PLURIPOTENT CELLS IN COMMON MARMOSET MONKEY TESTIS

BY

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I. Aeckerle, N., K. Eildermann, C. Drummer, J. Ehmcke, S. Schweyer, A. Lerchl, M. Bergmann, S. Kliesch, J. Gromoll, S. Schlatt, and R. Behr. 2012. The pluripotency factor LIN28 in monkey and human testes: a marker for spermatogonial stem cells? *Molecular Human Reproduction,* Oxford University Press. http://molehr.oxfordjournals.org/content/18/10/477.

II. Aeckerle, N., R. Dressel, and R. Behr. 2013. Grafting of neonatal marmoset monkey testicular single cell suspensions into immunodeficient mice leads to testicular cord neomorphogenesis. *Cells Tissues Organs*, S. Karger AG, Basel. http://www.karger.com/Article/FullText/355339.

III. Aeckerle, N., C. Drummer, K. Debowski, C.Viebahn and R. Behr. Pluripotency factor expressing pre-migratory primordial germ cells are in very close proximity to the prospective gonadal ridge in the monkey embryo. Submitted to *Development* (DEVELOP/2013/106682).

List of abbreviations

BPD	biparietal diameter
CRL	crown rump length
DAB	3,3'-diaminobenzidine
E	embryonic day
EGC	embryonic germ cell
ESC	embryonic stem cell
FROD	fronto-occipital diameter
HE	hematoxylin-eosin
HRP	horseradish peroxidase
ICM	inner cell mass
IF	immunofluorescence
IgG	Immunoglobulin G
IHC	immunohistochemistry
iPSC	induced pluripotent stem cell
MEF	mouse embryonic fibroblast
NHP	non-human primate
PAS	periodic-acid-schiff-staining
PGC	primordial germ cell
PGE	primitive gut epithelium
PND	postnatal day
PSC	pluripotent stem cell
RT-PCR	reverse transcriptase-polymerase chain reaction
SSC	spermatogonial stem cell
TSC	testicular stem cell
WPC	week post conception

Abstract

The aim of this project was to investigate whether primate testicular germ cells could provide a source of pluripotent stem cells (PSC), which may be used for cell replacement therapy.

After removal from their testicular niche, mouse testicular germ line stem cells, called spermatogonial stem cells (SSC), can spontaneously dedifferentiate *in vitro* to a pluripotent embryonic stem cell (ESC)-like state. The soundness of the corresponding data on human SSC were, however, not as convincing as for the mouse. Therefore, the aim of this study was to investigate whether PSC could be derived from the common marmoset monkey (*Callithrix jacchus*) as an experimentally accessible and bio-medically important non-human primate model.

Thus far, our attempts to generate PSC from cultivated marmoset monkey testicular cells were not successful, although we cultivated the cells according to published protocols for mouse and man. In order to identify possible reasons for this difference between mouse and primate SSC, we analyzed pluripotency marker expression in mouse and marmoset SSC. We found that the pluripotency factor LIN28 was strikingly differentially expressed between marmoset and mouse testis: in contrast to mouse testes, where LIN28 was present in all spermatogonia, LIN28 was only expressed in a very small subpopulation of adult monkey and human spermatogonia. This may provide an explanation for the difficulties in the generation of PSC from primate testes.

Male neonatal marmoset germ cells (gonocytes) are very immature and express many pluripotency-associated genes *in situ*. A functional test for pluripotency is the teratoma formation assay. We tested the teratoma-formation potential of gonocytes in a heterogeneous population of testicular cells. In the setting of our study, we did not observe any teratoma, but a rearrangement of the injected testicular cells to structures almost indistinguishable from neonatal testicular tissue. Future studies should repeat culture and teratoma formation experiments with purified gonocyte populations in order to test their developmental potential.

For human cells, the derivation of pluripotent embryonic germ cells (EGCs) from primordial germ cells has been shown. Therefore, we investigated the expression of the pluripotency factors OCT4A, NANOG, SALL4, and LIN28 during embryonic and fetal marmoset development. Particularly the expression of OCT4A and NANOG indicates an inherently pluripotent state of marmoset PGCs. Furthermore, we propose a novel model for PGC translocation during embryonic development which challenges the “dogma” of active long-range PGC migration. Our model is based primarily on passive translocation of PGCs from the gut-surrounding mesenchyme to the prospective gonad through intercalar expansion of the mesenchymal tissue harboring the PGCs. The data obtained in this project indicate that primate PGCs are the most promising source for the generation of PSC from primate germ cells.

1. Introduction

Germ cells are unipotent. *In vivo*, they exclusively give rise to sperm cells or oocytes. However, a close relationship between primitive germ cells and pluripotent stem cells has been shown for the mouse. If premeiotic mouse germ cells are removed from their natural histological environment and cultured under appropriate conditions, they switch from a unipotent state to a pluripotent one. These pluripotent germ line-derived cells are hardly distinguishable from pluripotent embryonic stem cells. The original aim of the present thesis was to investigate if the marmoset testis at any developmental stage contains a cell population that can be reprogrammed to a pluripotent state without genetic modification. The following introduction provides information about the different cell types in the developing marmoset testis, the current knowledge in this field of study and finally a description of the model organism that was used in this project, the common marmoset monkey (*Callithrix jacchus*).

1.1 Sources of pluripotent cells and their applications

The developmental potency of a cell defines its potential to differentiate into different cell types. Pluripotency refers to the ability of a cell to give rise to cell types of all three germ layers of the embryo: endoderm, mesoderm, and ectoderm, as well as the germ line. The very early embryo contains cells with pluripotent capacity from blastocyst to gastrula stages. But none of these cells establishes *in vivo* a permanent pool of pluripotent stem cells in the later embryo. In contrast, *in vivo* pluripotent cells are a transient cell population. However, when cells from the inner cell mass (ICM) of blastocysts are isolated and cultivated as embryonic stem cells (ESCs) they acquire the novel characteristic of unlimited self-renewal. ESCs are pluripotent, but in contrast to the ICM they are theoretically able to proliferate unlimitedly while remaining pluripotent.

Pluripotency of cells can be analysed in both functional and molecular terms. In mice, the gold standard for functional testing the pluripotency of stem cells is the chimera formation assay. In this assay, test-cells are injected into a recipient blastocyst. If the injected cells contribute to all three germ layers of the developing fetus, pluripotency has been proven. For ethical and biological reasons, this assay is not available for humans and non-human primates (NHP). Hence, pluripotency of human and NHP cells has to be demonstrated by another assay named the teratoma formation assay. In this assay, the test-cells are injected into an immunodeficient mouse, where xenogenic cells can survive, proliferate, and eventually differentiate for up to several weeks. The development of a teratoma – a tumor containing histologically normal endodermal, mesodermal, and ectodermal derivatives – from the injected cells provides an indication that these cells were pluripotent. This teratoma formation assay is the best test of pluripotency currently available for human and NHP cells.

The description of pluripotency using marker molecules requires the identification and presence of molecules that are strictly associated with the state of functionally pluripotent cells.

Pluripotent stem cells hold promise for regenerative medicine, since they theoretically provide an unlimited source of new tissue for therapeutic intervention. The use of ESCs, which are the biological gold standard of pluripotent cells, is ethically controversial, since the isolation of the inner cell mass results in destruction of the embryo. Therefore, scientists search for alternative sources of pluripotent cells for future regenerative approaches in humans.

Remarkably, it has been demonstrated that differentiated cells, thought being restricted in their developmental potential, can be induced to return to a pluripotent state (Takahashi and Yamanaka 2006). Besides the “classical” pluripotency transcription factors like OCT4A, SOX2 and NANOG, several other factors have been shown to be essential for the maintenance of the undifferentiated state of ESCs and / or for the induction of pluripotency (iPSC-generation). Among them are the transcription factors SALL4 (Elling et al. 2006; Tsubooka et al. 2009; Eildermann et al. 2012a), KLF4 (Jiang et al. 2008), Myc (Takahashi and Yamanaka 2006) as well as the RNA-binding protein LIN28 (Tomioka et al. 2010). The remarkable studies of Takahashi and Yamanaka were not the first which demonstrated the reprogramming of specialized cells into pluripotent stem cells (Matsui et al. 1992; Resnick et al. 1992; Stevens 1967). In fact pluripotent stem cells derived from germ cells were the first described in literature (Stevens 1967). In contrast to reprogramming of differentiated somatic cells, this process only required the addition of growth factors to the cells (Matsui et al. 1992; Resnick et al. 1992) and no genetic modifications. Therefore, testicular tissue is a very promising starting material for generation of pluripotent cells without genetic modifications.

The following paragraph provides an overview of the current knowledge about mammalian germ cells as possible source of pluripotent cells.

1.2 Testicular cells as possible source for pluripotent cells

Cells of the germ cell lineage have the role to pass genetic information from one generation to the next. After the fusion of a male and a female gamete an embryo develops, that consists of many cells with different characteristics. This implicates that germ cells conserve and transmit pluripotency.

Very early during embryonic development a small group of pluripotent cells is excluded from the formation of the soma. These cells are the primordial germ cells (PGCs; Figure 1) (Ginsburg et al. 1990).

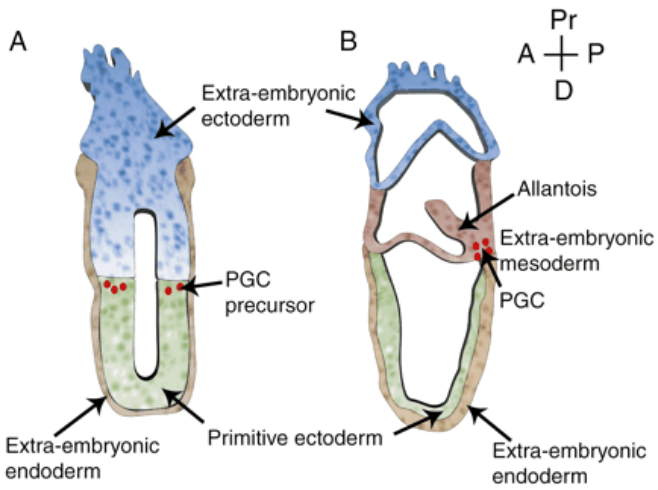

Figure 1: Early development of the mouse embryo.

A small group of cells, the primordial germ cells (PGC) is excluded from the formation of the soma. A: day 6.25 of embryonic development B: day 7.25 of embryonic development; A, anterior; P, posterior; Pr, proximal; D, distal (picture from Zwaka and Thomson (2005)).

Once these PGCs are specified at their extraembryonic site, they are translocated towards the developing gonadal ridge. When the PGCs have entered the male gonad they are generally referred to as gonocytes. Gonocytes represent the fetal and neonatal stages proceeding in the formation of spermatogonial stem cells (SSCs) (Culty 2009). They are considered as a transient population of germ cells disappearing prior to the onset of spermatogenesis when spermatogonia represent the only remaining premeiotic germ cell type. Gonocytes occupy the center of lumen-less seminiferous cords (Culty 2009). In the human and non-human primate (NHP) testis, gonocytes undergo a transformation to adult dark (A_{dark}) and pale (A_{pale}) spermatogonia that establish and later comprise the spermatogonial stem cell pool. Unipotent SSCs are the foundation for spermatogenesis and are capable of both self-renewal and production of daughter cells that differentiate into spermatozoa. However, since evidentiary assays and unequivocal markers are still missing in non-human primates and man, the identity of primate SSCs is unknown.

After the removal from the testicular stem cell niche, mouse spermatogonial stem cells can spontaneously dedifferentiate, leading to a pluripotent embryonic stem cell (ESC)-like state (Guan et al. 2006; Kanatsu-Shinohara et al. 2008; Seandel et al. 2007; Ko et al. 2009). Since the spermatogonial population of the primate testis is fundamentally different from the rodent testis (Ehmcke and Schlatt 2006), the promising mouse data may not simply be transferred to the adult primate. While the derivation of pluripotent cell lines has been unequivocally shown in the rodent model, an intense debate is currently questioning the reports on the derivation of pluripotent cell lines from the human testis (Conrad et al. 2008; Kossack et al. 2008; Chikhovskaya et al. 2012; Ko et al. 2010; Tapia et al. 2011; Eildermann et al. 2012b). Therefore, a non-human primate model would be useful to investigate whether the derivation of pluripotent cells from the primate testis is possible like in the mouse.

1.3 The Common marmoset monkey as model organism

The common marmoset monkey (*Callithrix jacchus*) is a new world monkey (*Platyrrhini*) belonging to the family of *Callitrichidae*. With a crown-rump-length (CRL) of 18-25 cm and a bodyweight of 300-500 g it is the smallest non-human primate commonly used in biomedical research. Marmosets have a high fecundity with short birth intervals (about five months) and a high reproductive rate (up to three neonates per litter). Their pregnancy lasts around 20 weeks (average 144 days), ranging from 140–150 days. Marmosets normally deliver twins (Windle et al. 1999). The bodyweight of a neonatal marmoset ranges from 25 to 35g.

It is known, that marmoset gonads are unusually immature at birth (Abbott and Hearn 1978; DeSouza 1988; Sharpe et al. 2003). The reason for this may be the fact that marmosets deliver twins or triplets which are often of different sex and develop a shared chorionic cavity and placental circulation (Hill 1932; Wislocki 1939). Therefore, co-twins of different sexes are XX/XY hematopoietic chimeras. Hence, it must be precluded that hormone-sensitive stages of gonad development occur in the presence of “wrong” sex hormones.

Puberty occurs over several months between 8 and 12 month of age. The adult body size is reached at about 12 months of age and adult weight and full social and sexual maturity between 18 and 24 months. Marmosets have a lifespan of 10 to 15 years.

Presently, the common marmoset is used in numerous areas of biomedical research (Okano et al. 2012). Since many characteristics of testicular development and histological appearance of the seminiferous epithelium and organization of epithelial stages closely resemble the situation in human (Millar et al. 2000; Weinbauer et al. 2001; Kelnar et al. 2002; Wistuba et al. 2003; Luetjens et al. 2005; Mitchell et al. 2008; Mitchell et al. 2009), the common marmoset monkey is a well-accepted model in reproductive biology and medicine (Li et al. 2005). Therefore, the common marmoset monkey was chosen as non-human primate model for the present PhD project.

Figure 2: Common Marmoset Monkey (*Callithrix jacchus*)

This photo shows a female marmoset monkey. It was taken in the breeding colony at the DPZ by Dr. med. vet. Charis Drummer, DPZ.

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2. Outline of the thesis

The working hypothesis of the project is that postnatal marmoset monkey germ cells can regain pluripotency after removal from their native niche within the testis.

To investigate this working hypothesis, the specific aims of this project were originally:

(I) **The *in situ* identification of potentially pluripotent stem cells in embryonic, fetal, neonatal, pubertal and adult marmoset testis via immunohistochemistry:** The differentiation process during the gradual development from a potentially pluripotent primordial germ cell (PGC) into unipotent spermatogonia had to be monitored. Main questions in this process were if and when germ cells lose their pluripotency during development, which cell populations arise during differentiation and if the potential of pluripotency remains in adult testicular stem cells.

(II) **The establishment of marmoset testicular stem cell (TSC) culture:** The *in situ* identified potentially pluripotent cells needed to be functionally characterized. Therefore, we attempted to establish *in vitro* culture systems according to published studies that promote the derivation of pluripotent stem cell from marmoset testis.

(III) **Characterization of marmoset TSCs *in vitro* and in bioassays:** The derived putative marmoset testicular stem cells were characterized *in vitro* using immunofluorescence, RT-PCR and western blot. As a functional bioassay to demonstrate pluripotency, the teratoma-formation assay should be performed.

Extensive experiments aiming at the long-term culture of gonocytes were performed under several different conditions. However, the establishment of a long-term TSC culture was not successful. Therefore, the following additional studies were performed:

(IV) **The analysis of differences between developing mouse and marmoset testes:** The generation of pluripotent cells from mouse spermatogonia was already proven, while this was not convincingly demonstrated for primate testicular cells. Therefore, we attempted to analyse differences between developing mouse and marmoset testes concerning the expression of pluripotency-associated genes.

(V) **The use of naïve neonatal marmoset testicular cells for the teratoma formation assay:** Since marmoset gonocytes from the neonatal testis express many pluripotency-associated markers, we attempted to analyse their teratoma-formation potential without previous culture.

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MHR Basic science of reproductive medicine

ORIGINAL RESEARCH

The pluripotency factor LIN28 in monkey and human testes: a marker for spermatogonial stem cells?

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ABSTRACT: Mammalian spermatogenesis is maintained by spermatogonial stem cells (SSCs). However, since evidentiary assays and unequivocal markers are still missing in non-human primates (NHPs) and man, the identity of primate SSCs is unknown. In contrast, in mice, germ cell transplantation studies have functionally demonstrated the presence of SSCs. LIN28 is an RNA-binding pluripotent stem cell factor, which is also strongly expressed in undifferentiated mouse spermatogonia. By contrast, two recent reports indicated that LIN28 is completely absent from adult human testes. Here, we analyzed LIN28 expression in marmoset monkey (*Callithrix jacchus*) and human testes during development and adulthood and compared it with that in mice. In the marmoset, LIN28 was strongly expressed in migratory primordial germ cells and gonocytes. Strikingly, we found a rare LIN28-positive subpopulation of spermatogonia also in adult marmoset testis. This was corroborated by western blotting and quantitative RT–PCR. Importantly, in contrast to previous publications, we found LIN28-positive spermatogonia also in normal adult human and additional adult NHP testes. Some seasonal breeders exhibit a degenerated (involuted) germinal epithelium consisting only of Sertoli cells and SSCs during their non-breeding season. The latter re-initiate spermatogenesis prior to the next breeding-season. Fully involuted testes from a seasonal hamster and NHP (*Lemur catta*) exhibited numerous LIN28-positive spermatogonia, indicating an SSC identity of the labeled cells. We conclude that LIN28 is differentially expressed in mouse and NHP spermatogonia and might be a marker for a rare SSC population in NHPs and man. Further characterization of the LIN28-positive population is required.

Key words: LIN28 / pluripotency / primate / spermatogonial stem cell / testis

Introduction

Mammalian spermatogonial stem cells are unipotent. They exclusively produce sperm cells over several decades in human and non-human primates (NHPs) and over years in rodents. However, mouse spermatogonial stem cells have been shown to spontaneously dedifferentiate after removal from the testicular stem cell niche (Kanatsu-Shinohara *et al.*, 2004; Guan *et al.*, 2006; Seandel *et al.*, 2007; Ko *et al.*, 2009), eventually leading to a pluripotent embryonic stem (ES) cell-like state. Although several groups aimed at translating these findings from the mouse model to the NHP model or directly to the human model (Conrad *et al.*, 2008; Golestaneh *et al.*, 2009; Kossack *et al.*, 2009; Mizrak *et al.*, 2010), it is still an open issue whether spermatogonia-derived pluripotent cell lines can be derived from the primate testis (Ko *et al.*, 2010, 2011; Tapia *et al.*, 2011). Since we failed in two independent laboratories (J.G. S.S.; Center for Reproductive Medicine and Andrology and K.E. and R.B.; German Primate Center) to establish pluripotent cell lines from marmoset monkey testes in numerous attempts over the last years, we started searching for possible differences in the expression of pluripotency factors between rodent and primate spermatogonial stem cells that may contribute to the difficulties during spontaneous human and monkey spermatogonial stem cell dedifferentiation to a pluripotent ES cell-like state.

Besides the 'classical' pluripotency transcription factors such as OCT4 and NANOG, several other factors have been shown to have essential roles for the maintenance of the undifferentiated state of ES cell and/or during induced pluripotent stem (iPS) cell generation. Among them are the transcription factors SALL4 (Elling *et al.*, 2006;

Tsubooka *et al.*, 2009), KLF4 (Jiang *et al.*, 2008) and the RNA-binding protein LIN28. In *Caenorhabditis elegans*, where *lin-28* was first investigated, it has been shown that the expression of this protein is strictly regulated during development and timely presence of the protein is essential for normal development of the larvae (Moss *et al.*, 1997). Also, the mouse orthologue of *C. elegans lin-28* exhibits strict temporal and cell-specific expression during development (Moss and Tang, 2003). Recently, it has been demonstrated that LIN28 acts at the top of the cascade of factors that orchestrate primordial germ cell (PGC) specification (West *et al.*, 2009), and in adult mouse testes, LIN28 is expressed in undifferentiated spermatogonia (Zheng *et al.*, 2009). In contrast, a recent paper reported complete absence of LIN28 in more than 50 post-pubertal healthy human control testes, while robust LIN28 expression was seen in human testicular germ cell tumors (Cao *et al.*, 2011). The absence of LIN28 in the adult testes was confirmed by Gillis and colleagues, while gonocyte-specific expression of LIN28 was detected in the fetal human testes (Gillis *et al.*, 2011). LIN28 has also been shown to significantly enhance human iPS cell generation (Yu *et al.*, 2007; Warren *et al.*, 2010) and in the common marmoset monkey (*Callithrix jacchus*), this factor was even essential for complete reprogramming of somatic cells to an iPS cell state (Tomioka *et al.*, 2010). Here, we confirmed that LIN28 is strongly expressed in adult mouse spermatogonia. Interestingly, we detected LIN28 also in a very small subpopulation of marmoset monkey spermatogonia. Moreover, in contrast to published data, LIN28 is also expressed in adult human testes and additional old-world NHP testes, where it is restricted to a very small subpopulation of spermatogonial cells. Numerous LIN28-positive germ cells in fully seasonally involuted hamster and monkey testes suggest that the LIN28-positive germ cells are spermatogonial stem cells.

Materials and Methods

Sample collection and processing were described recently (Eildermann *et al.*, 2012a).

Human material

Fetal human gonads and pediatric testis

Parental approval to perform autopsy at the Department of Pathology, University of Göttingen, Germany, was obtained for each fetus. This included the use of gonadal tissue for histopathological investigations. The fetal gonads of males were retrieved from the archive of the Department of Pathology. Human gonads of six males from second and third trimesters were obtained after spontaneous miscarriages (gestational ages ranging from 18 to 35 weeks). Fetal gonads were collected within 24 h of death and post-mortem examinations were carried out in the Department of Pathology, University of Göttingen. 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 with the foot length and the crown-heel length at autopsy. The biopsy of the pediatric testis from the 1-year-old boy exhibited an age-appropriate developmental stage and was obtained for diagnostic purposes.

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) to these investigations. Ten tumor-free histological control samples from patients with contralateral germ cell tumors were analyzed. Five additional tumor-free archival testes with normal spermatogenesis from prostate carcinoma patients undergoing orchiectomy for therapeutic purposes were also used. 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 all the testes.

Animal material

Marmoset monkey testis tissue samples

Testes of 15 adult, 14 pubertal (between post-natal weeks 20 and 42) and 13 newborn animals were analyzed. Five embryonal/fetal testes (between gestational days 68 and 117) were obtained after spontaneous miscarriage, or after timed surgical retrieval of the embryos (License # 33.9.42502-04/066/06). All studies were done in accordance with the German law on the protection of animals and were approved by the legal authorities.

Additional NHP species

The additional NHP testes were obtained from the DPZ tissue bank, in which many control tissue samples from different studies are archived. Furthermore, this tissue bank also includes material from Zoo animals that were autopsied at the DPZ Pathology Unit. All the tissues were obtained in accordance with the respective legal requirements.

Mouse tissues

Mouse (strain CD1) tissues were retrieved immediately after killing by cervical dislocation (adult animals) or decapitation (post-natal animals). The tissues were fixed in Bouin's solution and paraffin-embedded according routine procedures. Pregnancies were timed by checking the vaginal plugs. At least two embryos/animals were analyzed per developmental stage. All the embryos and tissues were obtained in accordance with the respective legal requirements.

Djungarian hamster tissue

Djungarian hamster (*Phodopus sungorus*) testes were obtained from an animal facility of the School of Engineering & Science, Jacobs University, Bremen. Testes from three animals with full spermatogenesis and three involuted testes with inactive spermatogenesis from short-day conditions (8 h light, 16 h dark) were used. The animals ranged in age from 6 to 13 months and were kept under the respective conditions for 3–7 months. The tissues were fixed in Bouin's solution and paraffin-embedded according to routine procedures. All tissues were obtained in accordance with the respective legal requirements.

Immunohistochemistry on sectioned tissues

Immunohistochemical staining for LIN28 was basically performed as described previously (Behr *et al.*, 2007). Tissues were fixed over night in Bouin's solution immediately after recovery. After several washes in 70% EtOH for at least 2 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). The LIN28 antibody (Cell Signaling, Cat. # 3978S) was produced by immunizing rabbits with a synthetic peptide corresponding to amino acid sequence surrounding Ala177 of human LIN28A. It was used at a 1:70 dilution (in provided antibody diluent from Cell Signaling) for immunohistochemistry (IHC). Other antibodies were used in the following dilutions in Tris-buffered saline plus 3% bovine serum albumin (BSA): VASA (DDX4 = DEAD box Protein 4) [R&D Systems, Cat.#AF2030, (0.2 mg/ml)] 1:100, Undifferentiated embryonic cell transcription factor 1 (UTF1) (Millipore, Cat. #MAB4337) 1:25, SALL4 (abcam, Cat. #ab57577) 1:200, Caspase-3 (Cell Signaling, Cat. #9661) 1:200 and PLAP (DakoCytomation, Cat. #M7191) 1:25. All incubation steps were done 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 horse-radish peroxidase (HRP)-conjugated streptavidin was employed for the detection of bound primary antibody. 3,3′-diaminobenzidine chromogen was used as substrate for the HRP and Mayer's hematoxylin as counterstain. Control stainings were carried out using non-specific rabbit immunoglobulin G (IgG) instead of the LIN28 antibody at the same protein concentration. Double staining was performed using the Envision Doublestain System, Rabbit/Mouse (DakoCytomation, Cat. #K5361) according to the manufacturer's instructions. Pictures were taken using the Nuance CRi multispectral camera (distributed by INTAS, Göttingen, Germany) which allows the detection of selected wavelengths also in the visible, i.e. immunhistochemical staining, spectrum.

Quantification of LIN28-positive cells in marmoset testes

The stained slides were scanned using the Pannoramic Midi and analyzed using the Pannoramic Viewer (3D Histech). The gonocytes were recognized by their uniform round nuclear shape, and the spermatogonia were recognized according the description given previously (Clermont, 1963; Weinbauer *et al.*, 2001). For each age (newborn, pubertal and adult), four animals were evaluated. For newborn and juvenile animals, the total number and the number of LIN28-positive germ cells per tubule were counted in 20 tubules per section. For adult animals, all tubules per section were counted and the percentage of tubules containing at least one LIN28-positive spermatogonium was evaluated. The numbers of counted tubules are given in Supplementary data, Table S1. As an internal control, a second section from an independent area of each testis was assessed for one animal of each age.

Quantification of LIN28-positive cells in human testes

The stained slides were scanned using the Pannoramic Midi and analyzed using the Pannoramic Viewer (3D Histech). For fetal and prepubertal testis, one section per testis was used, and for adult testis, three sections per patient. For the 6 fetal testis samples 100 tubules per sections were evaluated in each case, for 1 prepubertal and 15 adult samples, all tubules per section were evaluated. The numbers of counted tubules are given in Supplementary data, Table S2. The percentage of tubules containing at least one LIN28-positive spermatogonium was evaluated.

ES cell culture

Common marmoset monkey ES cells (line cjes001) were cultured as described previously by Muller *et al.* (2009).

Immunofluorescence staining

ES cells were grown on *x*-irradiated mouse embryonic fibroblasts [mouse embryonic feeders (MEFs)] in foil-bottom 24-well plates (Lumox™, Greiner Bio-One, Stuttgart, Germany) for 4 days, fixed for 30 min in 2% paraformaldehyde (PFA), 0.02% Triton X-100 and then washed twice in phosphate-buffered saline (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 h incubation with primary antibody (LIN28, Cell Signaling #3978S) dilution (1:100) at 4°C (or alternatively 1 h at 37°C), cells were washed twice in PBS and incubated for another 60 min with the anti-rabbit secondary antibody covalently linked to Alexa dye A488. Images were taken on a Zeiss Axio Observer Z1 microscope. Counterstaining reagent was 4(6-diamidino-2-phenylindole).

Western blot analysis

Protein from ~50 mg 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 μl resuspension buffer (0.15 M NaCl, 15 NP-40, 1% lithium dodecyl sulfate, 2% SARKOSYL (N-Lauroylsacosin natrium salt)). For western blot analysis, 15–20 μl of the protein-lysate (including 10 × DTT and 4 × loading buffer) and 5 μl Novex sharp prestained protein standard from invitrogen was loaded onto a NuPAGE Novex 4–12% Bis–Tris gel to separate proteins. Alternatively, mouse proteins were directly lysed in SDS and sample buffer and run on the gel. Proteins were then transferred to a nitrocellulose membrane. The membrane was washed in PBS-T (1 × 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. The LIN28 antibody (Cell Signaling #3978S 1:1000) was diluted in 5% skim milk/PBS-T. After washing in PBS-T, membranes were incubated with a secondary HRP conjugated antibody (goat-anti- rabbit-HRP from RandD #HAF008). Signal-detection was carried out using the ECL-Kit from Amersham (RPN2209) and an Intas Chemo Cam.

Quantitative real-time PCR

Relative quantitative real-time (qRT)-PCR for *LIN28* was performed on testicular RNA from four newborn, four 8-week-old and four adult monkeys and on testicular RNA from three newborn mice, three 9-day-old mice and three adult mice. For marmoset, the 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 ~85% between marmoset and human/mouse. Primers were designed with Primer Express®, crossing exon boundaries, 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 marmoset were forward—5′-GACGTCTT TGTGCACCAGAGTAA-3′ and reverse—5′-CGGCCTCACCTTC CTTCAA-3′. For mice the following primers were used: forward—5′-GGTGGTGTGTTCTGTATTGGGA-3′ and reverse—5′-AGTTG TAGCACCTGTCTCCTTTG-3′. Identity of the amplicon was confirmed by DNA sequencing. For qPCR a primer concentration optimizing run according to the Power SYBR® Green PCR Master Mix and RT–PCR Protocol from Applied Biosystems, including a dissociation curve, was performed for each gene. 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). The suitable primer concentration was determined experimentally. Six hundred nano molecules were found to be optimal for mouse forward and reverse as well as marmoset forward primers, while 900 nM were suitable for the marmoset reverse primer. The PCR program consisted of initial steps of activation and denaturation, which were run once for 10 min at 50°C and 5 min at 95°C, respectively, followed by 40 cycles of annealing (15 s at 95°C) and elongation (1 min at 60°C) steps. The extent of fluorescence of the SYBR green dye was detected and analyzed using the ABI Prism® 7000 SDS software. Each sample was assayed in triplicate und normalized to glyceraldehyde-3-phosphate dehydrogenase expression. Relative quantification was based on the $2[-\Delta\Delta C(T)]$ method according to (Livak and Schmittgen, 2001) using adult testicular RNA as calibrator.

Results

Validation of the LIN28 antibody for marmoset monkey LIN28

The specificity of the LIN28 antibody for the respective marmoset protein was tested by western blot analysis. The sample containing a mixture of marmoset ES cell and MEF protein showed one very predominant band at the expected size of ~28 kDa. The control sample lacking ES cell protein and consisting only of MEF protein showed no signal (Fig. 1A). Omission of the primary antibody resulted in no signal. Moreover, heterologously expressed marmoset LIN28 revealed the same western blot signal as shown in Fig. 1A (data not shown). Immunofluorescence showed the expected cytoplasmic LIN28 distribution in marmoset ES cells (Fig. 1B). In summary, these data indicate that the antibody used in this study specifically detects marmoset monkey LIN28 protein also.

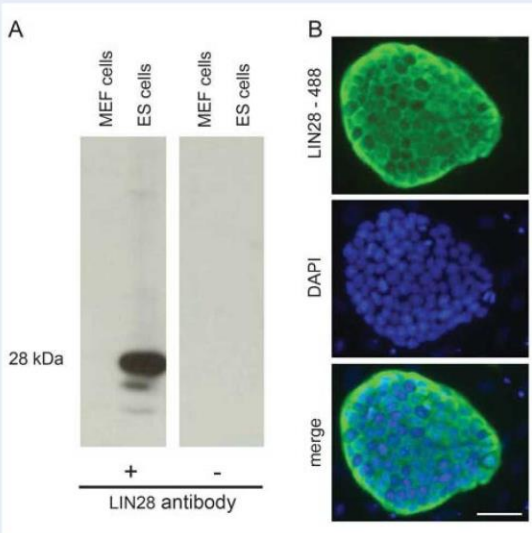

Figure 1 The LIN28 antibody detects marmoset monkey LIN28 protein in pluripotent ES cells. (**A**) Western blot analysis. Marmoset ES cells cultured on mouse embryonic feeder (MEF) cells showed a strong signal of the expected size of ~28 kDa. Protein from MEF cells alone contains no LIN28. Omission of the primary antibody also resulted in no signal verifying the specificity of the LIN28 antibody. (**B**) Immunofluorescent staining of LIN28 in an undifferentiated colony of marmoset ES cells cultured on MEF cells. LIN28 is specifically detected in the cytoplasm of ES cells. The scale bar represents 50 μm.

LIN28 expression in marmoset monkey testicular germ cells during development

Prenatal testicular development is severely delayed in the marmoset monkey compared with human (Li *et al.*, 2005). During the early stage of testicular organogenesis [embryonic day (E) 75], LIN28 was strongly detectable in PGCs during their late phase of migration (Fig. 2A, red arrows) and in germ cells at the transition from PGCs to gonocytes during the formation of the testicular cords (Fig. 2A, yellow arrow). Staining was seen in the cytoplasmic and in the nuclear compartment with greater intensity in the cytoplasm. At estimated fetal stage E90 [based on crown-rump length according to (Phillips, 1976) and head diameter], when the testicular cords were fully established (Fig. 2B), the gonocytes were enclosed in the cords. LIN28 was strongly expressed and localized predominantly to the cytoplasm of the germ cells (Fig. 2B, red arrows). Unexpectedly, at this stage, the majority of the gonocytes were descended to the basement membrane of the cord and only a few germ cells had a more central position. An interstitial cell population, most likely fetal Leydig cells, was also LIN28-positive, yet exhibiting lower staining intensity (Fig. 2B, yellow arrows). Basically, the same pattern was seen in the neonatal testis (Fig. 2C). However, the staining intensity

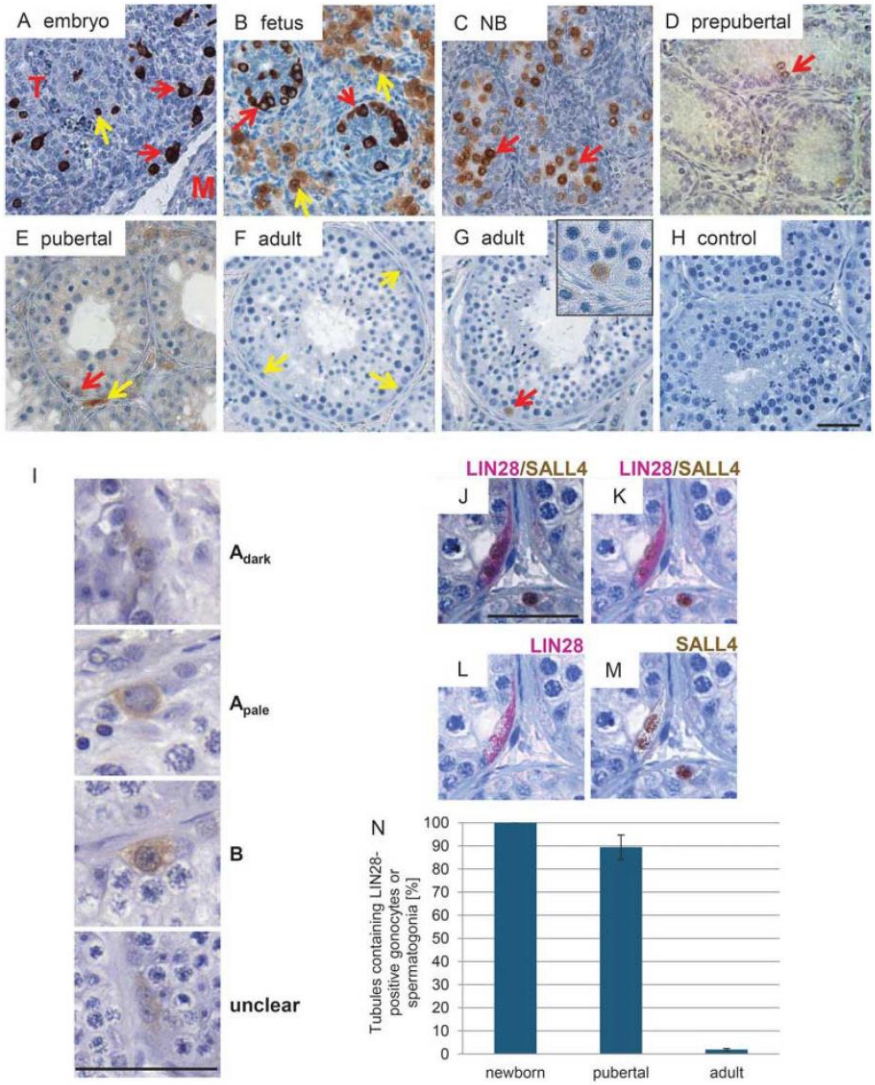

Figure 2 LIN28 expression in prenatal, post-natal and adult marmoset monkey testes. (**A**) LIN28-positive PGCs enter the developing testis at gestational day 75. Some stained cells are inside the forming testicular cords (yellow arrows), while other PGCs are still in the stromal compartment (red arrows). Many cells have extensions suggesting migratory activity. T, testis; M, mesonephros (**B**) Fetal testis at gestational day 90. Testicular cords clearly display an epithelium and the germ cells within the cords are LIN28-positive. Most of the germ cells have contact with the basal membrane of the developing germinal epithelium (red arrows). LIN28 signals in the cells of the interstitium (yellow arrows) become weaker compared with the germ cells' signal. (**C**) Newborn testis after ~145 days of gestation. Most germ cells detached again from the basal membrane (red arrow). LIN28 signal intensity became heterogenous with some germ cells showing strong signals and others exhibiting only very faint staining. (**D**) Prepubertal testis at post-natal week 20. LIN28 staining is restricted to few germ cells (red arrows). (**E**) Pubertal testis showing first meiotic cells. Few spermatogonia (red arrow) and some interstitial cells (yellow arrow) express LIN28. (**F**) Typical adult seminiferous tubule lacking LIN28-positive cells. Faint staining can be seen in some interstitial cells. (**G**) One of the rare tubular cross sections containing a LIN28-positive spermatogonium (for quantification see Fig. 2N and Supplementary data). The inset highlights the morphology of the stained cell. (**H**) Negative control. LIN28 antibody was replaced by non-specific rabbit IgG at the same protein concentration as the LIN28 antibody was used. (**I**) Representative examples of different spermatogonial subtypes in the marmoset monkey testis expressing LIN28. Shown are an A_{dark} spermatogonium, an A_{pale} spermatogonium, a B spermatogonium as well as LIN28-positive spermatogonia that cannot be assigned to one specific spermatogonial subtype. (**J–M**) LIN28-positive spermatogonia co-express SALL4. The brightfield-picture J) shows two LIN28^{+}/SALL4^{+} spermatogonia and one LIN28-negative spermatogonium expressing SALL4 (K-M) show the same picture false-colored as an overlay (K) or highlighting only LIN28 (L) or SALL4 (M) staining. All scale bars represent 50 μm.

between different individual germ cells varied. Furthermore, most germ cells that were localized at the basement membrane at E90 (Fig. 2B) had moved again to the central compartment of the forming seminiferous tuble (Fig. 2C, red arrows). In the pubertal testes, the differentiating germ cells are devoid of LIN28 (Fig. 2D and E) and only few spermatogonia remained LIN28-positive (red arrows). Additionally, there are some interstitial LIN28-positive cells (Fig. 2E, yellow arrows). The adult testis is almost negative for LIN28 and most of the tubule cross sections show no stained spermatogonia (Fig. 2F, yellow arrows). However, importantly, in very few tubular cross sections of the adult marmoset testis, there were specifically labeled LIN28-positive cells (Fig. 2G, red arrow, and inset). Weakly LIN28-positive cells could also be seen within the interstitial compartment. Those cells are most likely Leydig cells.

In the adult marmoset testes, we detected 39 clearly stained spermatogonia (see Supplementary data, Table S1). Noteworthy, staining was seen in all the three morphological spermatogonial subtypes (Fig. 2I): Two cells were clearly assigned to A_{dark} spermatogonia, 11 to A_{pale} spermatogonia and 8 to type B spermatogonia. Eighteen LIN28-positive spermatogonia could not be clearly assigned to a specific spermatogonial subtype. They were classified as 'unclear'.

A double staining of LIN28 and SALL4 revealed that all observed LIN28-positive spermatogonia co-expressed SALL4, whereas not all SALL4-positive cells expressed LIN28. Figure 2J shows the original bright-field picture of two spermatogonia co-expressing LIN28 and SALL4, while another SALL4-positive spermatogonium lacks LIN28 signals. Figure 2K–M shows the same picture false-colored to better distinguish between the LIN28 signals and the SALL4 signals.

Quantitative analysis of the LIN28-positive germ cells revealed that 100% of the tubules in the newborn testis contained LIN28-positive gonocytes (Fig. 2N). At this developmental stage, 30–80% of all germ cells were LIN28-positive. In pubertal testes, the percentage of tubules containing LIN28-positive (pre-) spermatogonia decreased to ~90%. In the adult marmoset testis only 0.5–3.3% of all evaluated tubules displayed any LIN28-positive spermatogonia demonstrating that in the adult testis the LIN28-positive population of spermatogonia is very rare. The absolute numbers of evaluated tubules and counted LIN28-positive cells are given in Supplementary data, Table S1.

LIN28 expression in mouse testicular germ cells during development

As a reference, we included fetal, neonatal, postnatal day 9 and adult mouse testes (Fig. 3A–D). As in the primate testis, mouse gonocytes and immature spermatogonia were LIN28-positive (Fig. 3A–C). However, in contrast to the adult monkey and human testes, the adult mouse testis still shows strong LIN28 expression in several spermatogonia per tubulular cross section (Fig. 3D).

LIN28 RNA and protein profiles in post-natal marmoset and mouse testes are divergent

To determine the relative abundance of *LIN28* mRNA during postnatal testis development in the marmoset and the mouse, we performed qRT-PCR. In the marmoset testis, we detected a constant decrease in relative *LIN28* mRNA abundance, which reflects the immunohistochemical findings: strong expression in the newborn, intermediate expression at 8-week-old testes and low expression in adult testes. In the mouse, the expression profile was different with a strong increase from birth to PND9 and subsequent decrease to adulthood. However, adult relative expression levels were still above the newborn levels (Fig. 3F). The mRNA data were corroborated by western blot analyses (Fig. 3G and H). In the newborn marmoset, we detected a clear band, while only an extremely faint band was visible in adult testis. Control organs such as newborn ovary or adult tissues including ovary, heart, liver and kidney showed only weak (newborn ovary) or no LIN28 signal (Fig. 3G). In the mouse, LIN28 was almost undetectable at birth. At PND9, there was a very intense signal and in adult testis, a rather weak one (Fig. 3H). Thus, mRNA and protein data correlate very well and corroborate the immunohistochemical data.

LIN28 is expressed in a rare population of adult human spermatogonia

On the basis of the findings in the adult marmoset monkey testis, we carefully analyzed a panel of adult human testis samples with qualitatively and quantitatively normal spermatogenesis ($n = 15$). We included fetal testes and a prepubertal sample as controls and to confirm the developmental expression of LIN28 in the human testis. The fetal samples were fixed in 10% PFA and not in Bouin's solution like the other samples used in this study. To exclude an influence of the different fixatives on the staining, testis samples from a marmoset monkey were fixed in Bouin's solution and 10% PFA, respectively. The LIN28 staining showed only a slight difference in the staining intensity between both fixatives (Supplementary data, Fig. S1). However, the basic staining patterns were fully comparable between both fixatives thereby excluding a significant effect of the fixation on our data. As shown in Fig. 4A and B, LIN28 is present in fetal gonocytes and spermatogonia of the postnatal testis. In contrast to previous papers (Cao *et al.*, 2011; Gillis *et al.*, 2011), a careful analysis revealed the presence of a very few clearly LIN28-positive spermatogonia in the adult testis also (Fig. 4C). However, the vast majority of all tubules showed no LIN28-positive spermatogonia (Fig. 4D). In the evaluated samples, the percentage of tubules showing at least one LIN28-positive spermatogonia ranged from 0 to 7.7% (Fig. 4E). Four out of 15 samples showed no LIN28-positive spermatogonia (Fig. 4E, details for the histological evaluation see Supplementary data, Table S2). The number of detected LIN28-positive cells showed no correlation with the age of the patient (data not shown).

LIN28 in additional NHP species

We analyzed testes of additional adult old world monkey species, which represent the closest phylogenetic relatives to man available to us. In the rhesus monkey (*Macaca mulatta*) and the lion-tailed macaque (*Macaca silenus*) as well as in the baboon (*Papio anubis*), we also found single or paired LIN28-positive spermatogonia (Fig. 5A–C).

LIN28 expression in hamster and monkey testes during seasonal involution

There are several mammalian (including primate) species that show severe testicular involution during the annual reproductive cycle. In these species, spermatogenesis is active only before/during the mating period to supply fertile ejaculates. After the mating period,

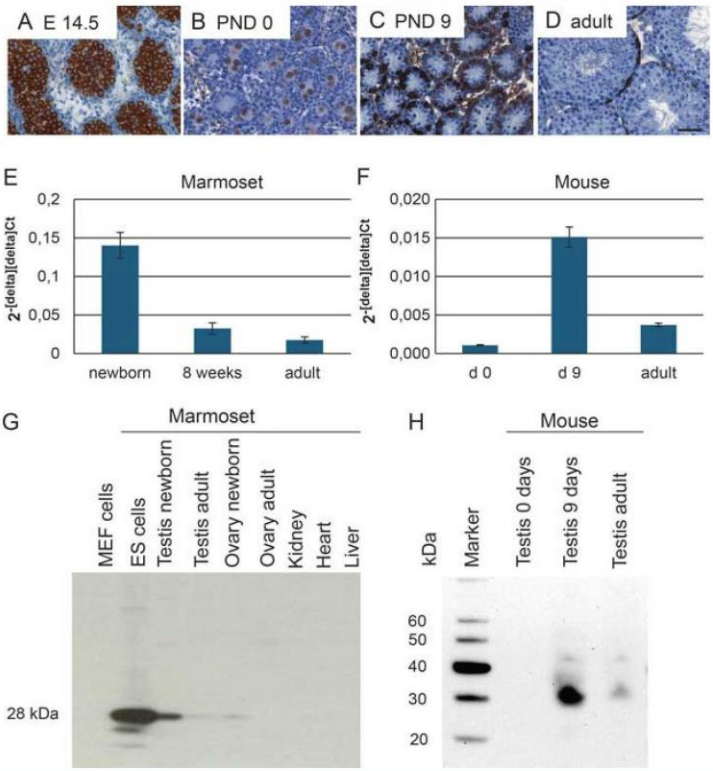

Figure 3 LIN28 protein and mRNA in marmoset and mouse testes. (**A**) LIN28 is present in the cytoplasm of mouse gonocytes and (**B** and **C**) immature spermatogonia, as well as in adult spermatogonia (**D**). The scale bar represents 50 μm. Quantitative RT–PCR with marmoset (**E**) and mouse (**F**) whole testis RNA. (E) Newborn, 8 weeks-old, and adult marmoset testes. There is a strong and significant decrease in the relative mRNA abundance during testicular development. (F) The expression profile of the mouse testis shows a strong increase from birth to PND9 and subsequent decrease to adulthood. The adult relative expression levels were still above the newborn levels. (**G**) Western blot analysis of LIN28 in newborn and adult marmoset monkey testis and control tissues. Omission of the primary antibody resulted in no signal (data not shown). (**H**) Western blot analysis of LIN28 in neonatal, post-natal day 9 and adult mouse testes.

spermatogenesis is down-regulated and the testis regresses to a rather fetal state, i.e. in extreme cases, the germ cell population consists only/mostly of the most primitive spermatogonial stem cells, while differentiating germ cells are mostly lacking. Starting from these spermatogonial stem cells, spermatogenesis is re-initiated prior to the next mating period. We analyzed LIN28 expression in involuted Djungarian hamster (*P. sungorus*) (Fig. 6) and NHP (Ring-tailed lemur; *Lemur catta*) testicular samples (Fig. 7). The testes showed strong testicular involution and had only very few germ cells in the seminiferous tubules. Very impressively, under these special circumstances, a significant fraction of germ cells in the hamster as well as in the NHP testis were LIN28-positive as evidenced by staining of the same samples for the general germ cell marker VASA. UTF-1 is an established spermatogonial marker in human and rat testis (van Bragt *et al.*, 2008; von Kopylow *et al.*, 2010). UTF1-expression by the germ cells of the involuted monkey tubules indicated their stem cell identity (Fig. 7). Importantly, in both species, we detected neither Caspase3 (apoptosis marker) nor placenta-like alkaline phosphates (PLAP; germ cell tumor marker) expression in the germ cells (data not shown), while the controls available to us (human testicular tumors and marmoset monkey placenta for PLAP and marmoset testis for Caspase3) showed the expected staining pattern. Altogether, these data strongly indicate that LIN28 is expressed in vital and functional stem cells, from which spermatogenesis is re-initiated after a physiological phase of spermatogenic quiescence.

Discussion

Primate SSCs are still enigmatic. Their exact identity and the functional roles of different morphologically characterized types of undifferentiated spermatogonia are even nowadays unclear (for review see, Hermann *et al.*, 2010). This is mainly due to the fact that the tools to study and identify spermatogonial stem cells are still very limited in primates, while in the mouse model transgenic and transplantation approaches and the combination of both were very instructive during the past two decades (Brinster and Zimmermann, 1994; Guan *et al.*,

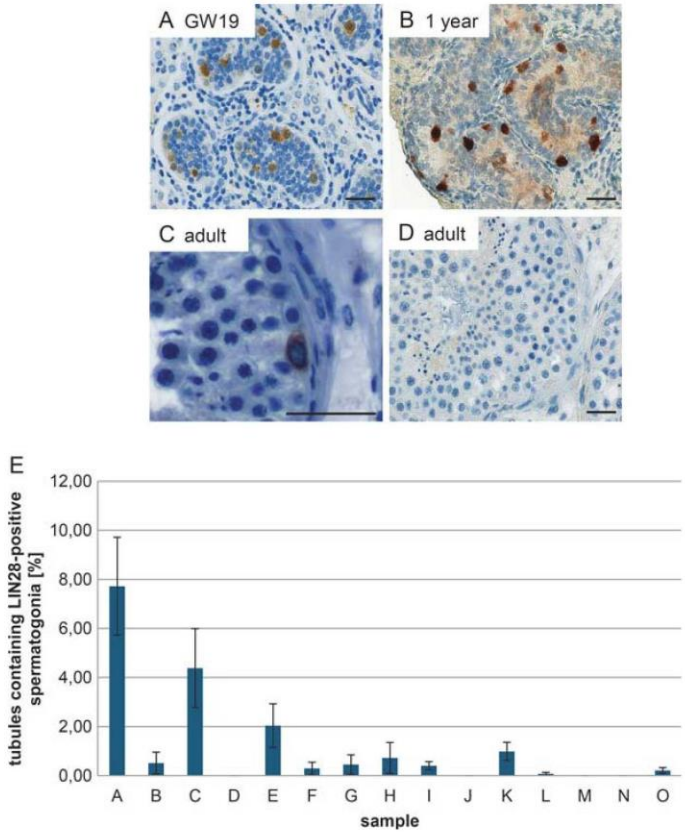

Figure 4 LIN28 expression in human germ cells during testis development. LIN28 is present in human gonocytes (**A**) and immature spermatogonia (**B**). Very few spermatogonia in the adult testis are LIN28-positive (**C**); for quantification see (**E**), however, the vast majority of all tubules showed no LIN28-positive spermatogonia. (**D**) Negative control. The scale bar represents 50 μm. (**E**) Percentage of tubular cross sections containing at least one LIN28-positive cell in adult testis. Bars A–J represent histologically normal samples from patients with germ cell tumors in the contra-lateral testis, K–O represent normal testis samples from prostate carcinoma patients without any evidence of germ cell tumors undergoing orchiectomy for therapeutic purposes.

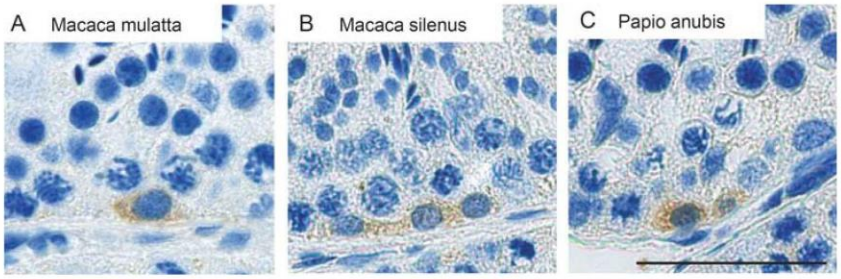

Figure 5 LIN28 in additional NHP species. LIN28 is present in single or paired spermatogonia in the rhesus monkey (*Macacca mulatta*) (**A**) and the lion-tailed macaque (*Macacca silenus*) (**B**) as well as in the baboon (*Papio anubis*) (**C**). The scale bar represents 50 μm.

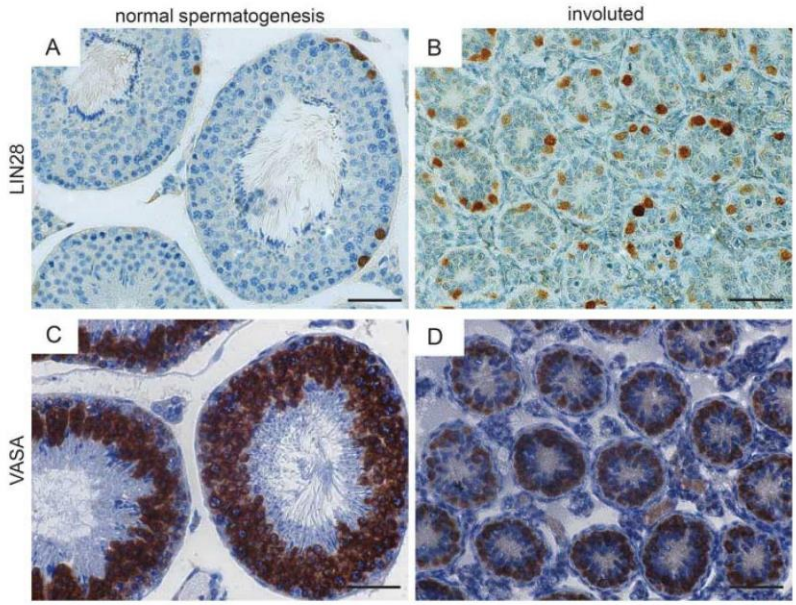

Figure 6 Spermatogonia in involuted hamster testes are LIN28-positive. (**A**) Djungarian hamster testis with normal full spermatogenesis. Few spermatogonia are intensely LIN28-positve. (**B**) Fully involuted hamster testis showing LIN28-positive spermatogonia, which are present at very high density in this condition. (**C**) Germ cells including most spermatogonia in the normal testis are also positive for VASA. (**D**) Also in the involuted testis the germ cells are VASA-positive. There are more VASA- than LIN28-positive cells. The scale bar represents 50 μm.

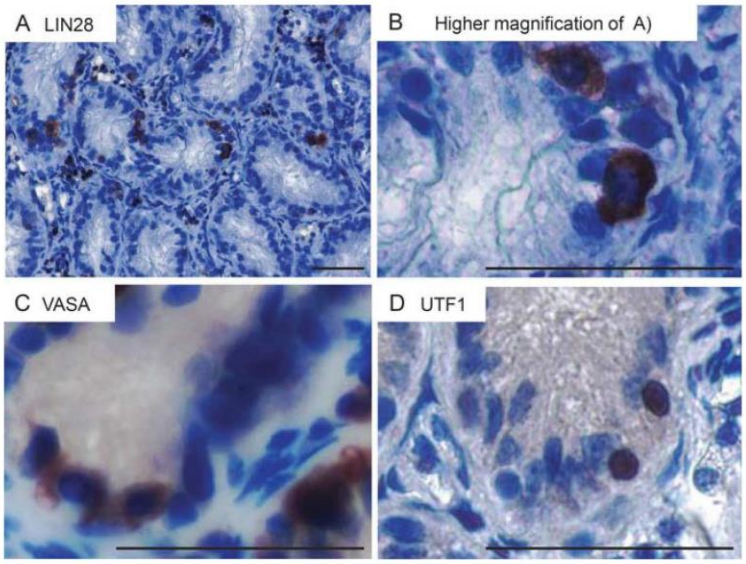

Figure 7 Spermatogonia in involuted primate testes are LIN28-positive. (**A**) Fully involuted monkey (*Lemur catta*) testis showing only very few germ cells in the inactive germinal epithelium consisting mainly of somatic Sertoli cells. However, many of the germ cells present are clearly LIN28-positive. (**B**) Higher magnification of (A). The germ cells also express VASA (**C**) and UTF1 (**D**). The scale bar represents 50 μm.

2006; Nakagawa *et al.*, 2007; Yoshida, 2008; Barroca *et al.*, 2009; Ko *et al.*, 2009; Suzuki *et al.*, 2009; Nakagawa *et al.*, 2010); for review see (Phillips *et al.*, 2010). Strikingly, in NHPs and human, where the SSCs are thought to be a subpopulation of the morphological populations of A_{dark} and/or A_{pale} spermatogonia, emerging data indicate that these spermatogonial populations exhibit a very heterogenous molecular phenotype (Hermann *et al.*, 2009) and even to date it is still a matter of debate whether A_{dark} spermatogonia contribute to sperm production under normal physiological circumstances or whether they only represent reserve stem cells which repopulate a germinal epithelium after a gonadotoxic insult (Ehmcke and Schlatt, 2006; Ehmcke *et al.*, 2006; Hermann *et al.*, 2010). Moreover, it is still not fully understood how and which type of spermatogonia replenishes the SSC pool. These fundamental gaps in our knowledge about SSCs in primates are very difficult or even impossible to close since the important tools which advanced mouse SSC research during the recent years, e.g. SSC transplantation and *in vivo*-imaging using transgenic mouse strains, will not be available in primates as routine within the foreseeable future. Therefore, analysis of SSCs in primates relies mainly on whole mount analysis of cultured tissue fragments and on *in situ* characterization of SSCs in fixed tissue sections using IHC.

For that purpose, it is important to establish a specific, meaningful and thus unequivocal panel of markers for the characterization and probably selection of specific types of spermatogonia in general and SSCs in particular. Some useful markers for different purposes have been described during recent years (Aponte *et al.*, 2005; Hermann *et al.*, 2010; Plant, 2010). However, additional markers would be of great advantage to better describe and characterize spermatogonial subpopulations.

To our knowledge so far, the LIN28-positive cells are a much rarer cell type than any other primate spermatogonial cell population described. Therefore, additional studies have to be performed using double (LIN28 together with e.g. PLZF, NGN3, c-KIT, respectively) and whole mount labeling, since the length of the LIN28-positive spermatogonial clones is of interest. However, the fact that those spermatogonia remaining in the testis during severe involution are clearly LIN28-positve in the NHP and rodent testis strongly argues for a spermatogonial stem cell identity of the LIN28-positive cells.

LIN28 is a pluripotency factor that is highly expressed in pluripotent mouse ES cells, mouse embryonal carcinoma and human teratocarcinoma cells (Moss and Tang, 2003). LIN28 was also very supportive or even necessary during iPS cell generation in human and NHP cells (Yu *et al.*, 2007; Tomioka *et al.*, 2010; Warren *et al.*, 2010). However, in contrast to most other reprogramming proteins, which are DNA-binding transcription factors, LIN28 is a well-conserved RNA-binding protein exhibiting unique molecular features as it is the only known mammalian protein that combines two specific domains: the cold shock domain, which binds to single-stranded nucleic acids, and the aminosäuren cystein/histidin zinc finger motif. The latter motif is known from nucleocapsid proteins of retro-viruses. It is involved in packaging the viral RNA into the virus particles [(Moss and Tang, 2003) and references therein]. Thus, LIN28 is an RNA-binding protein and as such, it predominantly localizes to the cytoplasm of the spermatogonia as shown in this study. A recent paper showed that LIN28 binds to and enhances the translation of mRNAs, including *OCT4*, that are involved in the growth and survival of human ES cells (Qiu *et al.*, 2010; Peng *et al.*, 2011). Since there are additional reports on LIN28 expression in different types of (cancer) stem cells, it is very likely that LIN28 has a general important function in stem cells.

During recent years, several publications reported the culture of spermatogonial stem cells from mouse testis and the subsequent derivation of multipotent/pluripotent ES cell-like cells (Guan *et al.*, 2006; Seandel *et al.*, 2007; Kanatsu-Shinohara *et al.*, 2008; Ko *et al.*, 2009). However, the attempts to derive the corresponding human cells were not as convincing as in the mouse (Conrad *et al.*, 2008; Golestaneh *et al.*, 2009; Kossack *et al.*, 2009; Mizrak *et al.*, 2010) and the origin and developmental potential of cultured cells from human testis is still under debate (Ko *et al.*, 2010; 2011; Tapia *et al.*, 2011; Chikhovskaya *et al.*, 2012; Eildermann *et al.*, 2012b). One major problem in the human system is that the final proof of pluripotency cannot be achieved, since chimera formation between preimplantation embryo and pluripotent stem cells appears impossible (Tachibana *et al.*, 2012). In addition to the debated data published for human testis cells, we failed to isolate and culture ES cell-like cells from the NHP testis (Eildermann *et al.*, 2012b). Taking this into consideration, we searched for biological differences between the mouse and the primate testes and analyzed the expression of several pluripotency factors in fetal and postnatal testes of man, macaques, marmosets and mice. While some pluripotency factors such as SALL4 showed expression in gonocytes and in adult spermatogonia in all the species analyzed (Eildermann *et al.*, 2012a), others were present in gonocytes (like OCT4), but were absent from spermatogonia of all the species. In general, the expression patterns of the factors analyzed in primates and mice were comparable. A very exciting exception of these conserved expression patterns of the pluripotency factors in the primate and mouse germ line was LIN28, as reported in this study and in parts also shown previously by others (Zheng *et al.*, 2009; Cao *et al.*, 2011; Gillis *et al.*, 2011). The data published by Zheng *et al.* (2009) were fully confirmed in our study. We also confirmed a huge discrepancy between LIN28 expression in the mouse and in the primate testes including human testes. However, while Cao *et al.* (2011) and Gillis *et al.* (2011) reported a complete absence of LIN28 from adult spermatogonia, we identified a very small subpopulation of spermatogonia clearly expressing LIN28 in the monkey and human testes. This discrepancy to previous reports could be due to the very low frequency of the LIN28-positive cells in primate testis. Indeed, there are human testis sections even in our own study that do not contain any LIN28-positive cells. If we had not detected LIN28-positive spermatogonia in the monkey testis, which we initially analyzed in more detail, it is possible that we (also) would have missed the stained spermatogonia in the human testis. Importantly, our data do not support the idea that the stained spermatogonia are undergoing apoptosis as it was suggested for LIN28-positive human gonocytes (Gillis *et al.*, 2011). They rather support the idea that the LIN28-positive cells may represent a spermatogonial stem cell population. The enormous difference between primate and mouse testis in the number of spermatogonia expressing LIN28 may contribute to the discrepancy regarding derivation of pluripotent ES cell-like cells from mouse and primate testis.

This discrepant LIN28 expression in mouse and marmoset testis also explains the differential expression profile during development (see Fig. 3E and F). In the marmoset testis, the percentage (and probably also the absolute number) of LIN28-positive cell is continuously

decreasing during development. In contrast, in the post-natal mouse testis, the population of LIN28 expressing cells, i.e. gonocytes and immature spermatogonia, is quickly expanding during the first days of post-natal testis development and only subsequently diluted by the more differentiated germ cells such as spermatocytes and spermatids.

In summary, LIN28 is expressed only in a very small subpopulation of spermatogonia in the adult monkey and human testis, which is in striking contrast to the mouse testis. Taking the functional role of LIN28 in pluripotent stem cells into account, it is likely that LIN28 is expressed in SSCs and may thus serve as a novel SSCs marker in NHPs and in man. Furthermore, since LIN28 is essential for germ cell specification in mice (West *et al.*, 2009), it is tempting to speculate that this RNA-binding protein is also involved in maintaining the identity of adult SSC. This assumption is supported by the fact that LIN28 is expressed in (i) spermatogonia of involuted rodent and primate testes (as shown in this study) and (ii) neoplastic germ cells (Cao *et al.*, 2011; Gillis *et al.*, 2011), which are known to express many pluripotent stem cell markers (Rajpert-De Meyts, 2006; Emerson and Ulbright, 2010).

Supplementary data

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

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Authors' roles

N.A. and K.E.: study design, collection and organization of data, data analysis and interpretation and final approval of the manuscript. C.D. and S. Schweyer: contribution of study material and final approval of the manuscript. J.E.: data analysis and interpretation and final approval of the manuscript. A.L., M.B. and S.K.: contribution of study material and final approval of the manuscript. J.G.: collection and assembly of data and final approval of the manuscript. S. Schlatt: contribution of study material, data analysis and interpretation and final approval of the manuscript. R.B.: study design, assembly of data, data analysis and interpretation, manuscript writing and final approval of the manuscript.

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Conflict of interest

None declared.

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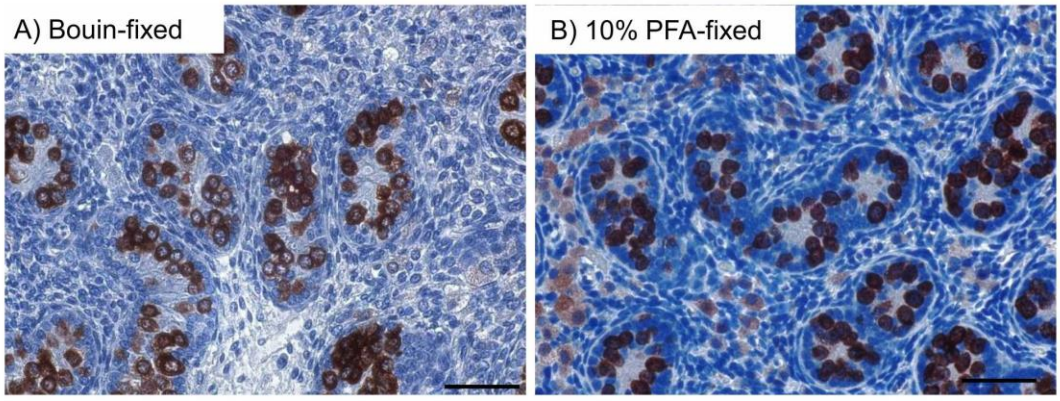
Aeckerle et al. Supplemental Fig.1



Comparison of LIN28-staining on differently fixed tissues shows no significant difference in the staining pattern between Bouin-fixed and 10% PFA-fixed tissue. A) Bouin-fixed testis from a newborn marmoset monkey. B) 10% PFA-fixed testis from a newborn marmoset monkey.

Aeckerle et al. Supplemental Tab. 1

sample	number of counted tubules	tubules containing LIN28-positive gonocytes/ (pre-) spermatogonia	number of LIN28-positive gonocytes/ (pre-) spermatogonia	% of tubules containing LIN28-positive gonocytes/ (pre-) spermatogonia
A	20	20	128	100
B	20	20	171	100
C	20	20	73	100
D	20 20	20 20	268 284	100 100
E	20	16	82	80
F	20	20	83	100
G	20	20	56	100
H	20 20	15 16	42 37	75 80
I	259	3	4	1.16
J	151	5	7	3.31
K	412	2	2	0.49
L	321 355	9 8	14 12	2.80 2.25

Quantification of LIN28 expression in marmoset monkey testis. A-D) Testis samples from newborn marmoset monkeys. 20 tubules per animal were evaluated. As an internal control a second section was evaluated for one animal (D). 100% ± 0 of tubules in the newborn testis contained LIN28 positive gonocytes. E-H) Testis samples from pubertal marmoset monkeys. 20 tubules per animal were evaluated. As an internal control a second section was evaluated for one animal (H). In average 89.38% ± 5.33 of tubules contained LIN28 positive spermatogonia. I-L) Testis samples from adult marmoset monkeys. All tubules per section were evaluated. As an internal control a second section was evaluated for one animal (L). In average 1.87% ± 0.56 of tubules contained LIN28 positive spermatogonia.

Aeckerle et al. Supplemental Tab. 2

sample	number of counted tubules	tubules containing LIN28-positive gonocytes/ (pre-) spermatogonia	number of LIN28-positive gonocytes/ (pre-) spermatogonia	% of tubules containing LIN28-positive gonocytes/ (pre-)spermatogonia
A	100	90	nd	90
B	100	82	nd	82
C	100	83	nd	83
D	100	79	nd	79
E	100	98	nd	98
F	100	78	nd	78
G	15	6	nd	40
H	141 179 186	7 22 11	13 39 16	4.96 12.29 5.91
I	83 65 42	0 1 0	0 1 0	0 1.54 0
J	171 194 215	8 2 16	11 2 17	4.68 1.03 7.44
K	86 153 165	0 0 0	0 0 0	0 0 0
L	45 72 60	0 2 2	0 2 2	0 2.78 3.33
M	114 93 140	1 0 0	1 0 0	0.88 0 0
N	63 25 73	0 0 1	0 0 1	0 0 1.37
O	31 46 31	0 1 0	0 1 0	0 2.17 0
P	161 171 169	1 1 0	1 1 0	0.62 0.58 0
Q	64 125 97	0 0 0	0 0 0	0 0 0
R	322 335 351	1 6 3	1 6 4	0.31 1.79 0.85
S	953 963 907	0 0 2	0 0 2	0 0 0.22
T	207 208 213	0 0 0	0 0 0	0 0 0
U	1054 1026 1021	0 0 0	0 0 0	0 0 0
V	1120 864 1087	2 4 0	3 7 0	0.18 0.46 0

LIN28 expression in human testis. A-F) fetal testis samples. 100 tubules per sample were evaluated. In average 85% ± 3.49 of tubules contained LIN28-positive gonocytes. G) Testis samples from a one year old boy. The section contained 15 tubules, from which 6 contained LIN28 positive (pre-) spermatogonia (40%). H-V) adult testis samples. H-Q are samples from patients with testis tumors on the contra-lateral side, R-V are samples from prostate carcinoma patients with no evidence of testicular malfunction. All tubules per section were evaluated. In average 1.19% ± 1.04 of tubules contained LIN28-positive spermatogonia. nd = not determined.

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Grafting of Neonatal Marmoset Monkey Testicular Single-Cell Suspensions into Immunodeficient Mice Leads to ex situ Testicular Cord Neomorphogenesis

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Key Words
Germ cells · Non-human primate · Pluripotency · Sertoli cell · Testicular morphogenesis

Abstract
Single-cell suspensions derived from immature rodent and ungulate testes can reconstitute testicular cords upon grafting into immunodeficient mice. In the present study, neonatal common marmoset monkey *(Callithrix jacchus)* testes were digested to a single-cell suspension, which was transplanted subcutaneously into immunodeficient mice. After 9 or 18 weeks of incubation, the derivatives of the grafted single-cell suspensions were retrieved and analyzed histologically and immunohistochemically. Three of 4 (75%) neonatal grafts exhibited reconstituted seminiferous cords strongly resembling seminiferous cords of the intact neonatal testis. The cords consisted of Sertoli cells, germ cells and peritubular myoid cells, which was confirmed by immunohistochemical marker analysis. Three-dimensional reconstruction models of the grafts revealed elongated tubules. Some of the tubules were branched, which occurs also in vivo, as we show here for the marmoset monkey. Importantly, no teratoma formation by immature pluripotency factor-expressing germ cells was observed. In summary, the reconstituted testicular cords were almost indistinguishable from the cords formed in situ, thereby impressively demonstrating a very high reconstructive potential of a single-cell suspension obtained from the neonatal marmoset monkey testis. To our knowledge, this is the first study demonstrating testicular cord neomorphogenesis for a primate species ex situ.

Introduction

Germ cells transmit the genetic information from one generation to the next. During male adulthood, spermatogonial stem cells preserve the male germ lineage. Stem cells are located in a specialized environment, the stem cell niche, which controls their proliferation and dif-

Abbreviations used in this paper

AMH	anti-müllerian hormone
DMEM	Dulbecco's modified Eagle's medium
F-12	Ham's F-12 nutrient mixture
HRP	horseradish peroxidase
MAGE-A4	melanoma-associated antigen 4
NB	new born
NHP	non-human primate
PGP	protein gene product
SALL4	Sal-like protein 4
α-SMA	α-smooth muscle actin

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ferentiation. Therefore, in the testes, the accurate spatial arrangement of the different cell types during embryogenesis is essential for the establishment of spermatogenesis in adulthood. During embryogenesis, an indifferent gonad arises, which is morphologically indistinguishable between males and females [Capel, 2000]. In the presence of SRY, the indifferent gonad develops into a testis. Histologically, testes and ovaries become distinguishable after formation of seminiferous cords in the testis. During cord formation, immature Sertoli cells aggregate with germ cells, differentiate and become polarized epithelial cells [Jost et al., 1981; Magre and Jost, 1991; Cupp and Skinner, 2005]. Concurrently, cells from the mesonephros migrate into the male gonad and surround the aggregating Sertoli and germ cells. It is assumed that these migrating cells become peritubular myoid cells and that this process is required for cord formation [Buehr et al., 1993; Tilmann and Capel, 1999]. A properly developed somatic environment is necessary to enable spermatogenesis during adulthood.

Researchers have long attempted to recapitulate the process of spermatogenesis in vitro [Kierszenbaum, 1994; Staub, 2001]. It was demonstrated that cocultured Sertoli cells and peritubular myoid cells isolated from pubertal rats aggregate and form structures resembling germ cell-depleted tubules, while both Sertoli or peritubular cells alone were unable to form these structures [Tung and Fritz, 1980]. The rearrangement of tubular structures was also observed after transplantation of testicular cells in immunodeficient mice. This transplantation approach has been modified and evaluated further using testis cells from neonatal pigs [Honaramooz et al., 2007], rodents [Dufour et al., 2002; Gassei et al., 2006; Kita et al., 2007; Gassei et al., 2008], bovine [Zhang et al., 2008] and sheep [Arregui et al., 2008]. The reconstructed tubules consisted mostly of Sertoli cells and peritubular myoid cells, but occasionally germ cells were also observed. Spermatogenesis up to and beyond the level of round spermatids was obtained in reconstituted tissue from testis cell suspensions of immature pigs, sheep and embryonic or neonatal rats and mice [Honaramooz et al., 2007; Kita et al., 2007; Arregui et al., 2008]. In the mouse model, even fertilization-competent spermatids were obtained in reconstituted tubules [Kita et al., 2007].

In the neonatal marmoset testis (i.e. first few days after birth), the number of OCT4-positive germ cells is on average 12% of all germ cells, which are in the range between 0.12 and 0.56 × 10^6 cells [McKinnell et al., 2009]. Moreover, besides OCT4, many marmoset monkey gonocytes express the well-established key pluripotency factors SOX2, NANOG, AP2γ, Sal-like protein 4 (SALL4) and LIN28 [Mitchell et al., 2008; Albert et al., 2010; Aeckerle et al., 2012; Albert et al., 2012; Eildermann et al., 2012a], suggesting a developmental potential similar to pluripotent embryonic stem cells [Boyer et al., 2005]. Pluripotency of human and non-human primate (NHP) cells can be demonstrated by an assay technically identical to the approach used in this study, the teratoma formation assay: the test cells are injected into an immunodeficient mouse, where the cells can survive, proliferate and eventually differentiate for up to several weeks. Development of histologically normal endo-, meso- and ectodermal derivatives in this assay is the best indication of pluripotency currently available for human and NHP cells.

In pathological cases, immature germ cells may undergo immediate reprogramming to become a pluripotent embryonic germ cell, which is the origin of (intragonadal) teratomas or yolk sac tumors (type I germ cell tumors) [Oosterhuis and Looijenga, 2005]. However, to our knowledge, human or NHP gonocytes were never tested for their ability to form teratomas after removal from their natural lineage-committing environment and subsequent transplantation into immunodeficient mice. Therefore, in addition to the reconstructive potential of isolated cells from the NHP testis, we were also interested in whether pluripotency factor-expressing gonocytes were able to form teratoma under the experimental conditions used in this study.

We show here for the first time testicular cord reconstitution for an NHP after full enzymatic dissociation of the testis to a single-cell suspension. Reconstituted cords were able to maintain germ cells for at least 18 weeks. They also exhibited branching, which we document here, to our knowledge, also for the first time for NHP cord (re)constitution. In contrast to these novel findings regarding primate testis cord development, we obtained no evidence of a teratogenic potential of NHP neonatal gonocytes.

Materials and Methods

Marmoset Monkey Testis Tissue Samples

This study was performed in strict accordance with the German Animal Protection Law. All surgery was performed under anesthesia, and every effort was made to minimize suffering. Animals were housed according to the standard German Primate Center practice for common marmoset monkeys. For castration, animals were deeply anesthetized with an intramuscular injection of 0.5 ml/kg body weight of Göttinger mixture II (50 mg/ml ketamine, 10 mg/ml xylazine and 10 mg/ml atropine) and 0.05 ml/kg body weight diazepam into the quadriceps femoris muscle. At this dose rate, the

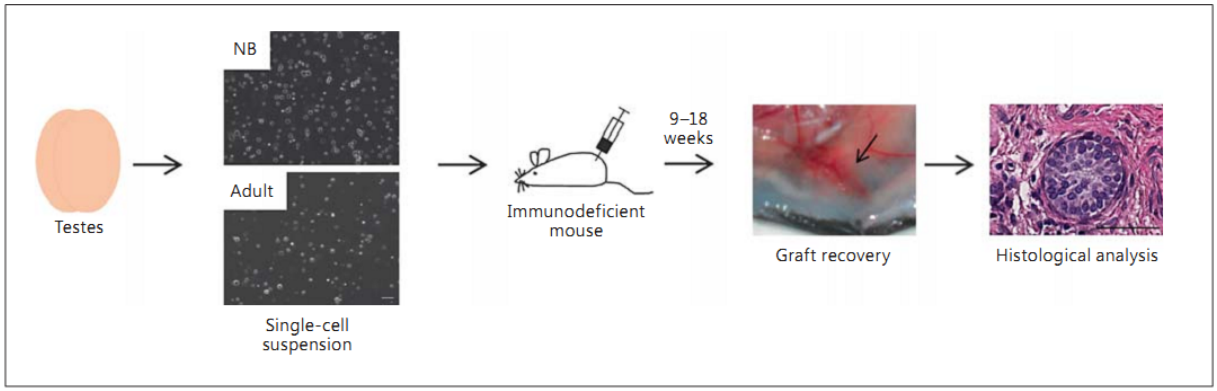

Fig. 1. Study design. Testes from marmoset monkeys of various ages were enzymatically digested to single cells. These single-cell suspensions were mixed 1:1 (vol/vol) with Matrigel and injected subcutaneously into an immunodeficient mouse. After incubation for 9 or 18 weeks, the grafts were recovered and prepared for histological and immunohistochemical analysis. Bar = 50 µm.

duration of anesthesia was usually 30–45 min. For euthanasia of neonatal and juvenile monkeys, 0.2 ml of Narcoren® were injected intraperitoneally. Nine marmoset monkey testes were used for the grafting experiments (the license number for adult testis retrieval was AZ11/0395). Neonatal and juvenile testes were obtained from animals that had to be killed due to animal husbandry reasons (inability of the mother to feed 3 neonates; no license necessary).

Derivation of a Single-Cell Suspension and Injection of Cells

Testes from 6 neonatal (1–2 days old, i.e. containing pluripotency marker-expressing germ cells), one 4-month-old and 2 adult (3.5-year-old) marmoset monkeys were used for cell preparation. A single-cell suspension was obtained using enzymatic digestion and subsequent filtering. In brief, testes were mechanically decapsulated and the seminiferous tubules were digested with 1 mg/ml collagenase I (Sigma, catalog No. C-2674), 1 mg/ml hyaluronidase (H-3506; Sigma) and 15 U/ml DNase I (Sigma, catalog No. D4263) in Dulbecco's modified Eagle's medium (DMEM; Gibco, catalog No. 31966021)/Ham's F-12 nutrient mixture (F-12; Gibco, catalog No. 31765027; 1:1) under mild rotation at 37°C with pipetting up and down every 5–10 min until a single-cell suspension was achieved after 30–45 min. The cell suspension was filtered through a 70-µm mesh to assure that no tubule fragments remained. The single cells were washed with DMEM/F-12 supplemented with 10% FBS, Pen/Strep and AmpB; 4×10^5 to 6×10^6 cells were resuspended in 100 µl DMEM/F-12 and mixed 1:1 (vol.) with Matrigel (BD Biosciences, catalog No. 356231). The fresh marmoset testicular cells were kept on ice prior to transplantation. Transplantation was performed within 1 h. Images of single-cell suspension were taken on a Zeiss Axio Observer Z1 microscope.

Grafting Procedure and Graft Recovery

Immunodeficient adult $RAG2^{-/-}\gamma c^{-/-}$ or SCID (C.B-17/Ztm-scid) mice (males and females) housed in the central facility for animal experimentation at the University Medical Center Göttingen served as hosts for xenografting. The license number for transplantation of cells into mice was 33.42502-04-113/09. SCID mice were used in 2 cases when due to the timing of the marmoset castrations no $RAG2^{-/-}\gamma c^{-/-}$ recipients were available. In contrast to $RAG2^{-/-}\gamma c^{-/-}$ mice, SCID mice have natural killer cells, but both strains are widely used in pluripotency assays and the presence of natural killer cells might delay but not prevent teratoma growth after injection of pluripotent stem cells [Dressel, 2011]. For grafting, 100 µl DMEM/F12 containing 4×10^5 to 6×10^6 marmoset testicular cells were mixed 1:1 with Matrigel. Extracellular matrix gel was co-injected because of its ability to support cord formation in a previous report [Hadley et al., 1985]. The resulting solution was injected subcutaneously in the left groin region of the host mouse using a 1.25-inch injection needle. The contralateral side served as control: 200 µl DMEM/F-12 without cells mixed with Matrigel (1:1) were injected. Grafts were allowed to develop for 9 or 18 weeks. An overview of the study design is given in figure 1. The recipient mice were palpated twice weekly to check for tumor formation.

For graft retrieval, recipient mice were killed by cervical dislocation. The ventral skin of the mouse was removed and grafts were located on the interior surface of the skin. Grafts were excised and fixed in Bouin's fixative overnight at room temperature followed by several washes with 70% ethanol. Tissues were routinely embedded in paraffin, sectioned at 5 µm and stained with hematoxylin-eosin for routine histological analyses.

Immunohistochemistry of Tissue Sections

For immunohistochemical staining, tissue sections were deparaffinized, 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 incubation with a peroxidase blocking reagent (DakoCytomation, Carpinteria, Calif., USA; LSAB+ system-HRP, K0679). Antibody specifications and dilutions are given in table 1. All incubation steps were done in a humid chamber and incubations with the primary antibody were performed overnight at 4°C. The DakoCytomation Universal LSAB+ kit including biotinylated secondary antibody polymer and

Table 1. Antibodies used in this study

Antigen	Source	Catalog No.	Host	Dilution
α-SMA	Sigma	A2547	mouse	1:1,000
LIN28A	Cell Signaling	3978S	rabbit	1:70
MAGE-A4	Giulio C. Spagnoli		mouse	1:25
AMH	Santa Cruz	sc-6886	goat	1:100
OCT4A	Cell Signaling	2890	rabbit	1:100
PGP9.5	DakoCytomation	Z 5116	rabbit	1:500
SALL4	Abcam	ab57577	mouse	1:200
SOX9	Millipore	MAB5535	rabbit	1:1,000
VASA	R&D Systems	AF2030	goat	1:100
Goat IgG isotype control	Antibodies-online	ABIN 376825	goat	1:2,500
Mouse IgG isotype control	Vector	I-1000	mouse	1:100
Rabbit IgG isotype control	Antibodies-online	ABIN 376827	rabbit	1:2,500

horseradish peroxidase (HRP)-conjugated streptavidin was employed for the detection of bound primary antibody. 3,3′-Diaminobenzidine chromogen was used as substrate for HRP and Mayer's hematoxylin as counterstain. Control stainings were carried out using suitable nonspecific IgG instead of the specific antibody at the same protein concentration. Pictures were taken using a Zeiss microscope and the Nuance™ multispectral camera.

Preparation of Seminiferous Tubules

Pictures of seminiferous tubules were taken after decapsulation of the testes and enzymatic removal of the interstitial cells using 1 mg/ml collagenase I (Sigma, catalog No. C-2674) and 15 U/ml DNase I (Sigma, catalog No. D4263) in DMEM/F-12 (1:1). Pictures of the tubules were taken using a stereomicroscope (Discovery.V8 SteREO; Zeiss).

3D Model of the Reconstructed Tubules

To construct a 3D model of the rearranged tubules we used the software Amira. With this software, the pictures of 29 adjacent sections were aligned. For construction of the model, some information on the voxel (3D pixel) size is needed. Since every section of the paraffin block is 5 µm thick and 1 µm of the picture correlates to 1 pixel, the voxel size was defined as 1 × 1 × 5 pixels. To reduce the amount of data needed for the calculation, the color of the pictures was reduced to a gray scale. With the segmentation editor, the outlines of all tubules were marked. Based on this information, the software was able to calculate the surface of the tubules. Since the display of all tubules included in the graft was confusing, only few selected tubules are shown in the 3D model.

Results

General Observations

During the incubation period of the injected cells in the host mice, no tumors were palpable and no other adverse effects of the cell injections became obvious.

Tissue Digestion Results in Single-Cell Suspensions

Single-cell suspensions were obtained from marmoset monkey testes by enzymatic tissue digestion using collagenase, hyaluronidase and DNase. Completeness of the tissue disaggregation was confirmed microscopically after filtration of cell suspensions through a 70-µm nylon mesh (fig. 1). Occasionally, few cells remained connected, but no tubular fragments were observed.

Histological Analysis of the Grafts

Derivatives of the transplanted cells from neonatal testes were found in 4 recipients (in total 4 recipients were successfully injected; 100% graft recovery). Three of 4 (75%; table 2) of these xenografts from neonatal testis cells contained tubular structures strongly resembling seminiferous tubules of the neonatal marmoset monkey (fig. 2). Tubular structures occurred irrespective of the sex of the recipient mouse (table 2). Pieces of Matrigel were detected in the tissue surrounding the tubules (fig. 2c), which were distinguishable by their pink appearance after hematoxylin-eosin staining (marked with an asterisk).

Comparison of grafts after 9 or 18 weeks of incubation in the host mice revealed some differences. Figure 2a, b shows tubules developed in a graft within 9 weeks. These tubules demonstrate almost no (fig. 2a) or only partial (fig. 2b) enclosure in peritubular myoid cells, which is reflected by the incomplete anti-α-smooth muscle actin (SMA) staining surrounding the forming tubules. In some developing tubules, aggregation of putative Sertoli cells is visible while a clear and well-structured epithelial layer is not visible (fig. 2a). In contrast, more developed tubules exhibit formation of a well-structured polar epithelium in the tubules (fig. 2b). The more the tubules became enclosed

Table 2. Overview of the grafting experiments performed in this study

Recipient mouse No.	Age of the donor marmoset	Cells grafted, n	Host mouse	Incubation time, weeks	Teratoma	Graft with tubular structures
1	neonatal (2 animals)	6×10^6	RAG2$^{-/-}$γc$^{-/-}$ female	18	no	yes
2	neonatal	2.5×10^6	RAG2$^{-/-}$γc$^{-/-}$ male	9	no	yes
3	neonatal	4×10^5	RAG2$^{-/-}$γc$^{-/-}$ male	18	no	yes
4	neonatal	1.9×10^6	RAG2$^{-/-}$γc$^{-/-}$ male	9	no	no
5	juvenile	1.2×10^6	RAG2$^{-/-}$γc$^{-/-}$ female	19	no	no
6	adult	3×10^6	SCID female	9	no	no
7	adult	3×10^6	SCID male	9	no	no

by peritubular myoid cells, the more mature they appear in their tissue architecture. This corresponds to normal tubule development in the testis. Figure 2c–e depicts tubules developed for 18 weeks. Histological appearance and tubule boundaries are more prominent. Tubules exhibit Sertoli cells, a basal membrane and surrounding cells with the typical flat morphology of peritubular myoid cells (fig. 2e). None of the tubules reconstituted in the grafts showed a central lumen. This is in agreement with the lack of a lumen in the native marmoset testis until puberty (fig. 3). Pieces of Matrigel were found inside the grafts (fig. 2c: asterisk). The tissues collected after control injection of DMEM/F12 mixed with Matrigel without cells revealed the presence of Matrigel in the subcutaneous adipose tissue of the host mouse. Apart from the Matrigel pieces, we did not observe any additional histological structure which does not occur in normal mice (data not shown).

Analysis of injection sites of older donor (pubertal and adult) tissue revealed the presence of Matrigel in the subcutaneous adipose tissue of the host mouse. This indicates that we analyzed the injection site. However, no cells in the recovered tissue could clearly be traced back to the donor cells, and formation of tubular structures was absent (data not shown).

Histological analysis of the recovered tissue (fig. 2c) revealed that no tumor with the histological features of a teratoma, i.e. histologically mature derivatives of the three embryonic germ layers (ectoderm, mesoderm and endoderm) developed after injection of testicular cells from neonatal (4 recipients), juvenile (1 recipient) or adult (2 recipients) animals (table 2).

Immunohistochemical Marker Analysis of the Graft Tissue

To investigate which cell types contribute to seminiferous tubule formation, we performed immunohistochemistry with established markers for different cell types present in the donor tissue (fig. 3).

Anti-müllerian hormone (AMH) is a cytoplasmic marker of immature Sertoli cells in human as well as mouse testes [Tran et al., 1987; Behringer, 1995]. This marker has also been shown to be valid in the marmoset monkey testis [Mitchell et al., 2008; Albert et al., 2012, own unpubl. data]. The testis-determining SOX9 gene is a downstream transcription factor of SRY and essential for Sertoli cell specification. In the testis, its expression is restricted to Sertoli cells of all developmental stages, including terminally differentiated Sertoli cells. Its localization is nuclear. The expression pattern of SOX9 is conserved among diverse vertebrate species, which suggests an evolutionary conserved role [Morais da Silva et al., 1996].

As a marker of peritubular myoid cells, we used α-SMA. It is known to be present in smooth muscle cells of blood vessels and in testicular peritubular myoid cells [Tung and Fritz, 1990; Dufour et al., 2002].

VASA (DDX4), LIN28, melanoma-associated antigen 4 (MAGE-A4), SALL4 and protein gene product (PGP) 9.5 were used as germ cell markers [Tokunaga et al., 1999; Castrillon et al., 2000; Aubry et al., 2001; Aeckerle et al., 2012; Eildermann et al., 2012a]. This marker panel detects premeiotic (VASA, LIN28, MAGE-A4, SALL4 and PGP9.5), meiotic (VASA and MAGE-A4) and postmeiotic (VASA) germ cells in mammalian testes.

OCT4A was used as marker for pluripotent and immature germ cells. Previously, OCT4 expression was noted in neonatal marmoset testis [Mitchell et al., 2008]. Our data (this study) generated with a novel OCT4A-specific antibody different from those analyzed previously [Warthemann et al., 2012] support the studies on OCT4 expression in marmoset gonocytes published by other groups.

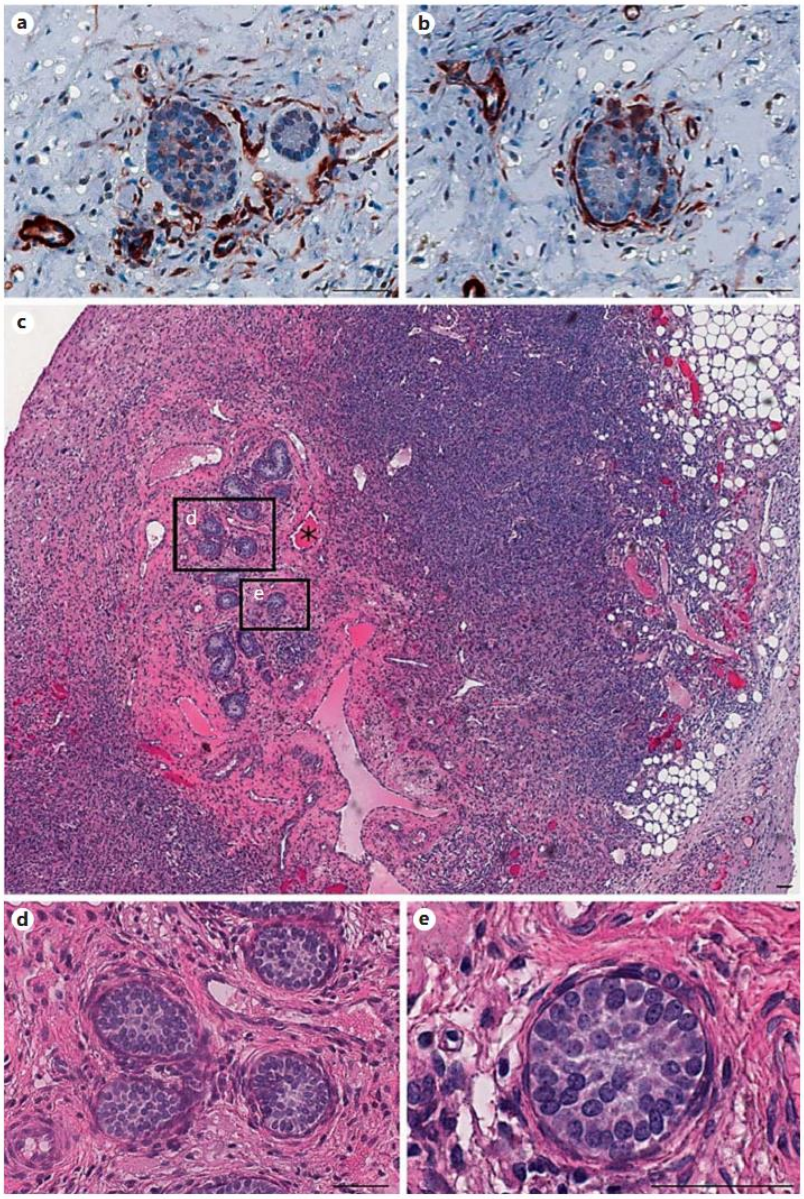

Fig. 2. Histological analysis of the grafts. Tubular structures developed inside the grafts, showing very similar morphology to intact testicular tissue of neonatal marmoset monkeys. **a**, **b** Graft after 9 weeks of incubation in the host mouse. Anti-α-SMA staining reveals that the grafts become surrounded by peritubular myoid cells during development. Some tubules showed no (**a**) and others only partial enclosure by peritubular myoid cells (**b**). Enclosure of the developed tubules seems to be associated with maturity of the tubules. The more a tubule is surrounded by peritubular myoid cells, the more organized are the cells within the tubule. **b** The tubule exhibits an obvious morphological polarity of the cells inside. **c–e** Graft developed within 18 weeks after injection of neonatal marmoset testicular cells into an immunodeficient host mouse. **c** Representative section of the graft. The tissue containing the reaggregated tubules is surrounded by adipose tissue of the host mouse. **d**, **e** Different magnifications of mature tubules which are almost indistinguishable from those in intact neonatal testes. They exhibit Sertoli cells, a basal membrane and surrounding cells with the typical flat morphology of peritubular myoid cells. None of the tubules reconstituted in the grafts showed a central lumen, which is in agreement with the lack of a lumen in the native marmoset testis until puberty. Pink areas show remaining Matrigel (*). **a**, **b**, **d**, **e** Bar = 50 μm. **c** Bar = 100 μm.

The donor tissue from neonatal testes used for the teratoma formation assay contained somatic cells, such as immature (i.e. not yet terminally differentiated and still proliferating) peritubular myoid (α-SMA+) and immature Sertoli cells (AMH+ and SOX9+). The prevalent germ cell type in the neonatal donor tissue was the gonocyte (LIN28+, PGP9.5+, MAGE-A4+, VASA+ and SALL4+), which represents the developmental stage between the primordial germ cells and the spermatogonial stem cells (fig. 3).

AMH immunostaining of the grafts revealed the presence of immature Sertoli cells in the cords. AMH was present in the cytoplasm of Sertoli cells for at least 18 weeks. This pattern is similar to AMH expression in pre-

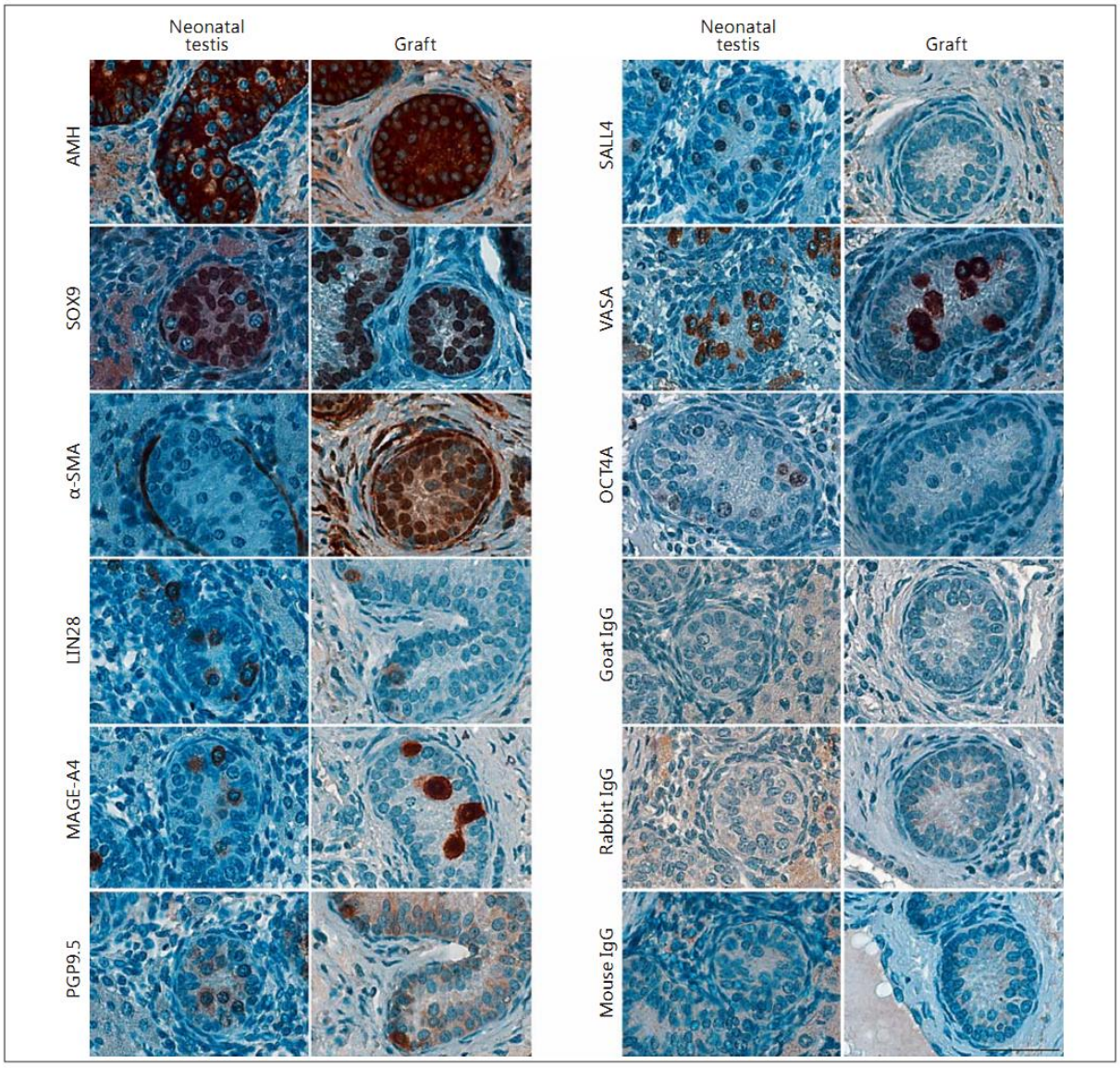

Fig. 3. Histological and immunohistochemical analysis of donor tissue and grafts. Reconstituted tubules in a graft after 18 weeks compared to intact neonatal testicular tissue. The testes of neonatal marmoset monkeys consist of seminiferous tubules and an interstitial compartment. The tubules contain Sertoli cells which express AMH and SOX9, and gonocytes which express LIN28, PGP9.5, MAGE-A4, VASA, OCT4 and SALL4. The tubules are surrounded by myoid peritubular cells which express α-SMA. The Leydig cells are located in the interstitial compartment. The tubules reconstituted in the grafts exhibit marker expression very similar to that in neonatal testes. The reconstituted tubules mainly consist of Sertoli cells, which is indicated by AMH and SOX9 expression of the cells. The number of Sertoli cells per cross section in the reconstituted tubules is comparable to that in normal testis tissue. The anti-α-SMA staining in the grafts was not restricted to peritubular cells. Many α-SMA+ cells are located in the interstitial compartment. Surprisingly, α-SMA signals were also obtained in nuclei of putative Sertoli cells inside the reconstituted tubules, which express AMH and SOX9 and exhibit the characteristic morphology of Sertoli cells. Germ cells are evidenced in the reconstituted tubules by the expression of VASA, MAGE-A4, PGP9.5 and LIN28. The number of germ cells present in the reconstituted tubules appeared lower than in intact testicular tissue. SALL4 and OCT4 expression was undetectable in all reconstituted tubules analyzed. For controls, unspecific IgG isotype controls were used. They show no specific signal. Bar = 50 μm.

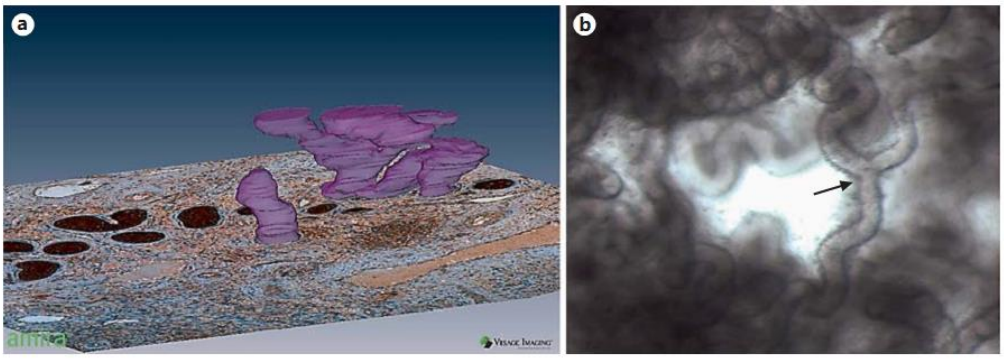

Fig. 4. Construction of a 3D model of the reconstructed tubules. **a** We used 29 adjacent sections for construction of the model. Since every paraffin section is 5 µm thick, the height of the model is 145 µm. The generated 3D model showed spherical structures as well as elongated tubules. Some of the elongated tubules are branched. Since the display of all tubules included in the graft may lead to confusion, only some selected tubules of the graft are shown. **b** Seminiferous tubules of a neonatal testis after removal of the interstitial tissue. Some tubules are branched (arrow).

pubertal marmoset monkey testes, suggesting that the transplanted Sertoli cells maintained their cellular identity. Sertoli cells in the periphery of the tubules appear polarized with their nuclei aligned along the basal membrane of the epithelium. The identity of the Sertoli cells was further confirmed by SOX9 expression. The specificity of the antibody for marmoset monkey SOX9 protein was corroborated by specific nuclear staining of pre-Sertoli cells in male gonadal primordia before the onset of sexual differentiation, while no staining was seen in gonadal primordia of female sibling embryos (data not shown). The peritubular myoid cells (α-SMA+) were surrounding the tubules. However, Sertoli cell nuclei in the graft as well as cells in the tissue between the tubules were also α-SMA+. α-SMA+ cells were additionally detected surrounding newly formed blood vessels (fig. 2a, b), which was also observed in intact neonatal testicular tissue.

One of 3 (33%) marmoset tissue grafts showing reconstituted tubules contained tubules with germ cells positive for VASA, LIN28, MAGE-A4 and PGP9.5 (fig. 3). VASA and MAGE-A4 exhibited stronger staining in the graft than neonatal testis. However, we are not able to discern whether this is due to biological differences in VASA and MAGE-A4 abundance or whether there are also technical factors, e.g. the grade of fixation, which may influence the staining intensity. One additional graft exhibited PGP9.5+ and MAGE-A4+ cells, but was negative for the other germ cell markers. In none of the reconstructed tubules, SALL4+ or OCT4A+ gonocytes were detectable. Control staining using the respective nonspecific IgG instead of the primary antibodies resulted in no staining. A comparison between neonatal testis and graft tissue (fig. 3) reveals that the morphology of the reconstructed tubules was almost indistinguishable from the appearance of normal testis tissue from neonatal marmoset monkeys. General tissue architecture appeared normal compared to neonatal testis cords. Some germ cells in the grafts were in a rather central position in the developing tubule, while other germ cells showed partial or even full contact with the basal lamina of the reconstructed epithelium. This suggests normal Sertoli-germ cell interaction. However, there was no indication (such as meiotic chromatin structure) that the surviving marmoset gonocytes further differentiated after injection. Their location within the epithelium and the cytoplasm/nucleus ratio in these cells identify them as gonocytes or (pre-) spermatogonia, which corresponds to the germ cell stages seen in situ. Marmoset monkeys exhibit a high Sertoli:germ cell ratio in the neonatal testis [Mitchell et al., 2008], which seems to be even higher in the graft. However, quantification of cell populations was not performed in this study.

3D Model of the Reconstructed Tubules

In order to get an idea of the extension and 3D structure of the reconstituted seminiferous tubules, we constructed a 3D model derived from consecutive histological sections using the Amira software (fig. 4a). The generated 3D model showed spherical structures as well

as elongated cords. Some of the reconstituted cords were branched, which is in accordance with the situation in vivo (fig. 4b, arrow). The height of the model is 145 µm.

Discussion

This study shows that cells from the postnatal NHP testis exhibit a remarkable reconstructive potential. The dispersed Sertoli cells realigned along a newly formed basement membrane and subsequently formed epithelium, while the peritubular myoid cells surrounded the polarized Sertoli cells forming distinct cords. The ability of isolated neonatal testis cells to recapitulate characteristic aspects of testicular morphogenesis appears to be initiated by interactions between Sertoli cells and peritubular myoid cells [Dufour et al., 2002]. One major difference between immature neonatal and juvenile or adult Sertoli cells is their proliferation rate. While neonatal Sertoli cells are highly proliferative, adult Sertoli cells are arrested in G_0 phase [Sharpe et al., 2003]. This might be the major reason why only neonatal Sertoli cells formed reconstituted tubules [Watanabe et al., 2009]. However, two different Ki-67 antibodies, which specifically label proliferating cells, did not work on these Bouin-fixed marmoset monkey tissues. Therefore, we were not able to further elucidate if the tubules in the grafts developed due to reassembly of injected preexisting cells or if the tubules formed by proliferation of small clusters of proliferating cells or even a single proliferating Sertoli cell. Nevertheless, a high proliferation capability or other unknown characteristics of neonatal Sertoli cells appear to be necessary, since grafting of Sertoli cells from older donors (juvenile or adult) did not result in cord formation. This also indicates that only immature somatic cells are capable of reorganizing cord-like structures for NHP.

The grafted neonatal cells mimicked the testicular development in vivo: the myoid cells in the graft migrated and surrounded the aggregating Sertoli and germ cells. As the graft matured, the myoid cells were located close to Sertoli cell nuclei located in the basal part of the cells. Nine and 18 weeks after injection, well-reconstructed fragments of seminiferous cords almost indistinguishable from the normal ones in the neonatal testis developed. Sertoli cells, peritubular myoid cells and gonocytes expressed all markers (except for SALL4 and OCT4A in the case of germ cells) tested in our laboratory to determine the cellular identity of different cell types in the normal testis.

The number of germ cells appeared to be relatively low in the grafts compared to natural cords, and not all reconstructed cords contained germ cells. We assume that this is due to the facts described in the following. Our unpublished experiments with cultured germ cells (gonocytes) showed that these cells are very vulnerable and vanish already after a few days in culture. Therefore, we assume that the disintegration of the testis before injection of the cell solution (which is the same procedure as for cell culture) is major stress for the cells leading to significant germ cell loss. Hence, we speculate that the single-cell solution available for tubulus reconstruction after injection does not represent the normal neonatal composition, i.e. the germ cells are underrepresented. This was also described for bovine cells [Honaramooz et al., 2007]. Furthermore, the Sertoli cells may exhibit a higher proliferation rate than germ cells, which leads to a dilution effect of the germ cells in an increasing Sertoli cell population after grafting.

It was evident that the Sertoli and peritubular myoid cells in the more mature grafts (18 weeks) were arranged strikingly similar to the seminiferous epithelial layer in neonatal marmoset monkey cords. The strong staining signal for α-SMA in peritubular cells suggests that these cells possess mature, functional contractile elements. The antibody against α-SMA detects only one specific band for marmoset α-SMA in Western blot analysis of marmoset monkey testis [Eildermann and Behr, unpubl. data]. Interestingly, the detection of nuclear signals for α-SMA within the cells of the reconstructed tubules is in accordance with the situation detected in 1 fetal marmoset gonad at GD95 [Aeckerle and Behr, unpubl. data]. If this represents any functionally significant condition remains to be determined.

The 3D model of the tubules in the graft showed spherical structures as well as elongated tubules. Some of the elongated tubules were branched. Branching of seminiferous tubules was previously reported for fetal and neonatal human testes [Hedinger and Weber, 1973], but it is generally a rarely described phenomenon. In this study, we detected tubular branching in reconstructed tubules as well as in the neonatal marmoset monkey testes. Thus, this indicates that, although destructed to a single-cell suspension, the testicular cells keep the inherent information to rebuild the complex histological 3D structure of a developing testicular seminiferous tubule consisting of at least two somatic cell types and germ cells. Previous studies reported cord formation at different transplantation sites, such as the renal subcapsular space, subcutaneous space, epididymal fat pad and omental pouch [Dufour et

al., 2002], suggesting that information inherent in the cells within the graft rather than instructive signals from the environment directs the neomorphogenesis of the cord structures.

Although we found germ cells in some tubules, it is not clear whether the testicular cords in the grafts are functional. The readout of functionality might be sperm production. In this study, germ cell differentiation apparently did not occur probably due to one of the following reasons: (i) the endocrine milieu was inappropriate for spermatogenesis; (ii) the time was not sufficient for entry into meiosis since even in vivo spermatocytes do not appear at 18 weeks of age in the marmoset testis, and (iii) a functionally abnormal germ cell-Sertoli cell interaction. Although previous studies in the marmoset monkey model reported grafting of immature testicular tissue [Schlatt et al., 2002; Wistuba et al., 2004], so far no single-cell suspensions (including isolated pluripotency factor-positive germ cells removed from their natural niche) were grafted xenologously. Importantly, in testicular tissue grafts from marmosets spermatogenesis did not proceed beyond the spermatogonial stage, although the germ cells grafted were present in their complete histological niche. The reason for this might be the absence of exon 10 of the marmoset luteinizing hormone receptor gene [Gromoll et al., 2003; Muller et al., 2004], which renders the marmoset sensitive to choriongonadotropin, but insensitive to luteinizing hormone. Since mice do not express choriongonadotropin, the host endocrine environment probably could not support androgen production in the graft. This lack of androgen production results in poor spermatogenesis after grafting [Schlatt et al., 2002; Wistuba et al., 2004]. However, other species-specific physiological reasons are also conceivable to be responsible for the arrest of testicular development in marmoset testicular grafts [Wistuba et al., 2004]. The resolution of this blockade is necessary to analyze the functionality of the reorganized cells and would allow the establishment of an in vivo model for postnatal primate testicular development. It would also offer the scientifically interesting possibility to (genetically) manipulate the germ cells prior to grafting.

Marmoset monkey gonocytes express many markers characteristic of pluripotent embryonic stem cells, e.g. OCT4, NANOG, AP2γ, SALL4 and LIN28. Moreover, it is accepted knowledge that teratomas arise from undifferentiated germ cells of the primordial germ cell or gonocyte/oogonia stage. Therefore, we hypothesized that neonatal marmoset monkey gonocytes may form teratomas. In parallel experiments to this study, we generated teratomas using marmoset monkey iPS cells in RAG2$^{-/-}$ γc$^{-/-}$ mice [Debowski and Behr, unpubl. data]. However, in the present experimental setting and in the limited numbers of experiments we were able to perform, we did not observe any teratoma. We were also not able to detect OCT4A in germ cells of the graft or in any other cell surrounding the tubules. The lack of OCT4 in germ cells may reflect the normal development of germ cells during postnatal development during 9–18 weeks. However, altogether, our study suggests that NHP gonocytes are not highly teratogenic at birth. We cannot exclude that some soluble factors from the co-injected somatic cells or direct somatic cell-germ cell interactions keep the gonocytes in a non-pluripotent and non-teratogenic state. Nevertheless, it is conceivable that cultured human and NHP gonocyte- or spermatogonia-derived cells (if their culture and propagation are possible) may revert or dedifferentiate to a developmental state that is characterized by pluripotency, as it has been shown for the mouse [Kanatsu-Shinohara et al., 2004; Guan et al., 2006]. Moreover, it would be interesting to purify marmoset monkey gonocytes to test their teratoma formation potential when separated from somatic cells. Unfortunately, to our knowledge, there is at present no useful marker for the specific enrichment of primate gonocytes available; all surface markers used previously and known to us are also expressed by testicular multipotent stromal cells [Eildermann et al., 2012b]. Hence, we were not able to efficiently enrich gonocytes (or even spermatogonia) from the neonatal or adult marmoset monkey testis, respectively [Eildermann et al., 2012b; Aeckerle and Behr, own unpubl. data]. Moreover, the very small size of the neonatal marmoset gonad (7.4 ± 2.99 mg; n = 35) prevents many approaches to purify and functionally characterize marmoset monkey gonocytes. Therefore, culture and significant propagation of these cells were so far impossible in our hands.

In summary, testicular cord-like structures almost indistinguishable from the normal testis cords were found upon xenografting of a single-cell suspension obtained from the neonatal NHP testis into mice. This impressively demonstrated the reconstructive potential of isolated testicular cells from the marmoset monkey and may represent a new system for the study of testicular morphogenesis and testis cell biology. Our study provides no evidence for a pluripotent and teratogenic potential of gonocytes from the neonatal marmoset monkey testis.

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5. Pluripotency factor expressing pre-migratory primordial germ cells are in very close proximity to the prospective gonadal ridge in the monkey embryo

Running title: Pluripotency factors in primate PGCs

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Key words: Primordial germ cell, pluripotency factor, embryo

Abstract

Primordial germ cells (PGCs) are the embryonic progenitors of sperm and egg cells. Mammalian PGCs are thought to actively migrate from the yolk sac endoderm over long distances across the embryo to reach the somatic genital ridge. General principles of PGC development were discovered in mice. In contrast, very little is known about primate PGC development due to extremely limited access to primate embryos. Here, we analyzed 12 excellently preserved monkey (*Callithrix jacchus*) embryos covering the phase from PGC emergence in the endoderm to the formation of the differentiating gonad. We show that pluripotency factors OCT4A and NANOG specifically mark PGCs. In contrast, SALL4 and LIN28 were first ubiquitously expressed and only later down-regulated in somatic tissues, while continuously expressed in PGCs. We further show endoderm-located PGCs in E50 embryos in unexpected and so far unknown spatial proximity to the prospective genital ridge making a long-range migration of PGCs dispensable. In E65, PGCs are present in the very primitive gonad, while significantly older stages still exhibit PGCs at their original endodermal site, revealing an unexpectedly wide spatio-temporal PGCs distribution window. Our findings challenge the “dogma” of active long-range PGC migration from the endoderm to the gonads. We therefore suggest an alternative model based primarily on passive translocation of PGCs from the gut-surrounding mesenchyme to the prospective gonad through intercalar expansion of the mesenchymal tissue harboring the PGCs. In summary, we (i) show differential pluripotency factor expression during primate embryo development and (ii) provide a novel model on embryonic PGC translocation.

Introduction

The germ line is constituted by all cells that have the potential to pass (half of) their genetic material to an individual of the next generation. The mammalian germ line can be considered as a periodically reoccurring cycle of cells holding different states of inherent pluripotency (Leitch and Smith, 2013). During the premeiotic phase the germ line comprises cells of the preimplantation embryo such as the blastomeres of the morula and the cells of the embryoblast (also called inner cell mass) of the blastocyst. These cells are toti- and pluripotent, respectively, and express characteristic pluripotency factors such as OCT4A (POU5F1) (Scholer et al., 1990) and NANOG (Chambers et al., 2007). The diploid phase of the germ line is continued by as yet undefined cells of the epiblast during germ layer formation. The first clearly specified germ cells are the primordial germ cells. Mouse primordial germ cells (PGCs) are segregated from neighboring non-germ line cells by the expression of Prdm1 (Blimp1), Prdm14, Tfap2c (AP2gamma), fragilis and stella (Saitou and Yamaji, 2010), and around E7.25 a founder population of about 40 PGCs has been established (Lawson and Hage, 1994). Human PGCs were originally identified based on their morphology (Politzer, 1933; Witschi, 1948) and later on by histochemical characteristics such as alkaline phosphatase activity (Danziger and Kindwall, 1953).

Since the invention of mouse transgenesis, mouse PGCs can be identified very impressively by germline-specific transgenic reporter constructs (e.g. Yeom et al., 1996; Molyneaux et al., 2001; Grabole et al., 2013). However, in the absence of respective transgenic tools in primates including humans, the detection and localization of PGCs in primates still depends either on cell morphology, which can be a rather ambiguous criterion with respect to the heterogeneous cell populations in an implantation embryo, or on validated and reliable endogenous germ line-specific markers. In general, it appears to be a characteristic of PGCs that they express pluripotency factors that are otherwise found predominantly or even exclusively in the pluripotent cells of the preimplantation embryo and that are immediately down-regulated in all the somatic and extraembryonic tissues of the implantation embryo (Zwaka and Thomson, 2005; Leitch and Smith, 2013). Embryonic stem cells are the prototype of pluripotent stem cells. Important key factors for the pluripotent state of cells include OCT4A (Nichols et al., 1998; Niwa et al., 2000; Boyer et al., 2005), NANOG (Chambers et al., 2007), SALL4 (Elling et al., 2006), and LIN28 (Yu et al., 2007; West et al., 2009). All four pluripotency factors have also been shown to play important roles for the establishment or maintenance of the mouse germ line (Kehler et al., 2004; Chambers et al., 2007; West et al., 2009;

Hobbs et al., 2012). Altogether, these data strongly indicate a close interrelationship between pluripotent cells and the germ line. In sharp contrast to the rapidly increasing knowledge of key events in mouse PGC development, only very little is known in humans. This is of special relevance since the tissues involved in PGC development in mice have in parts no clear counterpart in the human. Probably most important, there is no apparent structure in the human equivalent to the mouse extra-embryonic ectoderm (De Felici, 2013), which plays a crucial role for PGC specification in the mouse via BMP4 signaling (Hayashi and Saitou, 2013). Studies on human PGCs during their specification and migration are still rare (De Felici, 2013). Many studies on human PGC development were performed 35 - 100 years ago (Fuss, 1911; Politzer, 1933; Witschi, 1948; McKay et al., 1953; Fujimoto et al., 1977), and functional studies are impossible. At the time when the groundbreaking studies on human PGCs were done, even immunohistochemical tools for the detection of specific cellular epitopes were not available. Therefore, protein expression data on different stages of human PGC development, especially the early stages, are still extremely fragmentary (Mollgard et al., 2010; Mamsen et al., 2012). Moreover, it will also be difficult to extend such studies in the future due to practical and ethical limitations.

Human PGCs can first be identified by the end of the third week post conception (wpc) in the caudal wall of the yolk sac (De Felici, 2013). At that time no gonads exist. The prospective gonads may be recognized only by a vague condensation of the coelomic epithelium at the respective sites. To develop a functional gonad, the PCGs must be trans-located from their extra-embryonic site in the yolk sac endoderm to the intra-embryonic location of the forming gonads. Groundbreaking studies in the first half of the 20th century and also a few more recent studies strongly suggest that PGCs actively migrate in an amoeboid way over long distances across the embryo (Witschi, 1948). Their suggested trail starts through the endodermal epithelium of the yolk sac and the yolk sac stalk to the endoderm of the primitive gut, which is the dorsal continuation of the yolk sac endoderm. PGCs present in the endodermal gut epithelium were seen in human embryos from the 5th wpc onwards (Witschi, 1948; Mamsen et al., 2012; De Felici, 2013). Thereafter, the PGCs leave the endodermal epithelium and invade the surrounding mesenchyme. Then they apparently move through the gut mesentery continuing their migration towards the gonadal rigde (bipotential gonad). Active migration of PGCs appears to play an important role for the colonization of the mouse gonadal ridge (Molyneaux et al., 2001; Richardson and Lehmann, 2010). It has been shown that

the SDF-1 / SDF-1 receptor and the SCF / c-kit system play roles for PGC migration in vertebrates (Molyneaux et al., 2003). Recently, Mollgard et al. (2010) and Mamsen et al. (2012) provided data suggesting that peripheral nerve fibers innervating the gut may serve as an anatomical guiding structure for PGCs. However, numerous aspects of PGC development and colonization of the primitive gonad by PGCs, especially in primates, are not understood. On order to reduce the huge gap of knowledge regarding primate PGCs we decided to investigate PGC development in the common marmoset monkey (*Callithrix jacchus*) embryo. The marmoset monkey is a small new-world primate whose embryonic development was described in part already decades ago (Phillips, 1976; Merker et al., 1988). Interestingly, the marmoset's post-implantation embryonic development is, for unknown reasons, significantly delayed compared to the human (Phillips, 1976; Li et al., 2005). The marmoset recently gained increasing attention due to its frequent use in biomedical studies, especially in reproduction, stem cell research, and neurobiology. Moreover, the marmoset was the first transgenic non-human primate with germ line transmission (Sasaki et al., 2009). However, in sharp contrast to the relatively well-characterized postnatal testicular germ cell development (Chandolia et al., 2006; McKinnell et al., 2009; Aeckerle et al., 2012; Eildermann et al., 2012; Lin et al., 2012), embryonic germ cell development is poorly or even uninvestigated (Li et al., 2005).

Here, we studied PGC development in a set of 12 excellently preserved non-human primate embryos with special regard to pluripotency factor expression by and in situ distribution of PGCs. This study provides novel information on the expression of four pluripotency factors during primate embryonic development. We demonstrate a wide spatio-temporal window of PGC distribution and discovered an as yet unknown spatial proximity of endoderm-located PGCs to the site of the future gonad. This finding strongly questions the necessity of a long-range migration of PGCs. Based on this we propose a novel model of PGCs translocation from the endoderm to the gonad.

Results

Verification of the specificity and cross-reactivity of the antibodies

The antibodies used in the present study were generated against epitopes of human OCT4A, NANOG, SALL4, and LIN28, respectively. In order to verify the cross-reactivity and to test the specificity of the antibodies for the respective marmoset monkey pluripotency factors, we applied the antibodies to Bouin-fixed and paraffin-embedded marmoset monkey embryonic stem cells grown on mouse embryonic fibroblasts (MEFs) as feeder cells. Fig. 1A shows a hematoxilin-eosin-stained

section. Marmoset monkey ES cells (red arrow) with a cuboidal shape can be clearly distinguished from mesenchymal mouse embryonic fibroblasts (blue arrow). Fig. 1B shows a negative control (unrelated primary antibody at high concentration). Fig. 1C shows very intense nuclear OCT4A staining of ES cells, while the cytoplasm of ES cells is faintly stained and the mouse embryonic fibroblast (MEF) cells are devoid of signals. Other somatic tissues (see below for embryos and data not shown) also exhibited no staining. Very importantly, this antibody specifically detects the pluripotency-associated form of OCT4, i.e. OCT4A. The NANOG antibody revealed clear nuclear but also some cytoplasmic staining of ES cells (Fig. 1D). No NANOG staining was observed in MEFs. SALL4 showed very intense nuclear staining of ES cells and only very faint signals in MEFS (Fig. 1E). In contrast, LIN28 exhibited strong cytoplasmic staining of ES cells and also some nuclear signals (Fig. 1F). The LIN28 antibody also did not bind to MEFs. These data demonstrate the validity of the antibodies for the immunohistochemical detection of the marmoset monkey pluripotency markers OCT4A, NANOG, SALL4, and LIN28.

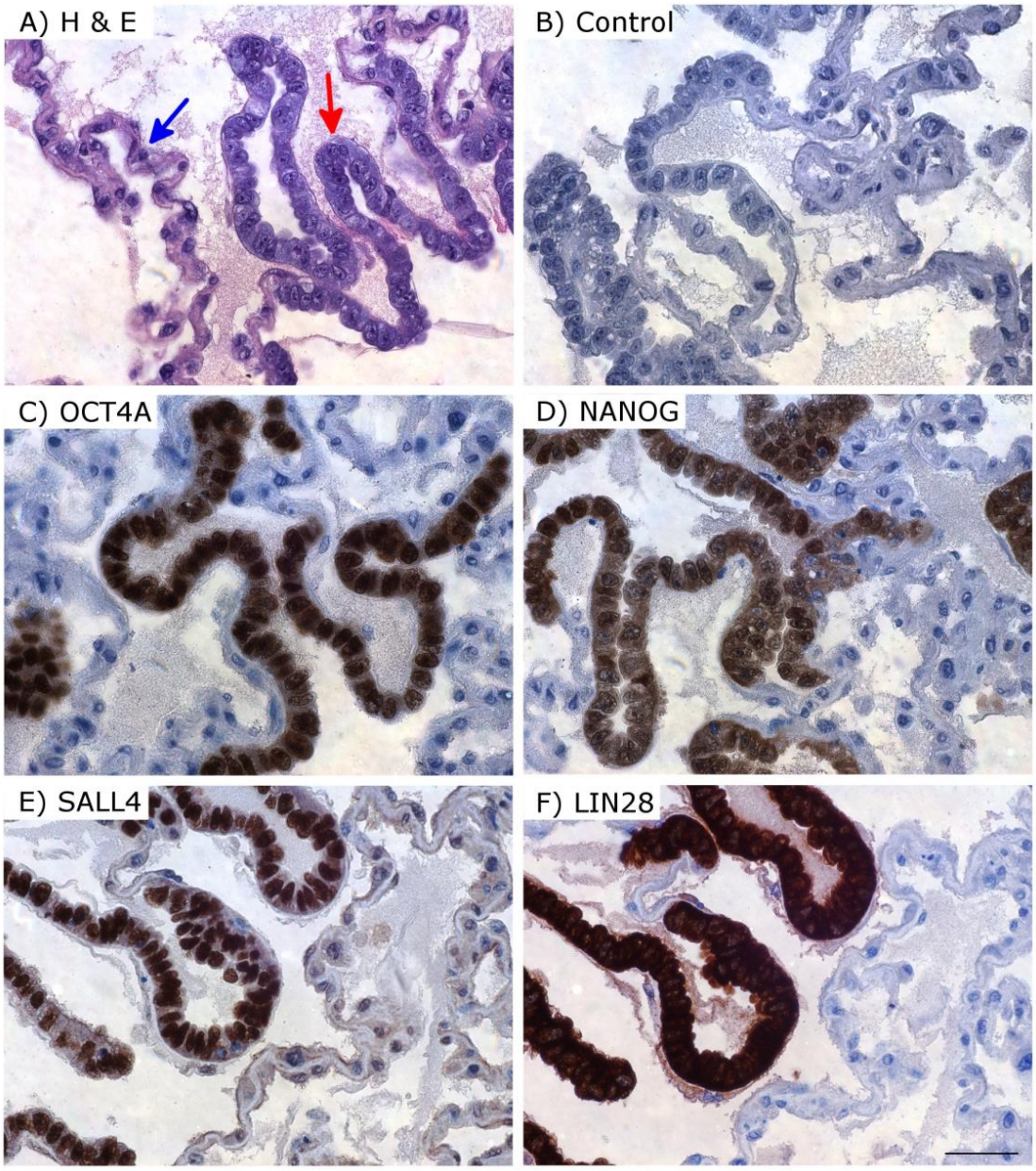

Figure 1: Validity of the antibodies for the marmoset monkey pluripotency factors. Marmoset money ES cells which were cultivated on mouse embryonic fibroblasts were used to verify the specificity and cross-reactivity of the antibodies used in this study. The cells were Bouin-fixed and paraffin-embedded. **A** shows a section stained with hematoxilin-eosin (HE): Epithelial ES cells with a cuboidal shape (red arrows) can be clearly distinguished from mesenchymal mouse embryonic fibroblasts (MEFs, blue arrows), **B** shows a negative control. **C-F** show stainings with antibodies against OCT4A, NANOG, SALL4 and LIN28, respectively. ES cells exhibit a clear nuclear signal for OCT4A, while the cytoplasm of ES cells shown only weak and the MEFs no signals (**C**). The NANOG antibody revealed clear nuclear, but also weak cytoplasmic staining of ES cells. No NANOG staining was observed in MEFs (**D**). SALL4 showed strong and specific nuclear staining of ES cells (**E**), while LIN28 exhibited strong cytoplasmic and weak nuclear staining of ES cells (**F**). Scale bar represents 50 µm.

Developmental stages of Marmoset monkey implantation embryos

Callithrix jacchus specimens available for analysis of the morphological and molecular status of PGC in the present study were from a post-implantation period between E50 and E75 (Fig. 2), which is roughly equivalent to the embryonic period in human development between Carnegie stages 10 and 17 (O'Rahilly and Muller, 2001). At E50, the embryo shows the first pharyngeal arch, an otic vesicle, 17 somites flanking a neural tube open at both ends (Fig. 2A, C), a straight primitive heart tube contained in a large pericardial cavity and showing first rhythmic contractions, and a large yolk sac with an open connection to the primitive gut via the vitellinointestinal (omphaloenteric) duct (Fig. 2D, see also Fig. 3A). The region of the future gonad anlage is recognizable as a short stretch of high columnar coelomic epithelium near the future root of the mesentery (Fig. 3, see below). A sexually indifferent gonad is found at E65, which is equivalent to Carnegie stage 16 (Fig. 2E, arrow), and at E68 to E75 (equivalent to Carnegie stage 17) the gonad is sexually differentiated (female in the specimen shown) and separated from the underlying mesonephros by a short meso (Fig. 2F, arrow).

General overview over the expression of OCT4A, NANOG, SALL4, and LIN28

We tested the expression of OCT4A, NANOG, SALL4, and LIN28 at different stages of implantation embryo development (Fig. 3 – Fig. 6). In general, OCT4A was detectable exclusively in primordial germ cells at all stages analysed. Basically the same was found for NANOG. In sharp contrast, the E50 embryos exhibited almost ubiquitous expression of SALL4 and LIN28. Interestingly, from E65 onwards, SALL4 and LIN28 were undetectable in the vast majority of somatic cells, while still strongly expressed in PGCs, indicating an abrupt switch-off of SALL4 and LIN28 in most somatic cells. Details are described in the following paragraphs.

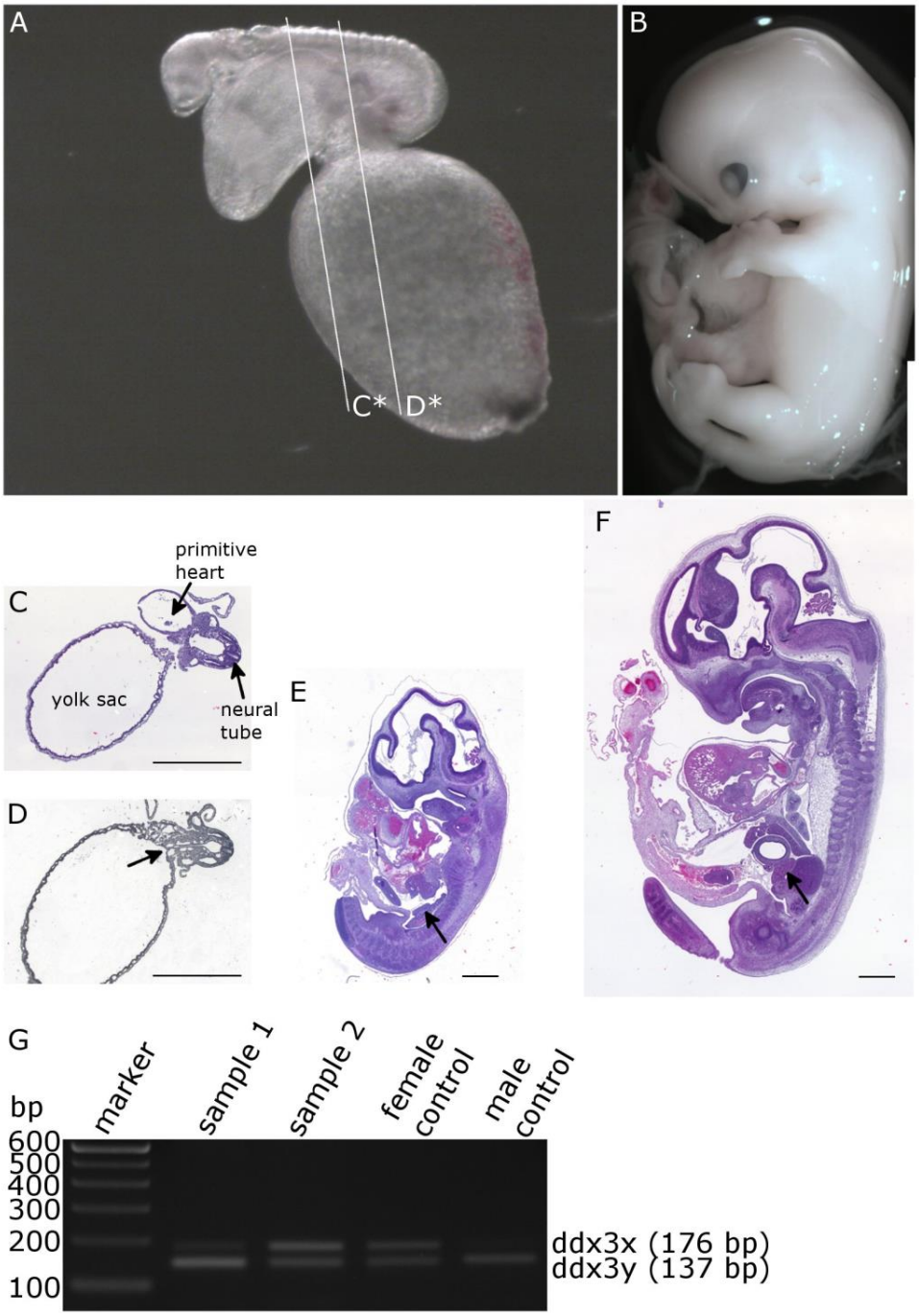

Figure 2: Characterization of marmoset monkey post-implantation embryonic development. Normal duration of pregnancy in marmosets is 143 days. **A** and **B** show external morphology of marmoset embryos at E50 (Carnegie stage 10) and E75 (Carnegie stage 17), respectively. White lines in A indicate plane of the sections shown in C and D. Image B (E75) was composed of two single pictures. **C** and **D** show sections of a male marmoset embryo at E50 (same embryo as shown in A). Characteristic structures are indicated. **D** highlights the duct connecting yolk sac and embryo (arrow). **E** and **F** show a sections of a male E65 and a male E75 (F shows the same embryo as B). The images reveal insights into the development of the organ primordia. At E65, the indifferent gonad is clearly visible (arrow). Crown-rump-length of the embryo is about 9 mm. At E75 crown-rump-length of the embryo is ~12 mm. Scale bar represents 1 mm in C-F. Sex of the embryos was determined by PCR on genomic DNA (**G**). Sample 1: male embryo, sample 2: female embryo. Skin biopsies from female and male neonatal marmosets were used as positive controls.

Pluripotency factor expression at E50

At gestational day E50, the embryo primarily consists of primitive derivatives of the three embryonic germ layers (Fig. 2 and 3), and no Chorda dorsalis had formed yet. Instead, the precursor cells of the Chorda are still intercalated into the roof of the primitive gut forming the chordal plate (cp). The only sign of presumptive gonad formation was a slight thickening of the coelomic epithelium lateral to the vitellinointestinal duct but ventral to the paired aorta (Fig. 3A). Primordial germ cells, not easily recognized by their “typical” morphology in the routinely stained sections (Fig. 3B), were specifically labelled by the OCT4A antibody. PGCs had a large, roundish nucleus, and the cells were present in the caudal endoderm lining the yolk sac stalk and the primitive gut (Fig. 3B). PGCs could be distinguished from the majority of the surrounding somatic cells, which also have large, but rather oval nuclei. However, OCT4A-positive cells mostly lacked the large pale cytoplasm that was described as a characteristic of human PGCs (Witschi, 1948). No other cell type was found to be labelled by the OCT4A antibody. Similarly, the NANOG antibody produced a nuclear staining and, like in the ES cells (Fig. 1), some cytoplasmic staining (Fig. 3C). In sharp contrast to OCT4A and NANOG, SALL4 and LIN28 did not exhibit a PGC-specific expression but a positive reaction in the vast majority of all cells of the E50 stage (Fig. 3D,E). Only few somatic cells were negative for SALL4 and LIN28, respectively.

With regard to the topographical proximity to the site of the prospective gonad, specifically labelled PGCs in E50 embryos were detected in the endoderm of the yolk sac stalk (red arrow in Fig. 3A) and of the primitive gut (blue arrow in Fig. 3A) and some of these PGCs were present in basal positions of the epithelium – i.e. close to the basement membrane - of the gut tube (Fig. 3A,B,C). In this position they are very near to the site where the gonadal ridge will form (Fig. 3B,C,D, brackets). Importantly, the distance between the two sites measures only about 50µm. PGCs leaving the endoderm would probably have to migrate less than 50µm to reach their final position in the genital ridge of the mesoderm at the dorso-medial wall of the embryonic coelom.

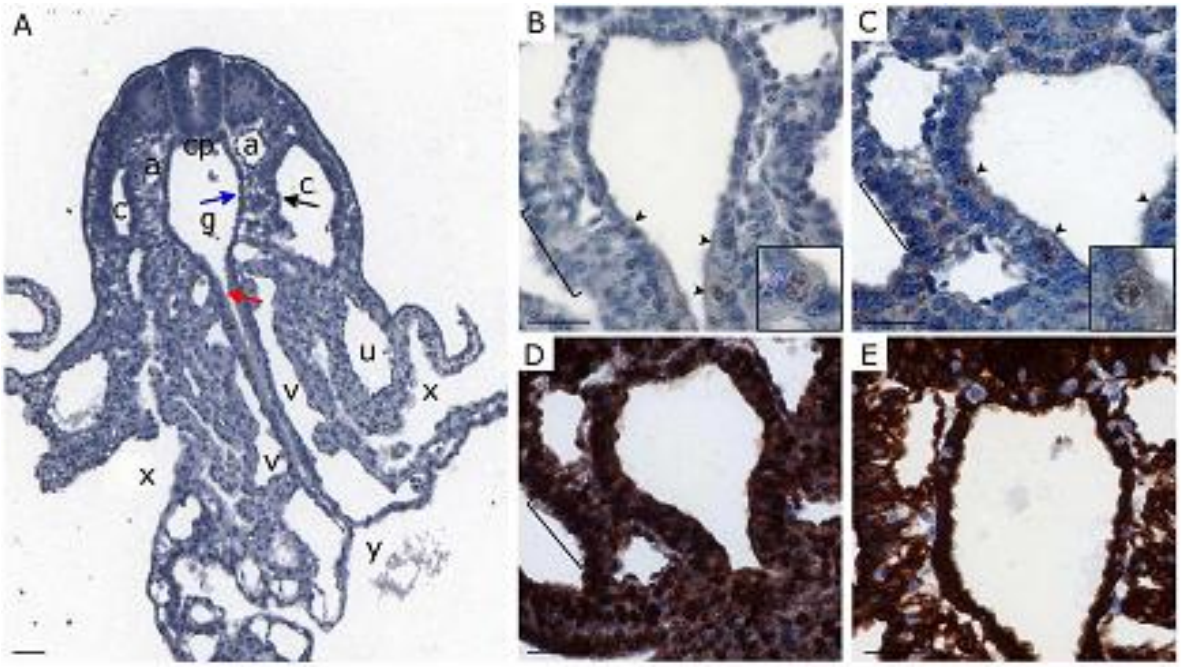

Figure 3: Pluripotency factor expression at E50. A shows the same embryo as Fig. 2C and D at a higher magnification. The red arrow highlights the endodermal epithelium lining the vitellinointestinal duct. The blue arrow highlights the endoderm of the primitive gut. The black arrow points at the site of the prospective gonadal ridge. **B-E** show a higher magnification of the area shown in A containing germ cells in the lateral wall of the primitive gut. Sections were stained for OCT4A (**B**), NANOG (**C**), SALL4 (**D**) and LIN28 (**E**). Brackets mark the coelomic epithelium of the future gonadal ridge. The OCT4A antibody specifically labelled nuclei of cells present in the endoderm at the junction between the yolk sac stalk and the primitive gut (arrowheads). The PGCs were also stained by the NANOG antibody (**C**, arrowheads). In sharp contrast to OCT4A and NANOG, SALL4 (**D**) and LIN28 (**E**) showed rather ubiqutious expression, including the PGCs. a: dorsal aorta, c: coelom, g: primitive gut, v: vitelline vessel, u: umbilical vein, x: exocoelom, cp: cordal plate. Scale bars represent 50 µm.

PGCs at E65

At E65 the gut had developed into a closed tube with a proper mesentery and the morphologically indifferent gonad was recognizable as a small, but distinct thickening of the intermediate mesoderm bulging from the posterior body wall (Fig. 4A, see also Fig. 2 for the overview) and covered by a single-layered epithelium, the future peritoneum. There is no histological evidence of epithelial organization within the gonadal primordium, and the male and female gonads are morphologically indistinguishable even though SOX9, a male (pre-) Sertoli cell-specific protein, was detectable in the male gonadal primordium, while this antigen was undetectable in the female (data not shown). OCT4A-positive PGCs were still detected in the endodermal gut epithelium (Fig. 4B) but no PGCs were detected in the densely packed mesenchyme underlying the gut epithelium. Some PGCs were localized in the root of the mesentery (Fig. 4C) as well as in the retroperitoneal mesenchyme. A substantial fraction of the PGCs had already reached the indifferent

gonad (Fig. 4D-G) occupying mostly the proximal part of the gonad, while the distal part contained only few scattered PGCs. PGCs positive for OCT4A, NANOG, SALL4 and LIN28 were mostly found in the gonads but, in addition, also at many extra-gonadal sites (data not shown for E65, but see Fig. 5 and Fig. 7).

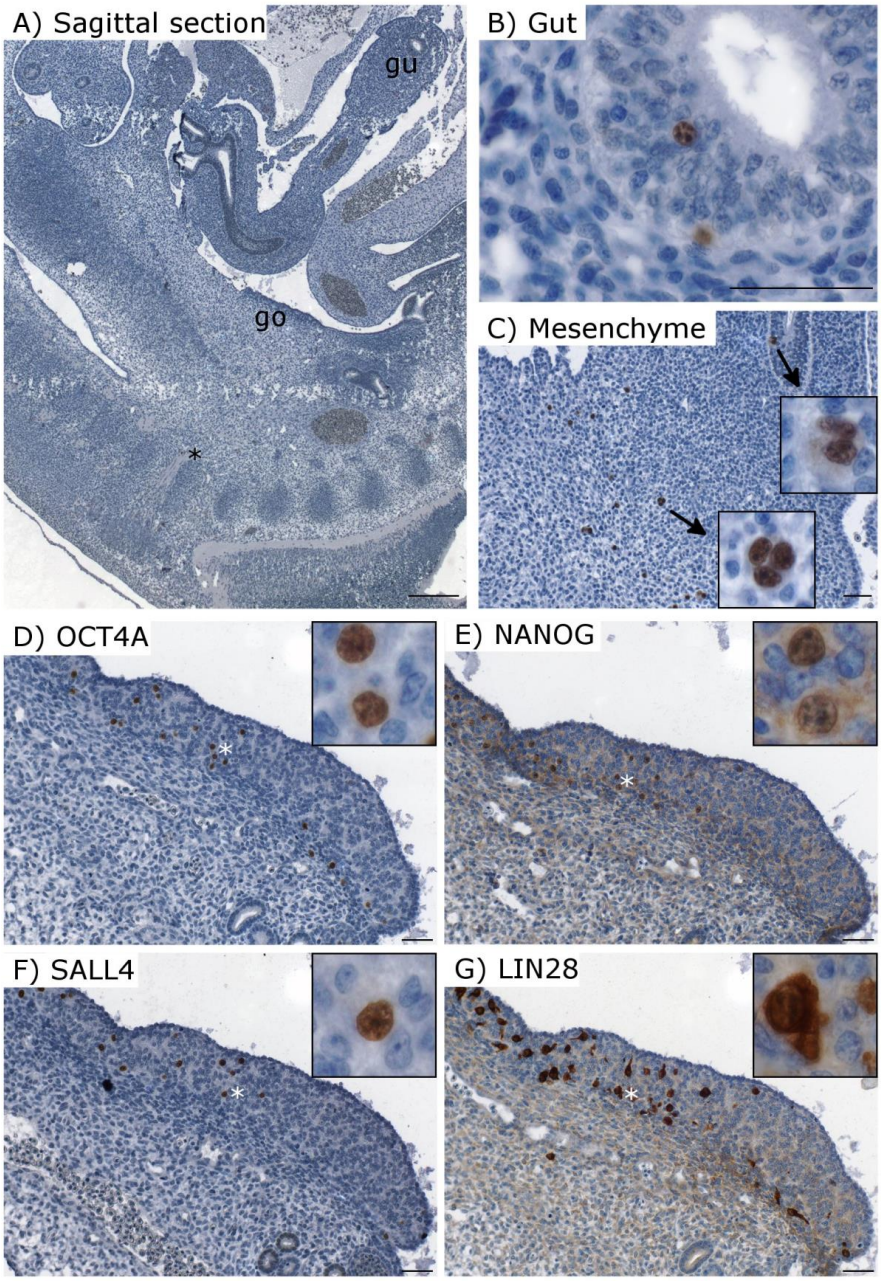

Figure 4: PGCs at E65. A shows an overview over a sagittal section through an E65. The gut is established as a closed tube (gu; see B for higher magnification) and the morphologically indifferent gonad is recognizable as a small but distinct thickening of the intermediate mesoderm (go; see D-G for higher magnification). OCT4A-positive PGCs were present in the endodermal gut epithelium, in the mesenchyme of the dorsal mesentery of the gut as well as in the retro-peritoneal mesenchyme. The asterisk highlights the position of an ectopic PGC associated with a peripheral nerve (see also Fig. 7B). **B** Higher magnification of (A) showing gut-epithelium-located PGCs. **C** Higher magnification of (A) showing gut-epithelium-and mesenchyme-located PGCs. **D-G** A substantial fraction of the PGCs already reached the indifferent gonad (bipotential gonad). **D** OCT4A, **E** NANOG, **F** SALL4, and **G** LIN28. Scale bars represent 50 µm.

PGCs at E68, E72, and E75

At E68 the gonads were still morphologically undifferentiated. Specifically labelled PGCs were detected, like at E65, at many parts of the developing gut including the mesentery, in retro-peritoneal tissue and in the gonads (Fig. 5A). At E72 PGCs were still seen in the gut epithelium as well as in the dense mesenchyme surrounding the gut epithelium (Fig. 5B), in the root of the mesentery and in the retro-peritoneal mesenchyme. PGCs expressed all four markers at all sites during all stages (Fig. 5C-L for E75; data not shown for E68 and E72). The female gonad at E75 contained PGCs positive for OCT4A, NANOG, SALL4 (all nuclear), and LIN28 (mostly cytoplasmic). OCT4A signals appeared most intense in the nucleoli of the PGCs (inset in Fig. 5C). In the E75 male gonad (inset in Fig. 5E), which exhibits formation of the prospective testicular cords (indicated by the white dotted line), PGCs were present inside and outside the forming cords. Remarkably, numerous PGCs were still detectable outside the gonads in the mesenchyme (Fig. 5G, H) or even in the gut epithelium (Fig. 5I-L). Besides single PGCs, several clusters of PGCs were seen in the mesenchyme (Fig. 5G, H). The wide distribution of PGCs at this stage is highlighted by red arrows on the sagittal section of the E72 in Fig. 5B. Sex-specific differences in PGC distribution or staining patterns were not found until the histological differentiation of the gonads (s. below).

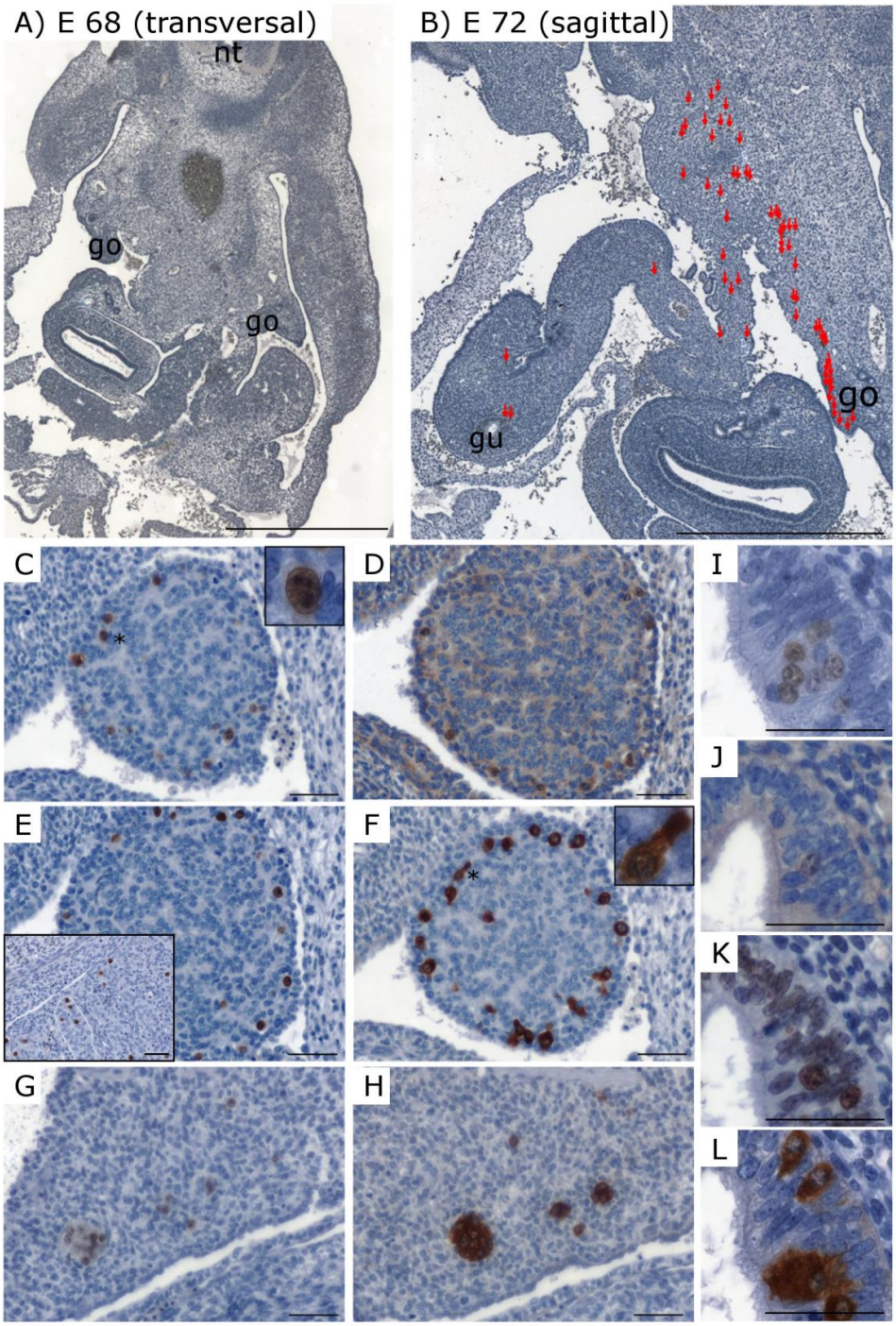

Figure 5: Aspects of the stages E68, E72, and E75. A shows a transversal section through an embryo at E68. The neural tube (nt) and the morphologically still undifferentiated gonads (go) are clearly visible. **B** shows a sagittal section through an E72 stained for OCT4A. Each single PGC is marked with a red arrow. PGCs are present in the gut epithelium (gu), in the mesenchyme surrounding the gut epithelium, in the loose mesenchyme of the mesentery, in the retro-peritoneum and in the gonad (go). **C-F** show transversal sections through a female gonad at E75. PGCs are located in the periphery of the gonad and express OCT4A (**C**), NANOG (**D**), SALL4 (**E**) and LIN28 (**F**). Inset in E shows an E75 male gonad. Formation of the prospective testicular cords was already initiated (indicated by the white dotted line). **G-H** show clusters of PGCs expressing OCT4A (**G**) and LIN28 (**H**) present in the retroperitoneal compartment of the female E75**. I-L** show PGCs present in the gut epithelium of an E75. They express OCT4A (**I**), NANOG (**J**), SALL4 (**K**) and LIN28 (**L**) and show no signs of apoptosis. Scale bar represents 1 mm (A-B) and 50 µm (C-L).

The male E95 gonad is a fetal testis

In male E95 the germ cells are generally enclosed in testicular cords; only few germ cell clusters were still located in the developing rete testis (Fig. 6). Intratubular germ cells, called gonocytes (if not in contact with the basal lamina of the cord) or pre-spermatogonia (if already in contact with the basal lamina) still express OCT4A, NANOG, SALL4, and LIN28 (Fig. 6A-D). OCT4A as well as NANOG also showed faint cytoplasmic signals in addition to robust nuclear signals, while SALL4 was strictly nuclear. LIN28 was almost exclusively cytoplasmic. No germ cells were detected outside the gonads or the rete testis at this stage.

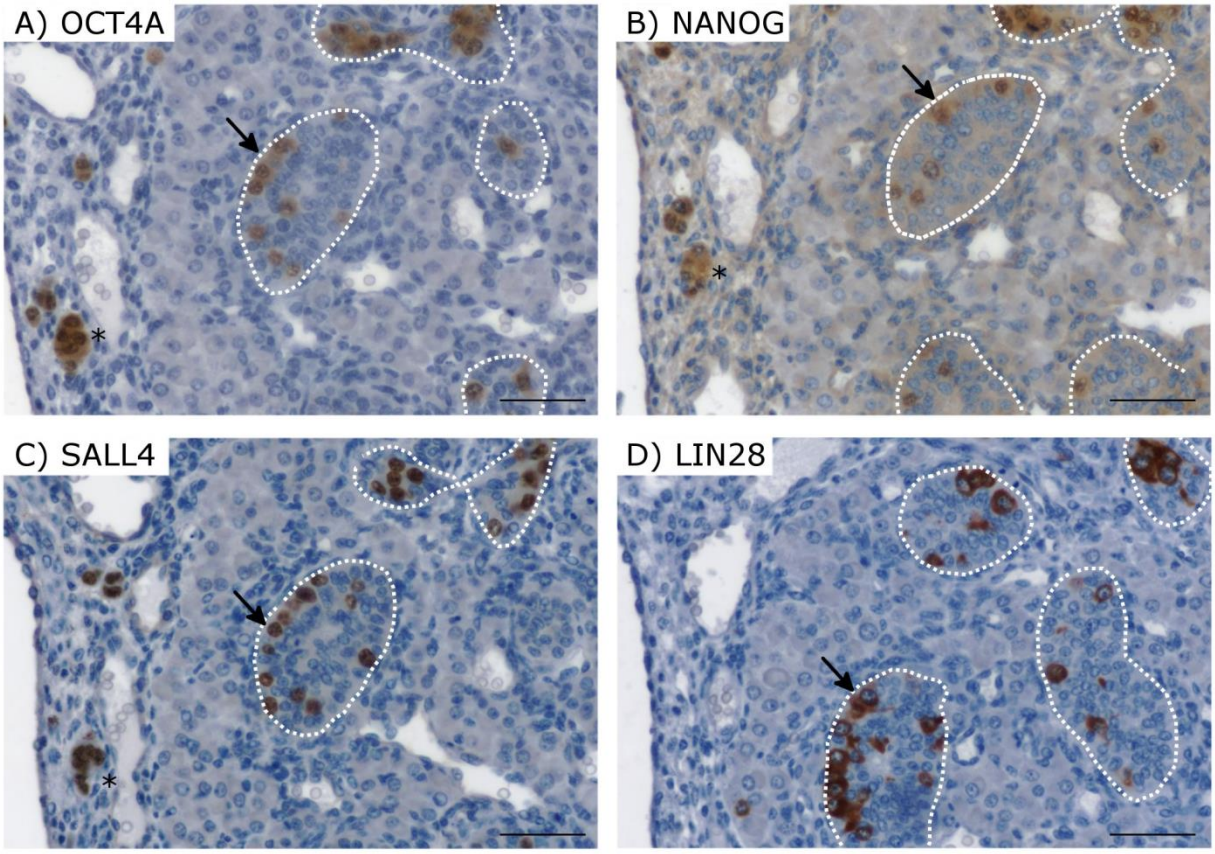

Figure 6: The E95 gonad is a fetal testis. At E95 the vast majority of germ cells are enclosed in testicular cords (indicated by arrows); only few germ cell clusters were still located at the junction between the testis and the prospective epididymis, i.e. the forming rete testis (indicated by *). Intratubular germ cells, called gonocytes (if not in contact with the basal lamina of the cord) or pre-spermatogonia (if already in contact with the basal lamina) still express high levels of OCT4A (**A**), NANOG (**B**), SALL4 (**C**), and LIN28 (**D**). However, there were also germ cells expressing little or even no OCT4A and NANOG (not shown). No germ cells were detected outside the gonads. Scale bar represents 50 µm.

PGCs at untypic/ ectopic sites

The assumed PGC migration route from the yolk sac epithelium to the gonads involves the endodermal epithelium, the mesenchyme directly surrounding the gut, the dorsal gut mesentery (root of the

mesentery), the retroperitoneal mesenchyme and eventually the gonads. While the vast majority of the PGCs detected in this study were present in one of the tissues constituting this route, we also localized some PGCs at untypical, i.e. ectopic sites. At E65 we found an OCT4A-positive PGC in the lumen of the gut with no contact to the epithelium (Fig. 7A). Another PGC was present in a developing peripheral nerve in the coccygeal region far "beyond" the gonad as physiologically final destination (Fig. 7B). In an E68 embryo we localized a PGC in a peripheral nerve in the cranial region of the embryo (Fig. 7C). Another PGC was found in the female E72 to be present in the Chorda dorsalis (Fig. 7D). Finally, an E75 embryo presented a PGC in the pancreas (Fig. 7E).

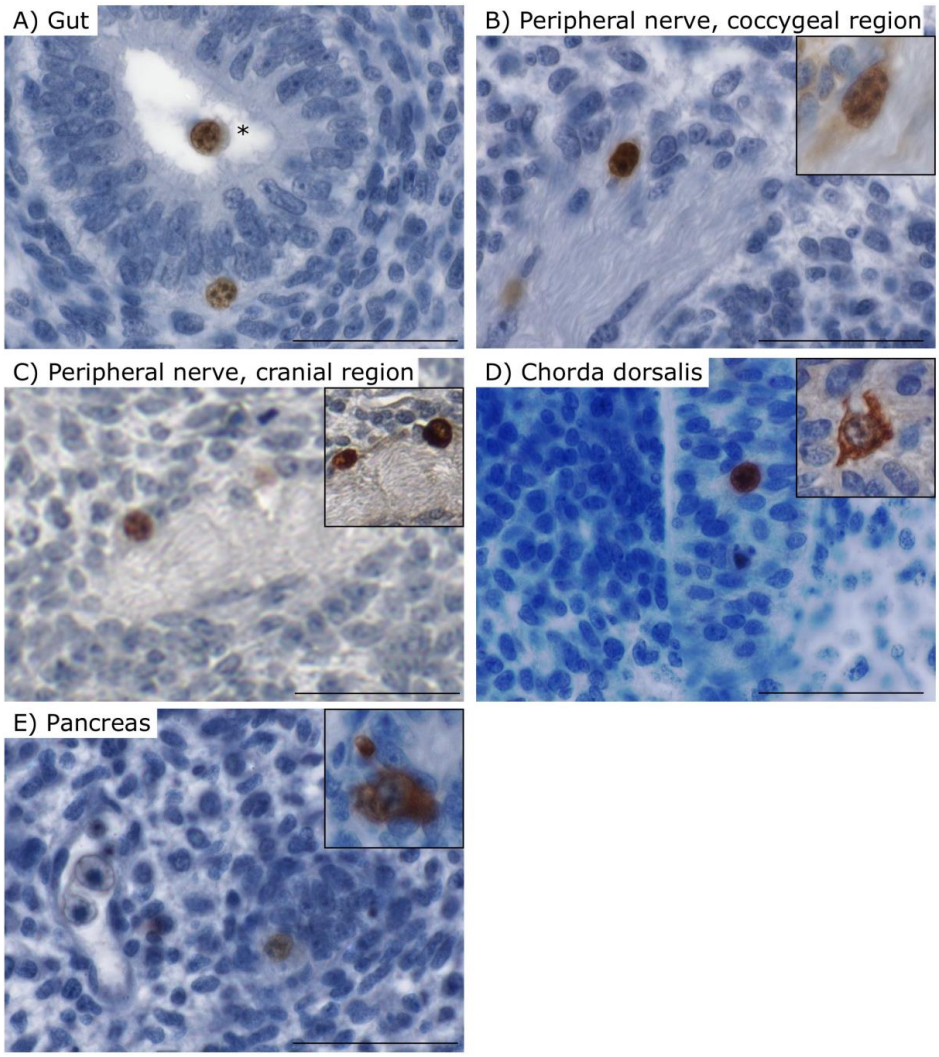

Figure 7: PGCs at unexpected/ectopic sites. In E65 one OCT4A-positive PGC was detected in the lumen of the gut with no evident contact to the epithelium (**A**). Another PGC was present in a forming ganglion in the coccyxial region and was clearly positive for OCT4A (**B**) and NANOG (inset in B). In an E68 embryo we localized an OCT4A and LIN28-positive PGC in a peripheral nerve in the cranial region of the embryo (**C**). Another PGC (OCT4A and LIN28-positive) found in the female E72 was present in the Chorda dorsalis (**D**). An E75 stage also presented one PGC in the pancreas (**E**). Scale bar represents 50 µm.

Discussion

This first systematic investigation of PGC development in a non-human primate provides detailed embryological and morphological data and combines them with novel insights into the spatio-temporal expression of a quartet of pluripotency factors during primate embryogenesis.

The timing of PGC development described in the present report is probably appreciated best in comparison with human gestation which is on average 267 days while marmoset monkey gestation is 143-145 days, the gestational period of marmoset monkeys amounting to only about 54% of the human. However, in the marmoset embryo, Carnegie stage 10 is reached at E50 while human embryos have reached this stage at around E23 already. Carnegie stage 10 is the stage where PGCs can first be identified in the human embryo yolk sac (De Felici, 2013). Similarly, the marmoset monkey embryo at E75 represents Carnegie stage 17 while this stage is reached in the human at around E41. These data are supported by findings reported previously (Phillips, 1976; Merker et al., 1988; Li et al., 2005) and indicate that the period of primordial germ cell specification and migration is significantly delayed in marmoset monkeys compared to the human despite the significantly shorter overall gestational period of the marmoset monkey. It remains to be analyzed when this delay is “ironed out” during further development.

In the absence of appropriate markers, PGCs were described in most publications as histologically easily identifiable cells because of their large spherical or oval shape and their clear cytoplasm in bright field microscopy (Politzer, 1933; Witschi, 1948; Fujimoto et al., 1977). They are also reported to have a large nucleus with usually one or two prominent nucleoli (Pereda et al., 2006). However, these generally accepted morphological characteristics of PGCs may apply only to later PGC stages in humans. Indeed, already Witschi emphasized that yolk sac-located human PGCs “differ little from associated somatic cells, and it is difficult to identify them with certainty” (Witschi, 1948). Our study confirmed that early PGCs are morphologically rather similar to the neighboring somatic cells and only the OCT4A and NANOG staining allowed us to certainly identify them. Interestingly, even the later PGCs in the marmoset embryos located in the mesenchyme of the mesentery or the retro-peritoneum are not always in agreement with the description of respective human PGCs. Indeed, while some PGCs exhibited the expected morphology, other clearly pluripotency factor-positive cells were rather small and had apparently oval nuclei making them morphologically hardly distinguishable from the neighboring somatic cells. A difference in the morphology of human PGCs during different phases of development and

migration has been also described by Fujimoto et al (1977).

The OCT4 gene is alternatively spliced (Wang and Dai, 2010). There is an OCT4A and an OCT4B version. Only OCT4A is specifically pluripotency-associated. In a previous study we analysed the specificity of different OCT4 antibodies and found that at least some of them are unspecific (Warthemann et al., 2012). In contrast to our previous experiences, the OCT4 antibody used in the present study produced very specific, robust and reproducible results in immuno-histochemistry. Therefore, the present study providing OCT4A-specific data on primate PGCs represents a significant progress regarding the analysis of OCT4 protein expression in the primate germ line.

Some data on OCT4 and NANOG in human PGCs is already available (Rajpert-De Meyts et al., 2004; Kerr et al., 2008). However, the information on LIN28 in primate PGCs is still very scarce (Gillis et al., 2011; Aeckerle et al., 2012; Childs et al., 2012) and that on SALL4 in PGCs to our knowledge even more preliminary (Eildermann et al., 2012). Furthermore, the data on the latter factors mainly refer to late PGCs. In contrast, we show here that both factors, SALL4 and LIN28, are already expressed during the very early phases of PGC development.

Considering that SALL4 as well as LIN28 are strongly expressed not only by pluripotent stem cells and PGCs, but also by some somatic (multipotent) progenitor cells, the finding of almost ubiquitous expression may rather reflect an undifferentiated progenitor stage than a pluripotent state of the E50 somatic cells. In contrast to SALL4 and LIN28, OCT4A and also NANOG are truly and strictly associated with pluripotency. Indeed, elegant studies in genetic mouse models showed that adult OCT4 does not play a role in adult stem and progenitor cells (Lengner et al., 2007). Our study extends this finding, at least on a descriptive level, to primates, since we were unable to detect any OCT4 protein in other cell types than in germ line cells of the marmoset monkey embryo. Hence, while all four factors investigated in this study are essential for pluripotency of ES cells, only OCT4A and NANOG can serve as really specific markers of pluripotent cells.

In the beginning of the era of human pluripotent stem cell research *in vitro* in the late 90´s of the last century, Shamblott and colleges reported the derivation of a pluripotent human cell line derived from PGCs isolated from 5-9 weeks old human gonadal primordia and mesenteries (Shamblott et al., 1998). These cultured cells were called human embryonic germ (EG) cells. Besides ES cells and iPS cells, EG cells may be another very interesting and instructive cell type regarding the investigation of the regulation of pluripotency. The epigenetic features of EG cells may differ from ES and iPS cells (Seisenberger et al., 2012), especially

when derived from different sites, e.g. gut epithelium, mesenchyme, and gonad of the embryo. This need to be addressed in future studies.

Upon completion of the physiological route and arrival in the gonad, male PGCs are enclosed by Sertoli cells during the formation of the fetal testicular cords. During this process, the PGCs start down-regulating the expression of OCT4A and NANOG in most germ cells (which are now called goncytes), and at birth these two key pluripotency factors are only detectable in a minor fraction of germ cells (data not shown for older stages). In adult primate testes (including human, baboon, rhesus macaque and marmoset monkey) we were neither able to detect OCT4A nor NANOG (data not shown). In contrast to these factors, LIN28 and SALL4 are expressed also postnatally at significant levels. While SALL4 can be robustly detected in all diploid male germ cells up to the Apale spermatogonial stage (Eildermann et al., 2012), LIN28 is seen in gonocytes and a very small subpopulation of spermatogonia in adult testis (Aeckerle et al., 2012). Hence, SALL4 and LIN28 can serve as germ cell markers from the migratory stage of PGCs up to the postnatal phase. The early down-regulation and subsequent lack of expression of OCT4A and NANOG during testis development suggests that the differentiating Sertoli cells may play a crucial role in the transition from the pluripotent state of the PGCs to the unipotent state of the gonocytes. However, the specific signals provided by the somatic environment influencing the germ cell potential remain to be identified. Interestingly, the histological male-specific embryonic rearrangements of the gonads were not associated with a detectable change in the expression of OCT4A, NANOG, SALL4, or LIN28, neither compared with earlier male stages nor with PGCs in the corresponding female gonads.

In general it was unexpected that in the E65 – E75 stages PGCs were seen at all sites analyzed (gut epithelium, root of the mesentery, mesenchyme of the retroperitoneum, gonad primordium), and no clear shift of the major PGC population from the gut endoderm to the gonad was really evident during PGC colonization of the gonad. Already in the E65 we found PGCs at their final destination in the gonadal primordium. On the contrary, we still found PGCs in the E75 and even the ~E90 gut endoderm, when gonadal differentiation was already at advanced stages. The gut endoderm-positioned PGCs did not exhibit evident signs of apoptosis, although this route of PGC depletion was suggested for the human (Mamsen et al., 2012) and the mouse embryo (Pesce et al., 1993; Stallock et al., 2003). Future analyses will address this issue also in marmoset monkey embryos. We found no PGCs in the coelomic epithelium as it was reported by McKay et al. (1953) for a 5 mm human embryo or by

Fujimoto (1977) for a human 5 wpc embryo. A relatively wide spatio-temporal distribution pattern of PGCs was also reported for human embryos ranging from the 4th week post conception (wpc) to the 14th wpc (Fujimoto et al., 1977; Mamsen et al., 2012, see also Fujimoto et al. 1977). This appears to be a physiologically remarkable situation: Why are there so many PGCs still in the gut epithelium, while the gonad has already formed and initiated differentiation? Do these gut PGCs still have the chance to enter the gonad and to contribute to adult gametogenesis? If not, why did they fail to reach the gonads timely? Indeed, as recently discussed (Byskov et al., 2011), it is still not clear when PGCs latest may enter the forming gonad to contribute to adult gamete production.

In contrast to the predominant opinion of a coordinated long-range migration of the PGC population from the endoderm to the gonad (Witschi, 1948; Fujimoto et al., 1977), we do believe and suggest that the PGCs are spatially allocated to the different tissues (endodermal epithelium versus mesodermal mesenchyme) very early during embryonic development (in the marmoset between E50 and E65) and that they are then further distributed and trans-located predominantly passively by the growth, proliferation and rearrangement of the respective tissue harboring the PGCs in the embryo. Indeed, in E50 embryos, the distance between endodermal PGCs and the site of the prospective gonad is only about 50µm. Fig. 8 provides a simple model for our hypothesis. This model is also in agreement with careful morphological observations in bovine embryos (Wrobel and Suss, 1998). Based on their findings, Wrobel and Süss already concluded that the assumptions of a long-range chemo-attraction of the PGCs by the gonadal ridge including active migration of PGCs is not necessary to explain the initial settlement of bovine primordial germ cells in the gonadal ridge. This view was further elaborated by Freeman (Freeman, 2003). However, future studies need to clarify whether the proliferation and apoptosis indices of PGCs and the somatic tissues at different sites in the embryo correspond to our model (Fig. 8). Nevertheless, PGC migration appears undoubtedly essential at least over short distances during the early embryonic phase, particularly for reaching the lateral position of the primitive gut as well as for crossing the basal lamina of the endodermal epithelium and the subsequent entry into the surrounding mesenchyme. The finding of PGCs in the Chorda dorsalis could be explained by PGC movements into a dorsal position of the primitive gut at that phase when the Chorda dorsalis is transiently intercalated into the roof of the gut. During re-separation of the Chorda from the dorsal gut PGCs may be co-translocated from the roof of the primitive gut to the Chroda dorsalis and eventually included in this structure.

Based on the recent findings by Mollgard et al (Mollgard et al., 2010) and Mamsen et al (2012) and the observations in the present study that ectopic PGCs were also in close association with developing peripheral nerves, it should be investigated whether the sprouting of the nervous system may play an as yet underestimated role in PGC guidance. Furthermore, there is increasing evidence that olfactory receptors may play a role in PGC guidance towards the gonad (Diedrichs et al., 2012).

The pluripotent state of PGCs in combination with a considerable chance of the PGCs to miss the default pathway to the gonad is also of clinical relevance since misguided PGCs are (possibly besides residual cells from the primitive streak) the progenitors of a special tumor entity called teratoma. Mature teratomas are by definition tumors exhibiting histologically mature derivatives of all three embryonic germ layers. They often even develop complex histological structures like teeth or hair. Such histopathological findings impressively illustrate the pluripotency of PGCs. Teratoma can be found most frequently intra-gonadally or extra-gonadally close to the body midline in the sacrococcygeal region or in the brain. However, other sites are also possible. The occurrence of teratoma at ectopic sites, especially in the brain, is difficult to explain without any PGC migration. Although teratomas occur very infrequently in primates (Haworth et al., 2003; Moore et al., 2003, and pers. comm. K. Maetz-Rensing), it may be worth investigating whether molecular differences exist between PGCs on their default route and those at ectopic sites.

Finally we observed strong clustering of PGCs in some embryos at different sites. To our knowledge this has not yet been described in the literature. At current it remains unclear whether this is a marmoset monkey-specific phenomenon or whether the absence of such PGC clusters is a human-specific characteristic. It will be interesting to analyze if isolated and clustered PGCs are different.

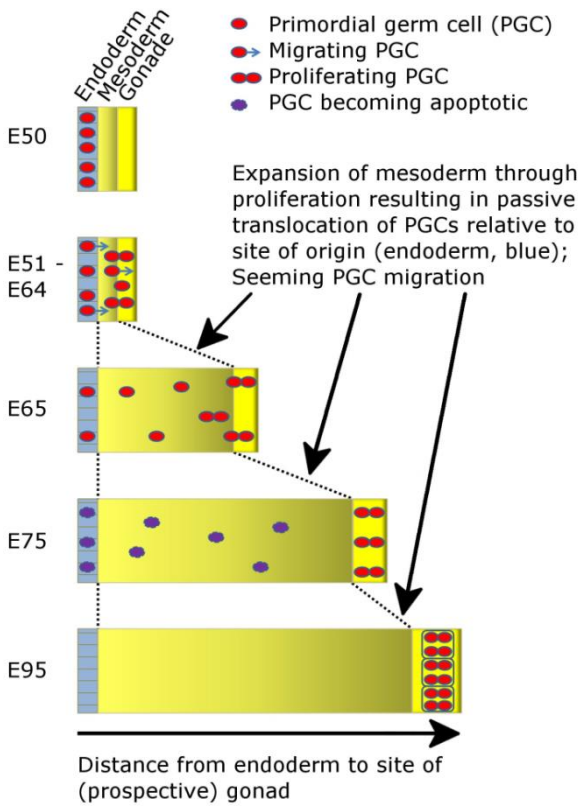

Figure 8: Model of PGC translocation from the endoderm to the gonad in the marmoset monkey. The model is mainly based on the one hand on PGC proliferation, survival, and apoptosis and on the other hand on growth of somatic tissues surrounding the PGCs rather than active PGC migration. At E50 the PGCs are exclusively present in the endoderm. Between E50 and E65 some PGCs actively leave the endodermal epithelium crossing the basal lamina thereby entering the surrounding mesenchyme. Importantly, at this early stage the site of the prospective gonad is very close to the endoderm. At E65, PGCs can be seen at all sites constituting the "migration" route of the PGCs: in the endoderm, in the mesenchyme as well as in the gonads. The distance between the endoderm and the gonads constantly and heavily increases by intercalary proliferation and growth of the mesenchymal structures linking the endoderm and the gonad. Thereby those PGCs located close to the site of the prospective gonad at earlier stages are passively and relatively translocated from the endoderm "towards" the gonad. Those PGCs close to or within the gonad proliferate and survive. In contrast, the model hypothesizes that PGCs distant from the gonad at > E75 stages are depleted by apoptosis, while gonadal PGCs survive and proliferate. At E95, only gonadal germ cells are left.

Summary and Conclusion

We provide the first detailed analysis of the expression of four key pluripotency factors during primate implantation embryo development. We show that OCT4A and NANOG are early and specifically restricted to primordial germ cells, while SALL4 and LIN28 are only useful for the detection of PGCs in later stages, since these factors can be detected also in somatic cells of E50 embryos. We further show that PGCs in the marmoset monkey can occur as clusters of PGCs. The spatio-temporal window of "PGC migration" is wide since PGCs can be found very early in the gonadal primordia and in late stages still in the gut endoderm. Most importantly, the distance between endodermal PGCs and the site of the prospective gonad in E50 embryos is very small. We interpret these findings as supportive of the view that there is no necessity for an active long-range PGC migration in marmoset monkey embryos. We suggest that predominantly intercalar mesenchymal tissue expansions contribute to the relative translocation of the PGCs from their origin at the endodermal epithelium to the gonadal primordium.

However, further detailed investigations are needed to better understand these morphogenetic processes as well as the molecular and cellular changes of the PGCs during different phases of development.

Material and Methods

Marmoset monkeys

All animal studies were performed according to the German Animal Protection Law. Animals were obtained from the self-sustaining marmoset monkey (*Callithrix jacchus*) colony of the German Primate Center (Deutsches Primatenzentrum; DPZ) and housed according to the standard German Primate Center practice for common marmoset monkeys. Embryonic and fetal stages were obtained surgically by hysterotomy (licence number 42502-04-12/0708) or hysterectomy. Hysterectomy was used to obtain the E50 embryos since these embryos are too fragile to be retrieved by hysterotomy. All surgical work on the monkeys was performed by a veterinarian with several years of experience in handling and operating marmoset monkeys. Samples taken before gestational day 80 are referred to as embryos, older stages as fetuses (Chambers and Hearn, 1985).

Pregnancy timing, hysterotomy, and embryo retrieval

Timed pregnancies (n=6 yielding 12 embryos / fetuses), were obtained from animals in which the stage of gestation was established from the post-ovulatory rise in progesterone (Harlow et al., 1984), which was determined after blood collection from female marmoset monkeys twice weekly. For hysterotomy, which delivers embryos/fetuses in an optimal histological condition, animals were anesthetized with an intramuscular injection of 0.5 ml/kg bodyweight Göttinger Mischung II (50 mg/ml ketamine, 10 mg/ml Xylazin, 10 mg/ml atropin) and 0.05 ml/kg bodyweight diazepam. The gravid uterus and the ovaries were delivered through a ventral midline incision in the abdominal wall under sterile conditions. The embryos or fetuses were removed through a horizontal incision in the uterine wall. The uterus and the abdominal wall were sutured surgically. To avoid postsurgical pain, 0.5 mg/kg bodyweight meloxicam were applied intramuscularly. In order to confirm the correct staging of the embryos before surgery, the development of the embryos / fetuses was observed via ultrasonography to ensure that the embryos / fetuses developed according to the expected growth curves. An overview over the embryos / fetuses used in this study is given in table 1. Embryos obtained before day 90 were immediately fixed in toto in Bouin's solution to preserve tissue integrity. After that, fixed embryos were measured. E95 was cut into three pieces before fixation to prevent tissue disintegration. The crown-rump lengths (CRL), biparietal diameter (BPD) and fronto-occipital diameter (FROD) were taken using a caliper.

Table 1: Embryos/fetuses used in this study.

No	CRL [mm]	BPD [mm]	FROD [mm]	GD/E	sex
1	ND	ND	ND	50	female
2	ND	ND	ND	50	female
3	ND	ND	ND	50	male
4	9.1	2.5	3.9	65	female
5	9.2	2.8	4.1	65	male
6	10	2.9	4.4	68	female
7	9.1	2.7	3.6	68	male
8	10.5	3	4.7	72	male
9	9.2	2.2	4.7	72	female
10	ND	ND	ND	75	female
11	ND	ND	ND	75	male
12	ND	12.8	15.5	95	male

CRL: crown-rump lengths, BPD: biparietal diameter, FROD: fronto-occipital diameter, GD / E: gestational day/ embryonic day

Tissue processing and immuno-histochemistry (IHC)

Retrieved embryos/fetuses were fixed overnight in Bouin's solution immediately after recovery resulting in excellent tissue preservation. After several washes in 70% EtOH for at least two days the embryos were embedded in paraffin and sectioned at 5 µm. During embedding the embryos were positioned to obtain either transversal or longitudinal sections. Tissue sections were deparaffinized, rehydrated and an antigen retrieval step was performed by microwaving the sections in 10 mM citrate buffer for 10 minutes. Endogenous peroxidase was inhibited by incubation with peroxidase blocking reagent (DakoCytomation Carpinteria, CA, USA, LSAB+ system-HRP, K0679). Antibody specifications and dilutions are given in table 2. All incubation steps were done 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 (DAB) chromogen was used as substrate for the HRP and Mayer's hematoxylin as counterstain. Control stainings were carried out using an unrelated primary antibody at high concentration instead of the specific antibody. Pictures were taken using a Zeiss microscope and the NuanceTM multispectral camera.

Table 2: Antibodies used in this study.

antigen	company	order number	host	dilution
OCT4A	Cell Signaling Technology	#2750	Rbt (monoclonal)	1:200
NANOG	Cell Signaling Technology	#4903	Rbt (monoclonal)	1:100
SALL4	abcam	#ab57577	Mm (monoclonal)	1:200
LIN28A	Cell Signaling Technology	#3978S	Rbt (polyclonal)	1:100

Marmoset monkey ES cell culture and ES cell samples for immuno-histochemistry

Marmoset monkey ES cells (line cjes001) were published previously (Muller et al., 2009). In order to obtain appropriate positive control samples for pluripotency factor immunohistochemistry, 10 cm dishes with undifferentiated marmoset ES cells cultured on mouse embryonic feeder cells for ~ 7 days were mechanically detached from the culture dish together with the feeder cell layer. Then the cells were transferred to a centrifuge tube and gently centrifuged at 200g for 1 minute. After centrifugation, the supernatant was replaced by Bouin's fixative, and the cells were fixed for one hour. Subsequent tissue processing is described above (Tissue processing and immuno-histochemistry). Finally, paraffin-embedded ES cells were sectioned at 5 µm.

Sex determination by PCR and immunohistochemistry

For sex determination of the embryos, genomic DNA was isolated from amnion, tip of the tail or a limb. Primers used for sex determination bind to conserved sites in the ddx3 gene thus making them suitable for sex determination in mammals in general. ddx3 is located on the X and the Y chromosome in variants of different lengths. Sequences of the primers are: fw 5'-GGWCGRACTCTAGAYCGGT-3', re: 5'-GTRCAGATCTAYGAGGAAGC-3'. The expected sizes for PCR products are 176 bp for ddx3x (female) and 137 bp for ddx3y (male and female). Because of the cellular chimerism in twin marmosets , even in females a weak male-"specific" band can occur if the co-twin was a male, which is frequently the case. Therefore, samples from neonatal male and female animals (where sexing is possible based on the external sex organs) were used as controls (Fig. 2G). In embryos at appropriate ages (≥ E65) the sex of the embryo was also determined by the expression (or absence) of SOX9. SOX9 is a Sertoli cell-specific protein marking Sertoli cells from the onset of differentiation till adulthood. The sexes of all embryos used in this study are listed in Table 1.

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6. Summary and concluding remarks

The hypothesis of this project was that postnatal marmoset monkey germ cells can regain an embryonic stem cell-like state after removal from their native niche within the testis. This was shown for mouse testis (Guan et al. 2006; Kanatsu-Shinohara et al. 2008; Seandel et al. 2007; Ko et al. 2009). However, the reported dedifferentiations of human germ cells into a pluripotent state were not as convincing as in the mouse (Conrad et al. 2008; Golestaneh et al. 2009; Kossack et al. 2009; Mizrak et al. 2010) and the origin and developmental potential of cultured cells from human testis is still under debate (Chikhovskaya et al. 2012; Eildermann et al. 2012b; Ko et al. 2010; Tapia et al. 2011).

In our experiments, the generation of pluripotent stem cells from marmoset monkey postnatal testes was not successful (Eildermann et al. 2012b, and own unpublished data). Therefore, we searched for biological differences between the developing mouse and primate testis and analyzed the expression of several pluripotency-associated factors in pre- and postnatal testes. In general, expression patterns were comparable in primates and mice (Eildermann et al. 2012a, and own unpublished data). An exception was the pluripotency marker LIN28, which attracted our interest because it was expressed in adult mouse spermatogonia and not, or only in very few adult primate spermatogonia. In detail, we demonstrated that LIN28 is expressed in marmoset and mouse PGCs and gonocytes. During puberty, the number of LIN28 positive cells decreases remarkably. Only very few LIN28-positive cells are detectable in the adult marmoset testis. In contrast, there were numerous LIN28 positive spermatogonia in the adult mouse testis. The data on LIN28 expression were published in the journal *Molecular Human Reproduction* entitled “The pluripotency factor LIN28 in monkey and human testes: a marker for spermatogonial stem cells?“ (Chapter 3). LIN28 is an important gene in gaining and retaining pluripotency. The lack of LIN28 in primate spermatogonia might therefore be a reason for the past failure of deriving pluripotent cells from adult primate testis. Exogenic expression of LIN28 in primate SSC could experimentally confirm or falsify this hypothesis. Nevertheless, the data obtained in this study indicates that LIN28 may be a suitable marker for a specific subpopulation of primate spermatogonial stem cells that persisted in a probably more primitive, i.e. undifferentiated state. It can be speculated that this very rare LIN28-positive population has a higher capability to dedifferentiate into a pluripotent state. At the same time it seems more likely to derive pluripotent cells from gonocytes of neonatal testis as these cells still strongly express LIN28. Based on these results, we speculate that neonatal gonocytes have a higher potential to revert to pluripotent state than adult spermatogionia have.

Germ cells are kept restricted to their unipotential state by the surrounding somatic cells. Our hypothesis was that release of the germ cells from their native tissue may release them also from their restriction to unipotency eventually resulting in pluripotency. Therefore, in the

second part of this thesis we tested this hypothesis. We subcutaneously injected a single cell suspension of whole neonatal marmoset testes tissue, i.e. including pluripotency factor-positive germ cells, into immunodeficient mice. The development of histologically normal endodermal, mesodermal and ectodermal derivatives in this assay indicates the pluripotency of injected cells. Ideally, isolated gonocytes should have been tested. Unfortunately, to our knowledge, there is no useful marker for the efficient and harmless enrichment of primate gonocytes currently available. All surface markers that have been used previously and that are known to us are also expressed by testicular multipotent stromal cells (Eildermann et al. 2012b). Therefore, we were not able to test isolated gonocytes. The results have been summarized in the manuscript “Grafting of neonatal marmoset monkey testicular single cell suspensions into immunodeficient mice leads to ex situ testicular cord neomorphogenesis“ (Chapter 4) which has been published in the journal *Cells Tissues Organs* in 2013. In the present experimental setting and in the limited numbers of experiments we were able to perform, we did not observe any teratoma. This suggests that NHP gonocytes are not highly teratogenic. However, we cannot exclude that some soluble factors from the co-injected somatic cells or direct somatic cell – germ cell interaction keep the gonocytes in a non-pluripotent and non-teratogenic state. It is conceivable that a pure fraction of gonocytes or cultured gonocyte- / spermatogonia-derived cells would be able to revert or dedifferentiate to a developmental state that is characterized by pluripotency. Interestingly, instead of teratoma formation we detected a reaggregation of the injected cells forming tubular structures resembling normal neonatal testicular tissue. These tubular structures mainly consisted of Sertoli cells surrounded by myoid peritubular cells, occasionally also gonocytes were detectable inside the tubules. All cell types expressed the cell-type specific markers (except SALL4 and OCT4A in the case of germ cells) that we use in our lab to determine the cellular identity of different cell types in the normal testis. This indicates that the rearranged testicular tissue highly resembles normal neonatal testicular tissue. This assay may represent a new system for the study of testicular morphogenesis and development.

For humans, the derivation of pluripotent stem cells (embryonic germ cells (EGCs)) from primordial germ cells (PGCs) has been demonstrated (Shamblott et al. 1998). In order to initially evaluate, whether marmoset PGCs may have a similar potential, we morphologically and immunohistochemically analysed PGC development in a set of 12 excellently preserved non-human primate embryos from the yolk sac stage to the sexual differentiation of the gonads. In this third part of the thesis, we paid special attention to pluripotency factor expression in PGCs to test potential differences between published data on human PGCs and marmoset PGCs. This study, entitled “Pluripotency factor expressing pre-migratory primordial germ cells are in very close proximity to the prospective gonadal ridge in the monkey embryo“ (chapter 5) and submitted to the journal *Development*, provides detailed

morphological data and novel insights into the spatio-temporal expression of the four pluripotency factors OCT4A, NANOG, SALL4, and LIN28, during primate embryogenesis. We were able to show that OCT4A is a very robust and the most specific marker of PGCs when compared to SALL4 and LIN28. However, importantly, all four pluripotency factors are expressed by PGCs. The study strongly indicates a close interrelationship between pluripotent cells and the germ line on the molecular and developmental level, and the expression of pluripotency factors, particularly that of OCT4A and NANOG, indicates a pluripotent state of the cells (Boyer et al. 2005).

In summary, it is plausible that cells that express pluripotency factors are potentially pluripotent. Therefore, as pluripotency factors specifically mark primordial germ cells after separation of the germ line from the somatic cells in post-implantation embryos, PGCs are potentially pluripotent (Leitch and Smith 2013). Although PGC-derived pluripotent cells will not provide a source for the generation of patient-specific pluripotent cells, they nevertheless may allow instructive insights into the regulation of pluripotency in primates. Based on our findings, we further suggested a novel model for the translocation of PGCs during embryonic development. This model challenges the “dogma” of active PGC migration from the endoderm to the gonads. Our model suggests a basically passive translocation of PGCs from the gut-surrounding mesenchyme to the prospective gonad through intercalar expansion of the mesenchymal tissue harboring the PGCs.

In summary, our initial aim, namely the derivation of pluripotent stem cells from marmoset testis, was not achieved. Despite of that, we put the hypothesis forward that a pure fraction of gonocytes is able to regain pluripotency under appropriate cell culture conditions. We also hypothesize that isolated marmoset PGCs are a promising germ cell type to initiate pluripotent cell cultures since marmoset PGCs very robustly express key pluripotency factors. Our studies provide an excellent basis for further analysis of testicular germ cells. They also provided the basis for novel experimental approaches for the derivation of pluripotent stem cells from primate testes.

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