Developmental Expression of the Pluripotency Factor Sal-Like Protein 4 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, which is highly expressed in embryonic stem (ES) cells and which is essential for mouse preimplantation 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 also a high expression of SALL4 in human testicular germ cell tumors. However, there is as yet no detailed analysis of SALL4 expression during mammalian testicular development. We analyzed SALL4 expression in ES cells, preimplantation embryos, and the developing and adult testis of a nonhuman 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 and newborn and adult testis by RT-PCR and Western blotting showed two different isoforms,

SALL4-A and SALL4-B. Immunohistochemistry 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 also exhibited SALL4 in undifferentiated spermatogonia, indicating a

Abbreviations used in this paper

ES	embryonic stem (cell)
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

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.

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Introduction

SALL4 (sal-like protein 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 [Jürgens, 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 led 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/only very limited numbers of atypical embryonic stem (ES)-cell-like cell lines could be established from *Sall4*-deficient mouse embryos; this proves 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 ES cells. 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; 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 the key factors for pluripotency. 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 cells [Tsubooka et al., 2009]. In another experimental setup, where somatic cells were fused with ES 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, 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 et al. [2002a] showed, 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 the strongest signals were obtained – apart from 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, there is still no detailed study of SALL4 expression in the developing and adult mammalian and specifically the primate gonad.

Here we demonstrate SALL4 expression in the nonhuman primate (NHP) preimplantation embryo and in ES 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 gonad 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 the second and third trimester) were obtained after spontaneous miscarriages. The gonads were collected with-

in 24 h after death and postmortem examinations were carried out in the Department of Pathology, 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 gonad 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 a 1-year-old boy exhibited an age-appropriate developmental stage and was obtained for 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, Medical Faculty of the University of Münster) for these investigations to be performed. Biopsies were carried out for diagnostic purposes during vasectomy reversal in 4 patients (5 testes samples) with obstructive azoospermia after vasectomy. Testicular tissue was fixed by immersion in Bouin's fixative and embedded in paraffin using standard techniques. For histological evaluation, 5- μ m paraffin sections were stained with hematoxylin. Histological evaluation revealed normal spermatogenesis in these testes.

Marmoset Monkey Material and Tissues

Collection of Preimplantation Embryo

The collecting of common marmoset monkey embryo was basically carried out as described earlier [Sasaki et al., 2005; Müller et al., 2009]. All experiments were performed according to the German Animal Protection Law. Animals were housed according to standard German Primate Center practice for the common marmoset. Briefly, marmoset preimplantation 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, nonextraction enzyme immunoassay using an antiserum raised in sheep against progesterone-11-hemisuccinate-BSA. The assay has been previously described for determining ovulation in the marmoset monkey by Heistermann et al. [1993] except that progesterone-3-CMO-peroxidase was used as enzyme conjugate.

Embryonic Stem Cell Culture

Common marmoset monkey ES cells (line cjes001) were cultured on γ -irradiated mouse embryonic fibroblasts (MEFs) in medium consisting of 80% Knockout DMEM and 20% Knockout Serum Replacement supplemented with 2 mM GlutaMAX™, 0.1 mM MEM nonessential amino acids, 0.1 mM β -mercaptoethanol (all obtained from Gibco), 100 IU/ml penicillin and 100 mg/ml streptomycin as described previously by Müller 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). 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

Preimplantation embryos were fixed for 30 min in 2% paraformaldehyde containing 0.02% Triton X-100. ES cells were grown on γ -irradiated MEFs in foil-bottom 24-well plates (Lumox™, Greiner Bio-One, Stuttgart, Germany) for 2–5 days, fixed for 30 min in 2% paraformaldehyde, 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-hour incubation in first antibody (SALL4, abcam #ab57577) dilution (1:200) at 4°C, cells were washed twice in PBS, incubated for another 60 min 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 h. After several washes in 70% EtOH for at least 2 days, the tissues were embedded in paraffin and sectioned at 5 μ m. 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 (Universal LSAB Plus kit, K0679; DakoCytomation, Carpinteria, Calif., USA). 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 1:200–1:300 dilution in 5% BSA in TBS. Identical staining results were obtained with another SALL4 antibody (abcam ab29112, rabbit polyclonal). All incubation steps were conducted in a humid chamber and incubations with the primary antibody were performed overnight 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 chromogen was used as a substrate for the HRP and Mayer's hematoxylin as a counterstain. Control stainings were carried out omitting the primary antibody and by replacing the specific primary antibody with the respective unspecific control immunoglobulin.

Western Blot Analysis

Protein from approximately 50 mg of tissue or cell culture material from up to one 9-cm 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 200 μ l RIPA 2 resuspension buffer [0.15 M NaCl, 15 NP-40, 1% LDS and 2% SARKOSYL (N-Lauroylsarcosine)-

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

SALL4 forward	5'-atgtcaggcgcaagcaggc-3'	marmoset SALL4-A: 3,160 bp
SALL4 reverse	5'-cttagctgaccgcaatctgttttc-3'	marmoset SALL4-B: 1,852 bp
SALL4 qFw	5'-aaggcaactgaaggttcactaca-3'	marmoset SALL4
SALL4 qRe	5'-gatggccagcttccttcca-3'	both isoforms: 77 bp
mmSALL4 qFw	5'-gcgggcgggccttca-3'	mouse SALL4
mmSALL4 qRe	5'-tgttgccccatgagtcacat-3'	both isoforms: 63 bp
ACTB forward	5'-cactcttcagccttcttcc-3'	marmoset ACTB: 175 bp
ACTB reverse	5'-gtgatctccttctgcatcctg-3'	
GAPDH forward	5'-tgctggcgctgagtatgtg-3'	marmoset GAPDH: 64 bp
GAPDH reverse	5'-agccccagccttctccat-3'	

trium salt)]. For Western blot analysis, 15–20 μ l of the protein lysate (including 10 $\times$ DTT and 4 $\times$ loading buffer) and 5 μ l Novex sharp prestained protein standard from Invitrogen were 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 (1 $\times$ PBS with 0.1% Tween-20) and blocked for 30 min in 5% skim milk/0.1% normal goat serum/PBS-T. Primary antibody incubation was performed for 1 h at room temperature or overnight at 4°C. All antibodies [mouse-anti-SALL4 (abcam, ab57577; dilution 1:3,000), mouse-anti-beta-actin (Sigma, A1978; dilution 1:1,000)] 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 approximately 50 mg frozen-tissue material or cell culture material from up to one 9-cm dish was isolated using the RNeasy mini Kit from Qiagen. Reverse transcription was performed using the Omniscript reverse transcriptase (Qiagen). PCRs (32 cycles) were performed with resulting cDNA using Taq Hot Start polymerase from Novagene. PCR products were analyzed using the QIAxcel System (Qiagen, Hilden, Germany) or standard gel electrophoresis. All PCR products were sequenced by linear congruential generator 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 4 newborn, 4 eight-week-old and 4 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 mar-

moset 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. Identity of the amplicon was confirmed by DNA sequencing. QPCR for each primer pair was 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 μ g of testicular RNA was reverse-transcribed, using random hexamers, by Superscript II (Invitrogen, Karlsruhe) to obtain cDNA; 2 μ l of 1:2-diluted cDNA was used for each 20- μ l PCR reaction with Power SYBR Green Mastermix (Applied Biosystems) and different primer concentrations ranging between 50 and 900 nM. The PCR programme consisted of an initial step of denaturation (5 min at 95°C) followed by 40 cycles of denaturation (15 s at 95°C) and annealing (1 min at 60°C) and elongation (1 min at 60°C). The extent of fluorescence of the Power SYBR green dye was detected and analyzed using the ABI Prism® 7000 SDS software (Applied Biosystems). Each sample was assayed in triplicate and normalized to glyceraldehyde-3-phosphate dehydrogenase expression. Relative quantification was based on the $2^{-\Delta\Delta C_T}$ method [Livak and Schmittgen, 2001].

Results

SALL4 Protein Is Highly Abundant in Marmoset ES Cells and in Newborn Testis

In order to obtain an overview of the general SALL4 protein expression and abundance in newborn and adult NHP testes, we performed Western blot analysis (fig. 1a). Protein from 4 different passages of the recently established pluripotent *Callithrix jacchus* ES cell line Cjes001 [Müller et al., 2009] was included as a positive control for this pluripotency factor. All 4 ES cell protein samples exhibited 2 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 about 140 kDa. A fourth band of minor intensity of about 100 kDa was detected only in ES cells. Three bands corresponding to the 160-kDa (SALL4-A), 140-kDa and 80-kDa (SALL4-B) 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 2 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 1 (PND1), PND9 and adult testis. Newborn and PND9 testes 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

Since Western blot analysis suggested the presence of different SALL4 isoforms in the testis, we also performed SALL4 RT-PCR expression analyses including newborn

and adult marmoset testes and ES cells. The *SALL4* gene has 4 exons (fig. 1b), and all exons contribute to the open reading frame (ORF). This gene structure is highly conserved in man, NHPs, mouse and other mammals (<http://www.ensembl.org/index.html>). Two SALL4 isoforms were detected in undifferentiated marmoset ES cells (fig. 1c). Besides the longest ORF (corresponds to isoform A; size of respective PCR product: 3,160 bp), there was an alternatively spliced transcript which encodes SALL4 protein isoform B (1,852-bp PCR product). The shorter ORF resulted from the use of an alternative splice donor site in the second exon thereby excluding the 1,308 nucleotides located at the 3' end of exon 2 (fig. 1b). Performing nonquantitative RT-PCR (32 cycles), both SALL4 transcripts were detected in the newborn and adult marmoset testis (fig. 1c).

In order to quantify the total SALL4 transcript abundance (isoforms A and B together) during postnatal testis development in the NHP and in the mouse, we performed quantitative real-time PCR on 4 newborn, 4 eight-week-old, and 4 adult marmoset testes (fig. 1d) and 4 newborn, 4 nine-day-old and 4 adult mouse testes (fig. 1e) using primers located in exons 3 and 4 (fig. 1b). SALL4 primer qFw (intron-spanning to prevent amplification of genomic DNA during PCR) and qRe detected approximately 20-fold more SALL4 transcripts in the adult marmoset testis than in the newborn testis. The 8-week-old testis showed an intermediate level with high variability between the different samples (fig. 1d). In contrast to the increasing transcript levels in the marmoset, the mouse testis exhibited a relative decrease of *Sall4* during testis development (fig. 1e).

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 preimplantation 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 also pluripotency-associated in the NHP *Callithrix jacchus*.

Fig. 1. SALL4 protein and transcript expression in neonatal and adult marmoset testes. **a** Western blot analysis demonstrates strong SALL4 expression in the undifferentiated pluripotent ES cells (ESC), which served as positive control. In ES cells, 4 different bands were seen. The strongest bands represent SALL4 isoforms A and B, respectively. The lower band between isoform A and B can be detected only in ES cells. SALL4 was undetectable in MEFs. Robust SALL4 signals were seen in the total protein preparations from 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** Nonquantitative RT-PCR reveals the presence of SALL4 isoforms A and B in undifferentiated ES cells as well as in newborn and adult testes. Only isoform B is weakly detectable in MEFs. **d** Quantitative real-time RT-PCR of marmoset testes. Primers (qFw and qRe) are indicated in **b** and amplify both SALL4 transcripts shown. In contrast to the protein, which is downregulated in the adult gonads, SALL4 transcripts are upregulated about 20-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 shows a relative decrease of *Sall4* expression during postnatal testis development.

Fig. 2. SALL4 in common marmoset preimplantation embryos. **a** SALL4 expression in a morula-stage embryo. The protein is detectable in all cells. **b** SALL4 expression in a blastocyst-stage embryo. SALL4 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 μ m.

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 (PGCs), gonocytes and (pre)spermatogonia in the fetal, newborn and prepubertal 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, i.e. PGCs (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 indi-

vidual gonocytes in the fetal and postnatal testes, and a subfraction of these germ cells exhibited no SALL4 signal at all (e.g. fig. 4a, d: yellow and blue arrows, respectively). In pubertal marmoset monkey testis (fig. 4f), the SALL4-positive germ cells became more and more scattered as the testis cords/seminiferous tubules grew (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 figure 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 signals or

Fig. 3. SALL4 expression in marmoset monkey ES cells (ESC). 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 .

only faint ones (fig. 5a: green arrows). The pediatric testis from a 1-year-old boy exhibited normal age-appropriate spermatogenesis with central prespermatogonia 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 the limitations of the sample size and the 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.

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 analyzed the 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 testes. All early gonocytes and PGCs outside the

cords in the forming mouse testes from embryonic day 12.5 were strongly SALL4-positive (fig. 5d). However, the signal distribution within the nuclei differed between different gonocytes. 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 3-day-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 post-meiotic spermatids sometimes showed very faint signals.

Spermatogonial SALL4 Expression Is Conserved in Additional Primate and Nonprimate Species and Exhibits No Stage Dependency

The expression pattern of SALL4 in marmoset monkey, human and mouse testes was confirmed in adult testes from Goeldi's marmoset monkey (*Callimico goeldii*,

Fig. 4. SALL4 expression in the prenatal and postnatal marmoset testis. **a** Early fetal testis during the transition from embryonic to fetal development (approx. gestational day 70; normal duration of pregnancy in marmosets is 143 days). The first testicular cords are forming (highlighted by the red dashed line), but they still lack differentiated peritubular cells. There were SALL4-positive and SALL4-negative (yellow arrow) germ cells within the cords. Some SALL4-positive germ cells with large nuclei and a large soma are still surrounded by stromal tissue, i.e. they are PGCs (red arrow). The green arrow points to the epithelium of the developing epididymis. **b** Higher magnification of **a** showing the large and prominent nucleus and the ring-like cytoplasm of the PGCs (red arrow). **c** Fetal testis (gestational day 100). SALL4 is specifically expressed in gonocytes (yellow arrows) and prespermatogonia

(red arrows), which are located in clearly delimited testicular cords. **d** Newborn testis. SALL4 is expressed in gonocytes (red arrows). Prespermatogonia were sometimes negative for SALL4 (dark blue arrows). **e** Prepubertal testis (21 weeks of age). Puberty starts at the age of 8–10 months. SALL4 was detected in different intensities in gonocytes and immature spermatogonia (red arrows). **f** Early pubertal testis (30 weeks of age) with first meiotic germ cells (blue arrow). SALL4 is restricted to spermatogonia (red arrows). **g** Adult testis. SALL4 was detected in spermatogonia. **h** Adult testis. Higher magnification showing details of SALL4-positive spermatogonia. Strongest SALL4 signals were detected in the nuclei of the spermatogonia. Weaker signals were also seen in the cytoplasm. **i** Newborn testis. Negative control. The scale bar represents 50 μm in all figures.

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 tree-shrew (*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 showed strong SALL4 expression in undifferentiated spermatogonia and no/only faint signals in later germ cell stages. In all the species analyzed, we did not observe any stage-dependent expression of SALL4 in spermatogonia.

Fig. 5. SALL4 expression in the human (a–c) and mouse (d–i) testis. **a** Fetal human testis (gestational week 21, formalin-fixed instead of with Bouin’s solution). SALL4 is present in gonocytes (red arrows). Prespermatogonia are only faintly stained (green arrows). **b** One-year-old testis showing nuclear stain in all (pre)spermatogonia. **c** Adult testis with complete spermatogenesis. SALL4 is strongly expressed in spermatogonia. **d** Mouse testis from embryonic day 12.5 (E12.5). **Inset** SALL4 exhibits strong and specific expression in the intragonadal gonocytes as well as in late extragon-

nadal PGCs. Some gonocytes have homogenous signal distribution within the nucleus (green arrows), while others show rather peripheral nuclear staining (red arrows). **e** PND3 testis. Spermatogonia were SALL4-positive. **f** PND9 testis. Spermatogonia show moderate or strong SALL4 expression. **g** PND27 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. hs = Human (*Homo sapiens*); mm = *Mus musculus*.

SALL4 Is Expressed in Type A Spermatogonia in the Adult Marmoset and Human Testis

All premeiotic 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 germi-

nal 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

Fig. 6. SALL4 expression in additional mammalian testes. **a** Goeldi's marmoset (*Callimico goeldii*). **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 representatives of nonprimate mammalian species. All species analyzed expressed SALL4 in undifferentiated spermatogonia (highlighted by red arrows). Cat and dog also showed weak signals in some later germ cell stages. The scale bar represents 50 μm in all figures.

in the SALL4 staining intensities indicating molecular heterogeneity between the cells belonging to one specific histologically identifiable type of spermatogonia.

Discussion

In this work we analyzed the expression of the pluripotency-associated transcription factor SALL4 during male germ cell development in common marmoset mon-

key as well as in preimplantation embryos and ES cells. Recently, Cauffman et al. [2009] found SALL4 expressed in the nuclei of human preimplantation embryos and in undifferentiated human ES cells. We confirmed strong nuclear SALL4 expression in the NHP preimplantation embryo at the morula and the blastocyst stage. Strong SALL4 expression in undifferentiated ES cells is also 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

Fig. 7. SALL4 expression in spermatogonial subtypes of marmoset monkey and human. **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.

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 proof 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 for this species, functional studies using common marmoset embryos are currently also not possible. Nevertheless, 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 NHP embryo.

Testicular germ-line stem cells (spermatogonia) are unipotent in vivo. They produce exclusively 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 induced pluripotent stem 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 reviews, see Zwaka and Thomson, 2005; Rajpert-De Meyts, 2006]. Among these pluripotency markers are OCT4, NANOG,

AP2 γ , SSEA4 and TRA-1-81, which are also present in the premeiotic germ cells of the marmoset testis [Mitchell et al., 2008; Müller et al., 2008; Albert et al., 2010]. Presenting the data on SALL4, we added an additional pluripotency marker to the list of proteins that are shared by ES cells, the preimplantation embryo and testicular germline stem cells. In addition to the expression of SALL4 in ES cells and adult spermatogonia, for the first time, we show here 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 et al. [2004] who showed that the gonocytes in the human fetal testis are not a homogeneous population of cells as appears to be the case in the mouse. However, it can generally 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 a conserved expression pattern.

A_{dark} spermatogonia are undifferentiated reserve spermatogonia. A_{pale} spermatogonia are the mitotically active undifferentiated spermatogonia, which support continuous 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 type to another (i.e. A_{dark} to A_{pale} 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 reenter a functional stem cell niche and thus regain full stem cell potential. In general, A_{pale} spermatogonia appeared to exhibit stronger SALL4 signals than A_{dark} spermatogonia, although both spermatogonial populations contained SALL4-positive and SALL4-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 the 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 differ-

ent SALL4 isoforms. Since this study was 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 the first hints at the functional differences of the SALL4-A and SALL4-B isoforms in pluripotent mouse ES cells. SALL4-B binds preferentially to promoters of genes showing a high expression in undifferentiated ES cells. In contrast, DNA binding of SALL4-A occurred preferentially in the 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. Functional studies are needed to determine whether this control of gene expression by SALL4 isoforms is also true for primate (germ) cells.

Functional in vivo analysis of SALL4 in germ cells was not possible for 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 this study. A possible explanation for this basically unexpected finding could be that translational efficiency and/or SALL4 transcript stability are significantly different in the 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 de-

velopment, *Sall4* mRNA is (relatively) downregulated 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, NHP and mouse testes as well as in marmoset monkey preimplantation embryos. Testicular PGCs, gonocytes and 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 the 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 has yet to be analyzed in future studies.

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