on TMSC morphology and proliferation. (**A**) TMSCs cultured with 0, 1, 5, 10 and 50 ng/ml bFGF, respectively. (**B**) The effect of bFGF on cell proliferation. Cell numbers determined after ~7 days of culture. Seeding density was 1×10^4 cells/cm^2. Mean value and standard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

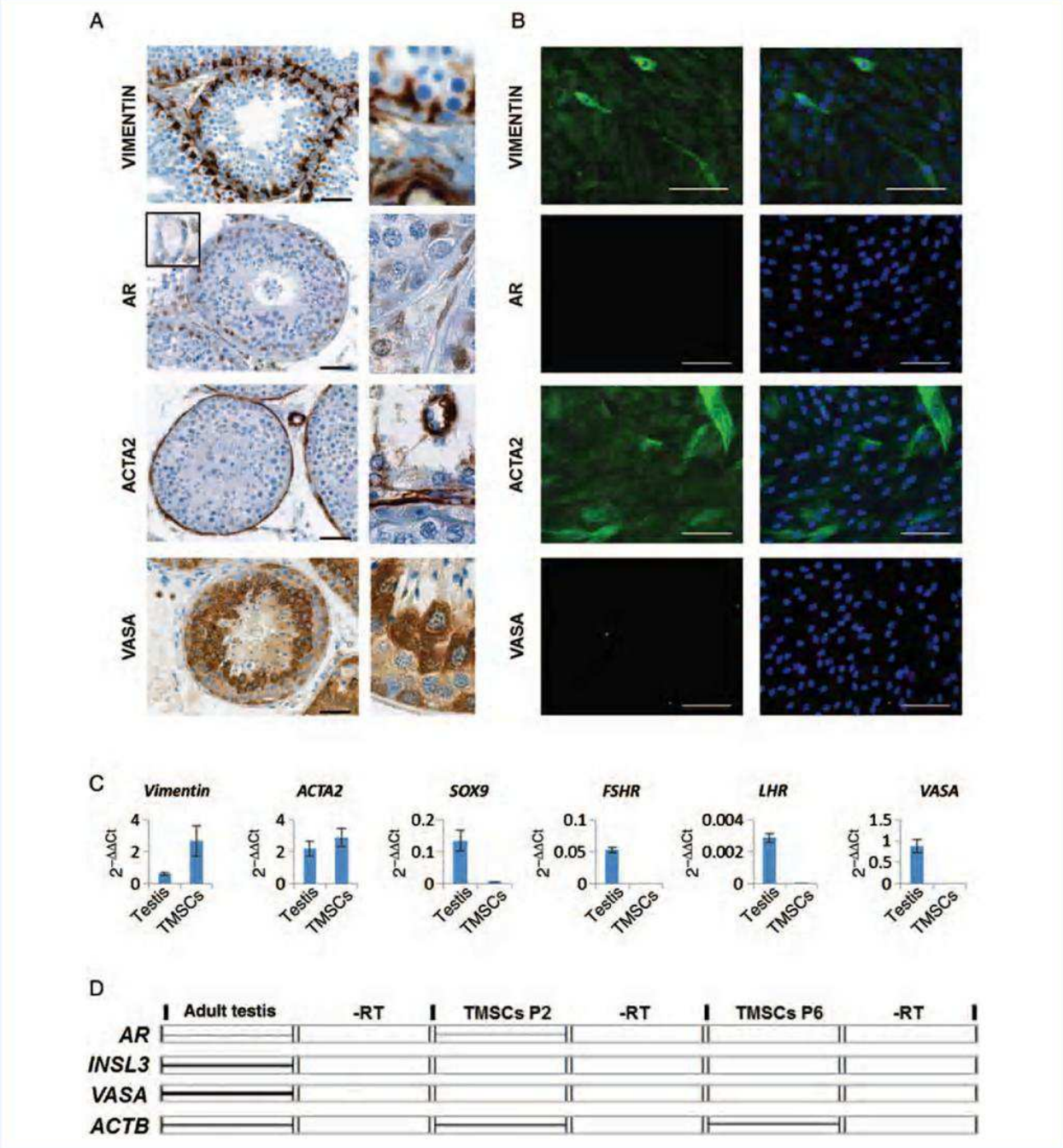

Figure 3 Analysis of the cellular origin of TMSCs. (**A**) Immunohistochemical detection of Vimentin, AR, ACTA2 and VASA in marmoset testis. Scale bar: 50 μm (**B**) Immunofluorescence detection of Vimentin, ACTA2, AR and VASA in TMSCs. (**C**) qRT–PCR for Vimentin, *ACTA2*, *SOX9*, *FSHR*, *LHR* and *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

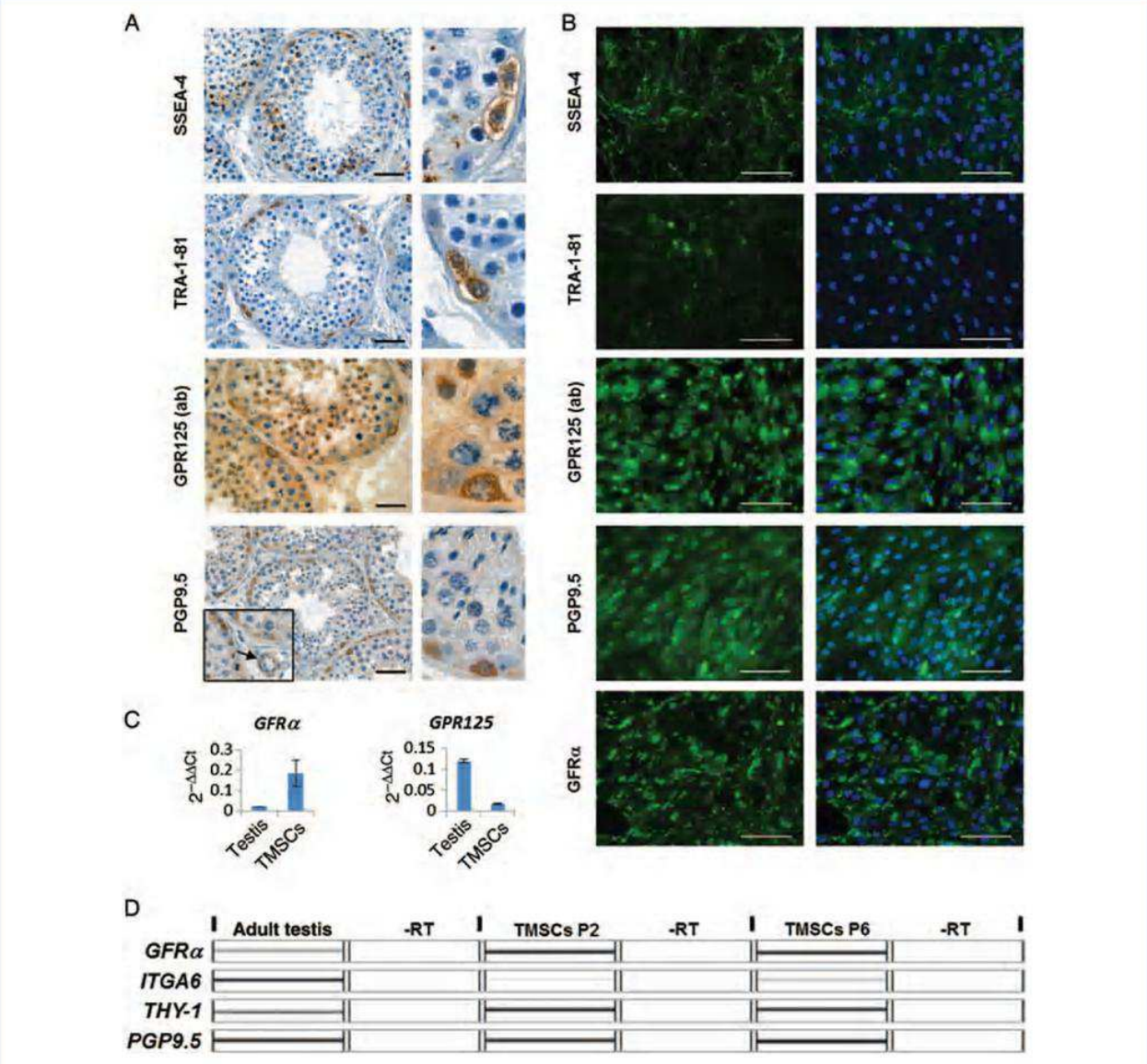

Figure 4 Several spermatogonial markers are also expressed by TMSCs. (**A**) Immunohistochemical detection of SSEA-4, TRA-1-81, GPR125 and PGP9.5 in marmoset testis. Scale bar: 50 μm (**B**) Immunofluorescence detection of SSEA-4, TRA-1-81, GPR125, PGP9.5 and GFR-α in TMSCs. (**C**) qRT–PCR for *GPR125* 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.

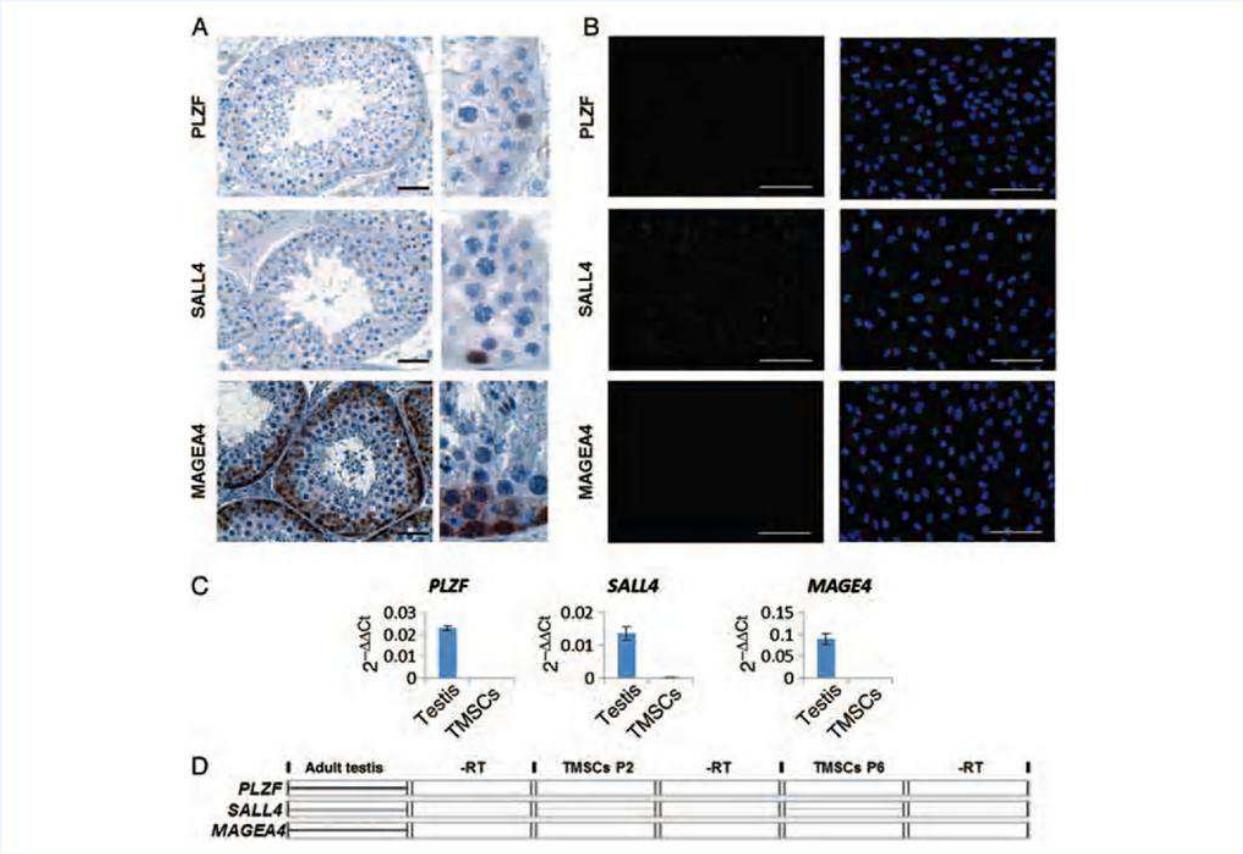

Figure 5 Spermatogonia-specific markers not expressed by TMSCs. (**A**) Immunohistochemical detection of PLZF, SALL4 and MAGEA4 in marmoset testis. Scale bar: 50 μm (**B**) Immunofluorescence detection of PLZF, SALL4 and MAGEA4 in TMSCs. (**C**) qRT–PCR analysis of *SALL4*, *MAGEA4* and *PLZF* in marmoset 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

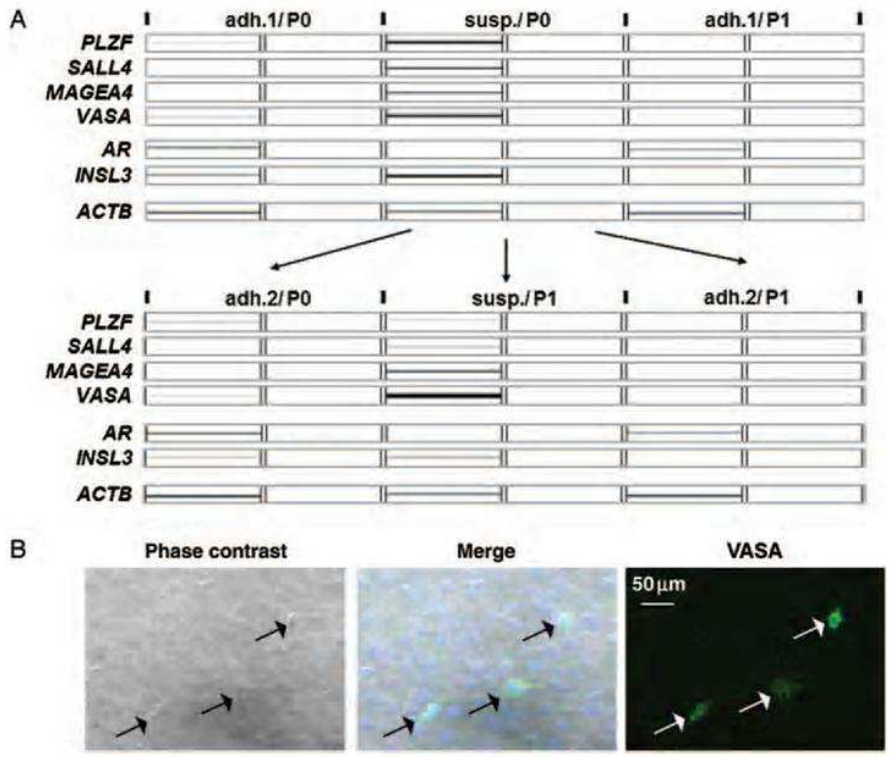

Figure 6 Identification of spermatogonia within heterogenous testicular cell populations. (**A**) RT–PCR for PLZF, SALL4, MAGEA4, VASA, AR, INSL3 and ACTB. For retrieval of the different samples, see Supplementary Fig. S1. (**B**) Immunofluorescence staining for VASA visualized all germ cells that adhered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

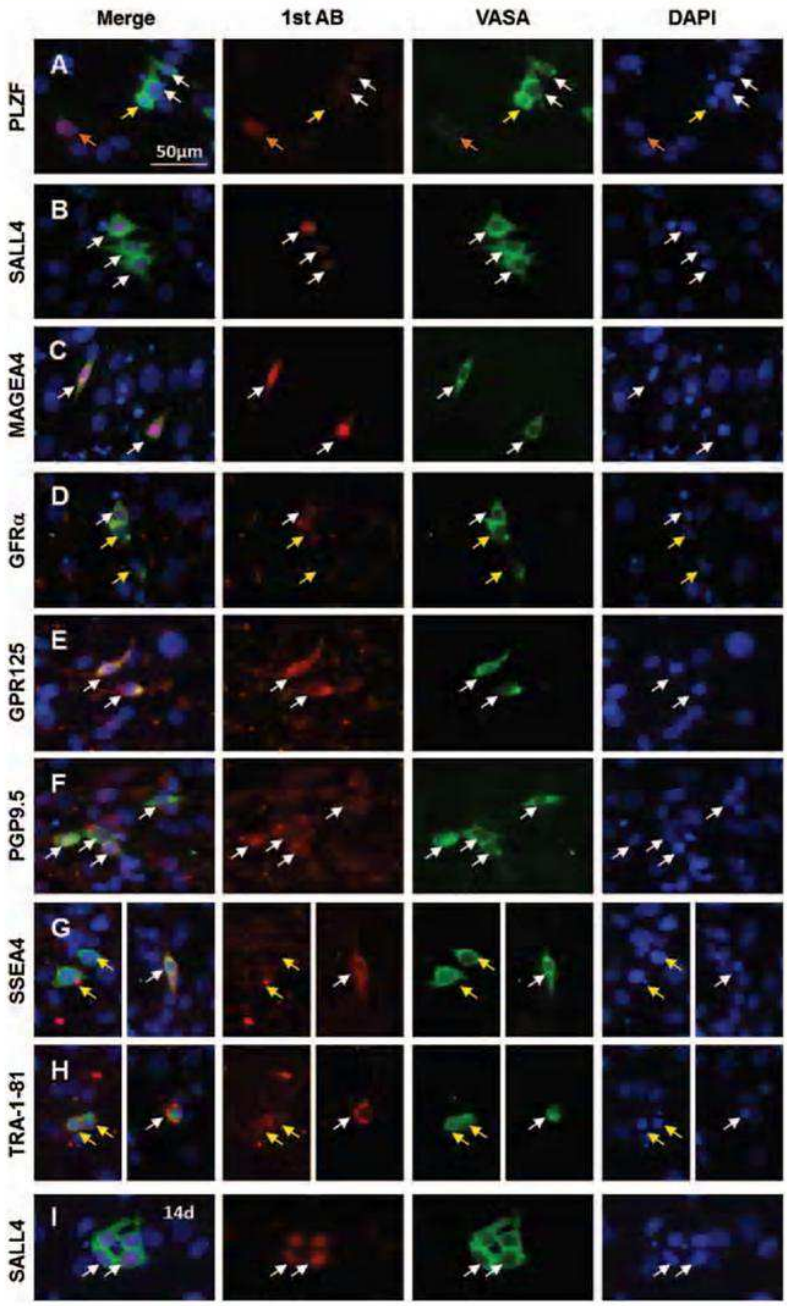

Figure 7 Characterization of marmoset monkey germ cells cultured on irradiated TMSCs. Suspension cells were seeded onto proliferation-inactivated TMSCs and cultured for 3 (**A**–**H**) or 14 (**I**) days. (**A**–**H**) Double staining of VASA and the respective spermatogonia markers. (**I**) Spermatogonia 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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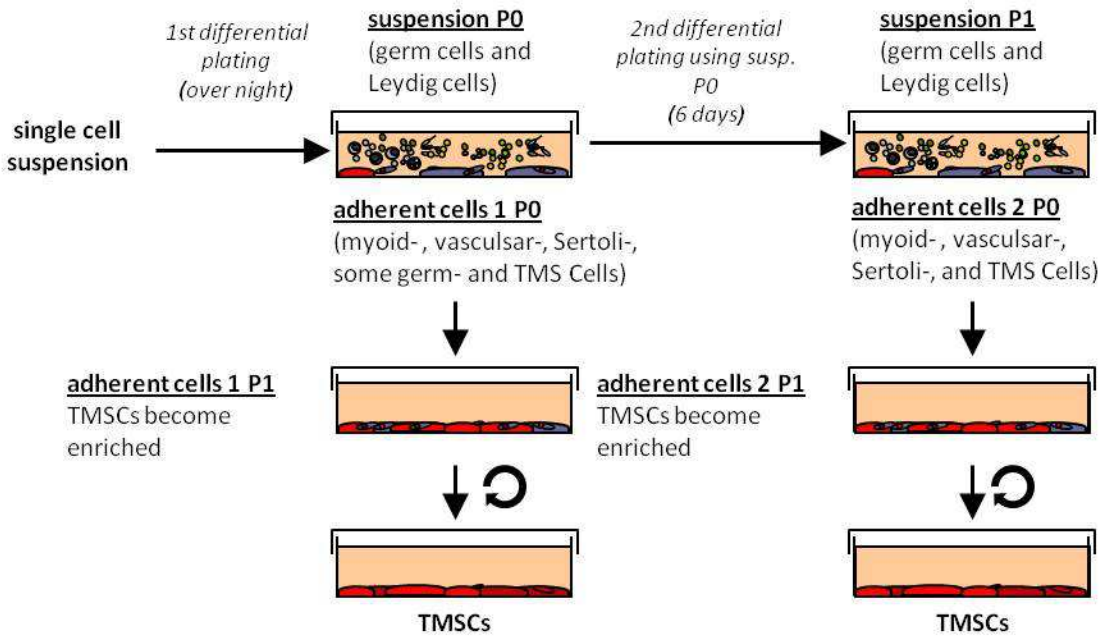
Supplement, SI: Scheme of the different adherent and none-adherent cell fractions that occur during TMSC derivation.



Supplement, SII: Composition of spermatogonia-medium (modified from Ko *et al.*)

Non-Growth Factor Components	Final Concentration
BSA (Calbiochem, #126609)	5mg/mL
d-(+) Glucose (Sigma, #G7021)	6mg/mL
NEAA (100x) (Invitrogen #11140-035)	1X
GlutaMAX-I (Invitrogen, #35050-038)	2mM
Penicillin/Streptomycin (GIBCO #15140-122)	100U/mL Penicillin 100µg/mL Streptomycin
Fetal Bovine Serum (GIBCO 10270-106)	1%
MEM Vitamins (Invitrogen #11120-037)	1X
N-2 Supplement (Invitrogen #17502-048)	1X
Fungizone, Amphotericin B (GIBCO #15290-026)	0,25µg/mL
d-Biotin (Sigma #B4639)	10µg/mL
Pyruvatic acid, sodium salt (Sigma P4562)	30µg/mL
dl-Lactic acid (60% solution) (Sigma #L7900)	0.06%
Ascorbic acid (Sigma, #A4034)	100µM
Progesterone (Sigma, P8783)	60ng/mL
ß-Estradiol 17-cypionate (Sigma, E8004)	30ng/mL
2-Mercaptoethanol (GIBCO, #31350-010)	10µM

Growth Factors	Final Concentration
ESGRO(human Lif) (Chemicon, #LIF1010)	10^3 U/mL
Forskolin (Calbiochem, #344273)	10µM
rHuGDNF (ProSpec Tany, #CYT-305)	10ng/mL
SCF (ProSpec Tany, #CYT-255)	20ng/mL
TGFß-1 (Sigma, #T7039)	0,12ng/mL

Basic Medium
StemPro34 SFM (Invitrogen #10639-011)
40x StemPro34 nutrient supplement

Supplement, SIII: List of primary antibodies used in this study.

Antigen	Company	Cat. No.	Diltuton in IF	Isotype
CD105	R&D Systems	#SC017	1:10	mm IgG
CD90	R&D Systems	#SC017	1:10	mm IgG
CD166	R&D Systems	#SC017	1:10	mm IgG
CD44	R&D Systems	#SC017	1:10	mm IgG
STRO-1	R&D Systems	#SC017	1:10	mm IgM
CD19	R&D Systems	#SC017	1:10	mm IgG
CD45	R&D Systems	#SC017	1:10	mm IgG
Vimentin	santa cruz	#sc-32322	1:50	mm IgG
P27	NCL	#NCL-p27	1:20	mm IgG
AR	santa cruz	#sc-816	1:200	rbt IgG
ACTA2	Sigma	#A2547	1:500	mm IgG
SSEA-4	Millipore	#MAB4304	1:50	mm IgG
TRA-1-81	eBioscience	#14-883	1:50	mm IgM
GPR125	Abcam	#ab51705	1:100	rbt IgG
GFRα	R&D Systems	#MAB7141	1:50	mm IgG
PGP9.5	Dako	#Z5116	1:500	rbt IgG
VASA	R&D Systems	#AF2030	1:100	gt IgG
PLZF	Sigma	#HPA001499	1:250	rbt
SALL4	Abcam	#ab57577	1:300	mm IgG
SALL4	Abcam	#ab29112	1:50	rbt IgG
MAGEA4	Giulio C. Spagnoli	alpha MAGE-A4 57B	1:30	mm IgG

Supplement, SIV: List of secondary antibodies used in this study

Antigen	Linked Fluorophore	Host Species	Company	Cat. No.
anti-mm IgG	Alexa fluor 488	donkey	Invitrogen	#A21202
anti-gt IgG	Alexa fluor 488	donkey	Invitrogen	#A11055
anti-rbt IgG	Alexa fluor 488	donkey	Invitrogen	#A21206
anti-mm IgM	Alexa fluor 488	Gt	Invitrogen	#A10680
anti-mm IgG	Alexa fluor 594	donkey	Invitrogen	#A21203
anti-rbt IgG	Alexa fluor 594	donkey	Invitrogen	#A21207

Supplement, SV: Primers used for RT-PCR in this study

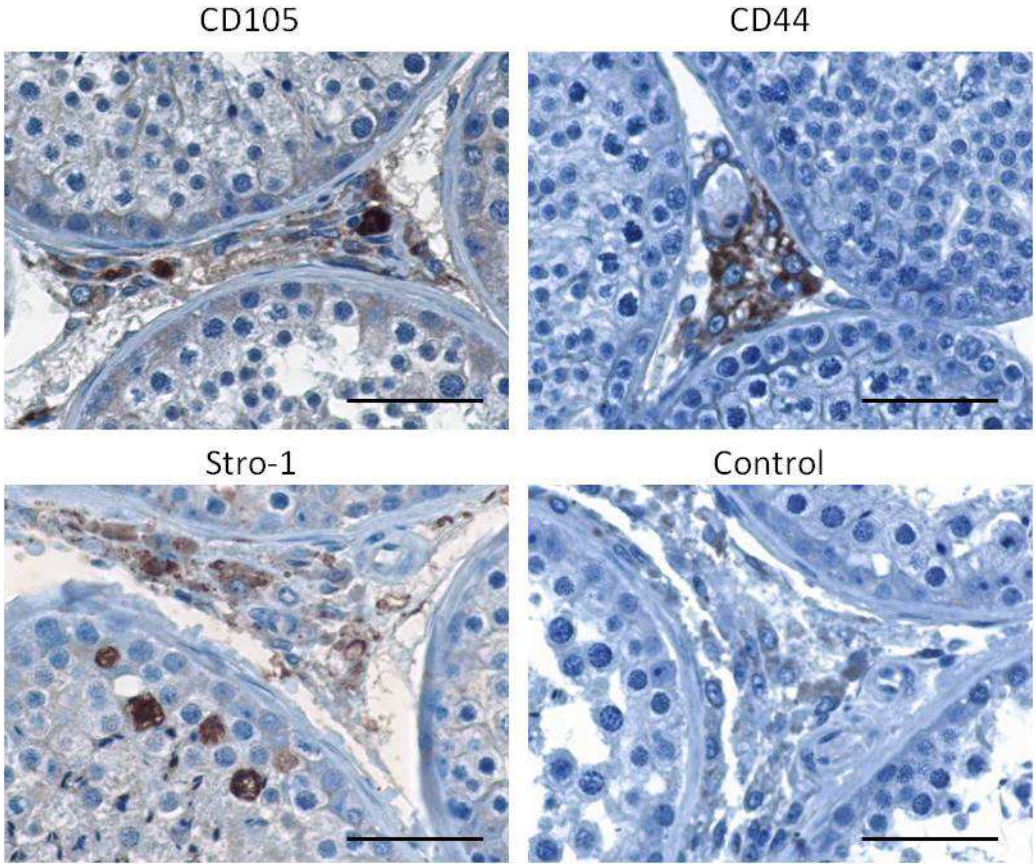
gene	forward primer	reverse primer	bp
ACTB	5'- CACTCTTCCAGCCTTCTTTCC - 3'	5'- GTGATCTCCTTCTGCATCCTG - 3'	175
AR	5'- GGCACCTCTCTCAAGAGTTTG - 3'	5'- GGCTTGACTTTCCCAGAAAGG - 3'	376
CD166	5'- CTACGTCTGCGAAACTGCTC - 3'	5'- TCATCACTTATCTCGTCTGCCTC - 3'	384
ERAB	5'- TGGTGGCGGTAATAACTGGAG - 3'	5'- TCGGCTAGGAAAGGGTACTTG - 3'	647
GFRα	5'- CAGCCAGAGTCAAGGTCTGTC - 3'	5'- GGTTATGTGGCTGGAAGCACC - 3'	495
INSL3	5'- GAGATGCGTGAGAAACTGTGC - 3'	5'- TGGTGTGGGGTAGATAGTGAG - 3'	450
ITGA6	5'- CCCATTCCCATAACTGCTTC - 3'	5'- CTTGATTTCCTTCTCGGGTG - 3'	680
Magea4	5'- GCTGAGTGTGATGGAGGTGTATG- 3'	5'- TCCTGGGCTCCCCAAAG- 3'	59
Nanos1	5'- TGCAGGTGTGCGTGTTCTG- 3'	5'- TGAGGATGTGGGTGGTGTAGAG- 3'	66
p27	5'- GCGACCTGCAATTCTTCTACTC - 3'	5'- CGTCTGTCTTCCTTGCTTCATC - 3'	205
PGP9.5	5'- CCGCGAAGATGCAGCTCAAGCCG - 3'	5'- TTAGGCTGCCTTGCAGAGAGCCAC - 3'	214
PLZF	5'- CAGTCTCCACCTCTTTTGGTC - 3'	5'- TACGTCTTCATCCCACTGTGC - 3'	739
SALL4-A	5'- GCATCAAGTCAAAGTCTCCCG - 3'	5'- CTTCTCCTTCCATGCACGTTC - 3'	803
THY-1	5'- GACCCGTGAGACAAAGAAGC - 3'	5'- GGAGCAGCAGTAGCCATGAG - 3'	278
Vasa	5'- TTGGGACTTGTGTAAGAGCTG - 3'	5'- CCCGATCACCATGAATACTTG - 3'	556
VCAM1	5'- CGAACCCAAACAAAGGCAAAC - 3'	5'- TACACCCCTGCATCCTCCAAC - 3'	516

Supplement, SVI: Primers used in qRT-PCR in this study

gene	forward primer	reverse primer	bp
ACTA2	5'- CGTGAGAAGATGACGCAGATCA- 3'	5'- CAGCCTGGATGGCCACAT- 3'	70
FSHR	5'- GAAAGTGTGACTCTATGGCTGAATAAGA- 3'	5'- CTCATCTAGTTGGGTTCCATTGAA- 3'	81
GAPDH	5'- TGCTGGCGCTGAGTATGTG- 3'	5'- AGCCCCAGCCTTCTCCAT- 3'	64
GFRα	5'- AGGACTCCTGCAAGACAAATTACA- 3'	5'- TGGCTGGCAGTTGGTAAAAAA- 3'	68
GPR125	5'- CCGCAGCAGCGAACATTA- 3'	5'- CACGCCATCCAGCAATAGG- 3'	64
LHR	5'- GAGCCGGGAGCATTTACAAA- 3'	5'- TGGAAACTTTCTAATGCCTGTGTTA- 3'	75
Magea4	5'- GCTGAGTGTGATGGAGGTGTATG- 3'	5'- TCCTGGGCTCCCCAAAG- 3'	59
Nanos1	5'- TGCAGGTGTGCGTGTTCTG- 3'	5'- TGAGGATGTGGGTGGTGTAGAG- 3'	66
PLZF	5'- GAGACGCACAGACAGACCCATA- 3'	5'- TCCCACACAGCAGGCAGAA- 3'	61
SALL4	5'- AAGGCAACTTGAAGGTTCACTACA- 3'	5'- GATGGCCAGCTTCCTTCCA- 3'	77
SOX9	5'- TGGGCAAGCTCTGGAGATTT- 3'	5'- TCCGCCTCCTCCACGAA- 3'	60

Vasa	5'- AAGTATTAACAGATGCTCAACAGGATGT- 3'	5'- TGAAGCCAGGAATGTATGCACTA- 3'	78
VIM	5'- TCCCTGAACCTGAGGGAAACT- 3'	5'- CGTCTTAATCAGAAGTGTCCTTTTTG- 3'	81

<u>Supplement, SVII:</u> IHC of multipotent stromal cell (MSC) markers on marmoset testis tissue sections. All MSC markers stained the interstitial cells. Scale bar 100µm.



6 SUMMARY AND CONCLUDING REMARKS

When this work started in April 2008 it had been demonstrated for mice (Guan *et al.*, 2006; Seandel *et al.*, 2007) that pluripotent stem cells can be derived from adult spermatogonia. Similar findings were published for the human by the end of 2008 (Conrad *et al.* 2008). Therefore, deriving pluripotent stem cells from marmoset spermatogonia seemed quite promising.

Clear identification of the different testicular cell types was of major importance for the realization of this project. *In situ*, a cell can be identified inter alia through its location within a tissue. This criterion is missing *in vitro*. Furthermore, several typical morphological criteria get lost in culture due to the changes of physical forces that affect a cell. Functional assays for the identification of spermatogonia or pluripotent cells were also not available for the common marmoset. Consequently, reliable identification of the different testicular cells could only be provided with unequivocal markers. One part of the thesis was therefore to establish a detailed *in situ* characterization of the different testicular cell types followed by the establishment of a feasible marker-panel that enabled the identification of cultured testicular cells (Table 2, panel 1-5).

Marker / cell type	spermato-gonia	later germ cells	Leydig cells	Sertoli cells	myoid cells	TMSCs	ESCs
Vimentin	-	-	-	X	X	X	ND
AR	-	-	X	X	X	-	ND
ACTA2	-	-	-	-	X	X	X
SOX9	-	-	-	X	-	-	X
FSHR	-	-	-	X	-	-	-
LHR	-	-	X	-	-	-	X
INSL3	-	-	X	-	-	-	-
VASA	X	X	-	-	-	-	-
GPR125	X	-	-	-	-	X	ND
PGP9.5	X	-	-	-	-	X	X
GFRα	X	-	-	-	-	X	ND
THY-1	X	-	-	-	-	X	ND
SSEA4	X	-	-	-	-	X	X
TRA-1-81	X	-	-	-	-	X	X
TRA-1-60	X	-	-	-	-	X	X
PLZF	X	-	-	-	-	-	X
SALL4	X	-	-	-	-	-	X
MAGEA4	X	X	-	-	-	-	-
OCT4	-	-	-	-	-	-	X

Table 2: Summary of the marker-panel that enables reliable discrimination of different testicular cell types, cultured TMSCs and pluripotent embryonic stem cells (X: gene is expressed; -: gene is not expressed; ND: not determined or inconclusive).

Over the course of characterizing marmoset spermatogonia, several pluripotency-related genes were found to be expressed, such as TRA-1-60, TRA-1-81, SSEA4 (Müller *et al.*, 2008) and SALL4. Furthermore, SALL4 was detected in marmoset pre-implantation embryos, embryonic stem cells and prenatal and newborn germ line stem cells. These findings suggest that although spermatogonia are determined very early in development they remain in a rather undifferentiated state. We also found that the expression of SALL4 in germ line stem cells and spermatogonia was highly conserved in different mammalian species, indicating that the presence of SALL4 in spermatogonia might be fundamental for these cells. The data on SALL4 are contained in the manuscript "Developmental expression of the pluripotency factor SALL4 in the monkey, human, and mouse testis: restriction to premeiotic germ cells." (Chapter 4) which was accepted for publication in the journal "Cells Tissues Organs" (DOI: 10.1159/000335031; publisher: S. Karger AG, Basel) in 2012.

The second part of this thesis included the culture and propagation of spermatogonia to eventually derive pluripotent stem cells. However, all attempts to accomplish this goal resulted in the enrichment of a fibroblast-like cell type. When these mono-layered cells were cultured up to confluency or passaged without full dissociation they generated colonies that morphologically resembled the claimed pluripotent colonies described for the human (Conrad *et al.*, 2008; Kossack *et al.*, 2009, Golestaneh *et al.* 2009; Mizrak *et al.*, 2010). These morphological characteristics suggested that the colonies described in earlier publications and the ones seen in this study both consisted of the fibroblast-like cells. Initial immunofluorescence experiments suggested the expression of the transcription factor OCT-4, indicating that the cells were pluripotent. Further analysis using three different OCT-4 antibodies as well as quantitative RT-PCR lead to the conclusion that false-positive signals were obtained with two of the tested antibodies. This potential source of data misinterpretation was summarized in the manuscript "False-positive antibody signals for the pluripotency factor OCT-4A (POU5Fl) in testis derived cells may lead to erroneous data and misinterpretations" (Chapter 5). This manuscript was submitted to the journal Molecular Human Reproduction and completed in collaboration with Rita Warthemann, a PhD student in the Stem Cell Biology Unit of the German Primate Center.

Acknowledging that the fibroblast-like cells were not pluripotent stem cells, the question of their possible identity was addressed. Using the established marker panel (Table 2) it was found that many markers considered germ cell- or spermatogonia-specific within the testes by *in situ* analysis (e.g. SSEA4, TRA-1-81, GFRα, GPR125, THY-1 (CD90), and ITGA6) were also expressed in these unknown cells while others, namely VASA, SALL4, PLZF and MAGEA4 were missing. Interestingly,

using a standardized human marker-panel and standardized human differentiation assays, these cells were identified as TMSCs.

The identification of the fibroblast-like cells as TMSCs was in accordance with data that was very recently published by van Pelt group (Chikhovskaya *et al.*, 2012). The authors demonstrated that their own previously published htES-like cells (Mizrak *et al.*, 2010) were also no pluripotent cells as suggested previously but rather TMSCs.

The present study was the first to demonstrate the significant overlap in marker expression of TMSCs and spermatogonia. One major consequence was that there is now a complete lack of spermatogonia-specific cell surface markers leading to a lack of targets that enable specific enrichment of spermatogonia.

Acknowledging this overlap of spermatogonia and TMSC expressed genes, markers were selected that were not expressed in TMSCs (i.e. VASA, SALL4, MAGEA4 and PLZF) were selected to distinguish TMSCs from cultured spermatogonia. With this method it was possible to establish a culture method with TMSCs as a feeder layer. Although spermatogonia were not significantly propagated, with this system the cells were cultured for up to 14 days. All data on TMSCs and the culture of spermatogonia are published in the journal Human Reproduction in the manuscript "Misleading and reliable markers to differentiate between primate testis-derived multipotent stromal cells and spermatogonial stem cells in culture" (http://humrep.oxfordjournals.org/content/early/2012/03/22/humrep.des091.full.pdf+html).

Summarizing the results achieved in this theses, we succeeded in (I) the establishment of an *in situ* characterization of the different testicular cell types (Chapters 4 and 6) and (II) the establishment of a feasible marker-panel that enables a clear identification of the different testicular cell types in culture (Chapters 4, 5 and 6). (III) Although we were not able to propagate common marmoset spermatogonia we established a system to culture these cells for up to 14 days (Chapter 6). (IV) We were not able to reprogram common marmoset spermatogonia into a pluripotent state. However, the recent literature suggests that this was likely also never achieved for the human (Ko *et al.*, 2010; Tapia *et al.*, 2011; Chikhovskaya *et al.*, 2012). We further (V) established a culture-system for common marmoset TMSCs and were able to (VI) characterize them according to standard procedures that are also used for human MSCs (Chapter 6).

Furthermore, we discovered a great overlap in marker-expression of spermatogonia and TMSCs (Chapter 6). That provides a potential explanation for the past failure of deriving pluripotent stem cells from primate spermatogonia and importantly reveals a lack of spermatogonia-specific cell-surface markers that can be used to specifically select and enrich spermatogonia.

The data achieved in this thesis provides a good basis for future studies on germ cell culture. The established marker-panel should be used to clearly identify the different testicular cell types in culture. Most importantly, combinations of the markers PLZF, VASA, SALL4 and MAGEA4 should be used to distinguish spermatogonia and TMSCs. With this tool future spermatogonia-culture approaches can be evaluated and eventually a protocol can be selected that allows the propagation of spermatogonia.

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7 STATUTORY DECLARATION

I, Katja Eildermann, hereby declare that I have written this PhD thesis independently, unless where clearly stated otherwise. I have used only the sources, the data and the support that I have clearly mentioned. This PhD thesis has not been submitted for conferral of degree elsewhere.

place, date

Katja Eildermann

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