From stem cells to male germ cells: Experimental approaches for the *in vitro* generation of mouse and human spermatogonial stem cells

Dissertation

for the award of the degree
“Doctor rerum naturalium”
of the Georg-August-University Göttingen

within the doctoral basis program Biology
of the Georg-August University School of Science (GAUSS)

submitted by

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Göttingen, 2015

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Day of the oral examination: 29.05.2015

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Abbreviations

2-ME	β-Mercaptoethanol
3-β-HSD	3-β-Hydroxysteroid Dehydrogenase
7-AAD	7-Amino-Actinomycin

A

A_1, A_2, A_3, A_4	differentiating spermatogonia
A_{al}	aligned A-type Spermatogonia
ABP	Androgen Binding Protein
ACR	Acrosin
Amp	Ampicillin
AP	Alkaline Phosphatase
A_{pr}	paired A-type spermatogonia
APS	Adenosine 5’ Phosphosulfate
AR	Androgen Receptor
ART	Assisted Reproduction Treatment
A_s	single A-type Spermatogonia
AT1R	Angiotensin II Type I Receptor
ATP	Adenosine Triphosphate

B

B18R	B18R Recombinant Protein
BCL6	B-Cell Lymphoma 6 protein
bFGF	basic Fibroblast Growth Factor
BJs	Stemgent® BJ Human Fibroblasts
BMP4	Bone Morphogenetic Protein 4
Bp	base pair
BrdU	5-Bromo-2′-deoxyuridine
BSA	Bovine Serum Albumine

C

°C	Centigrade
CAL	Calponin
cDNA	complementary DNA
^{m}C	methylated Cytosine

cond	conditioned
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSF-1	Colony Stimulating Factor 1
CYP26	Cytochrome P450, Family 26 family

D

d	day
DAZL	Deleted In Azoospermia-Like
DCN	Decorin
ddH_2O	bi-distilled water
DDX4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 8 (aliases: VASA)
DMEM	Dubecco's Modified Eagle Medium
DMR	Differentially Methylated Region
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside triphosphate
dpc	days post coitum
dTG	double transgene

E

EB	Embryoid Body
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGFP	Enhanced Green Fluorescent Protein
EGR2	Early Growth Response 2
ELISA	Enzyme Linked Immunosorbent Assay
EM	Electron Microscopy
EPCAM	Epithelial Cell Adhesion Molecule
ESC	Embryonic Stem Cell
ESR1 / 2	Estrogen Receptor 1 / 2
et al.	et alteres
EtOH	Ethanol
ETV5	Ets Variant 5

F

FACSorting	Fluorescence-Activated Cell Sorting

FB	Fibroblast *or* Fibroblast Medium
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
Fig.	Figure
FKBP6	FK506 Binding Protein 6
FSH	Follicle-Stimulating Hormone
FSHR	Follicle-Stimulating Hormone Receptor

G

gDNA	Genomic Deoxyribonucleic Acid
GDNF	Glial cell line-Derived Neurotrophic Factor
Gel	Gelatine
GFRα1	GDNF Family Receptor α1
GPER	G-Protein coupled Estrogen Receptor
GPR125	G-Protein coupled Receptor 125
GPX4	Glutathione Peroxidase 4

H

h	hour *or* human
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric Acid
H&E	Hematoxylin-Eosin
hiPSC	human induced Pluripotent Stem Cell
hiPSC-M	hiPSC Medium
hMGSCs	human Multipotent Germline Stem Cells
hOKSML	mRNA endcoding for the transcription factors hOct4, hKlf4, hSox2 and hc-Myc
HPRT	Hypoxanthine Phosphoribosyltransferase
HRP	Horseradish Peroxidase
HTF	Human Testicular Feeder
HTPC	Human Testicular Peritubular Cell

I

ICR	Imprinting Control Region
ICSI	Intracytoplasmic Sperm Injection
IG	Intergenic
IGF I/II	Insulin-like Growth Factor I/II

IGF2R	Insulin-like Growth Factor 2 Receptor
IgG	Immunglobulin G
iPSC	induced Pluripotent Stem Cell
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
IUI	Intrauterine Insemination
IVF	*In Vitro* Fertilization

K

kb	kilo base
KCL	Potassium Chloride
kDa	kilo Dalton
KLF4	Kruppel-Like Factor 4
KO™-SR	KO Serum Replacement

L

l	liter
LB	Luria-Bertani
LHCGR	Luteinizin Hormone/Choriogonadotropin Receptor
LHX1	LIM Homeobox Protein 1
LIF	Leukemia Inhibitory Factor

M

µ	Micro (10^{-6})
m	mouse
m-	Milli (10^{-3})
M	Molar
MACSorting	Magnetic Activated Cell Sorting
MAGE A4	Melanoma-Associated Antigen 4
maGSCs	multipotent adult Germline Stem Cells
MEF	Mouse/Murine Embryonic Fibroblasts
Meg3	Maternally expressed gene 3
MESA	Microscopic Epididymal Sperm Aspiration
$MgCl_2$	Magnesium Chloride
mGSCs	multipotent Germline Stem Cells
min	minute
miRNA	micro RNA
mJ-score	modified Johnsen Score

MOV10l1	Moloney Leukemia Virus 10-like 1
mRNA	messenger RNA
MTF	Mouse Testicular Feeder

N

n	Nano (10^{-6})
NaAc	Sodium Acetate
NaCl	Sodium Chloride
NEAA	Non-Essential Amino Acids
Ngn3	Neurogenin 3
NLS	Nuclear Localization Signal
NGF	Nerve Growth Factor
nm	Nanometer
NOA	Non-Obstructive Azoospermia
NuFF	Neonatal Human Foreskin Fibroblasts

O

OA	Obstructive Azoospermia
OAT	Oligoasthenoteratozoospermia
OCT4	Octamer-binding Transcription Factor 4
ORF	Open Reading Frame
OVOL1	OVO Homolog-like 1

P

p	Pico (10^{-9})
P	Passage
Pa	Pascal, unit of pressure
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PE	Phycoerythrin
PESA	Percutaneous Epididymal Sperm Aspiration
PFA	Paraformaldehyde
PGC	Primordial Germ Cell
PGCLC	Primordial Germ Cell-Like Cell
PGP9.5	Protein Gene Product 9.5
pH	negative decimal logarithm of the hydrogen ion concentration

PLZF	Promyelocytic Leukemia Zinc Finger Protein
PMSF	Phenylmethylsulfonfluorid
PP_i	Pyrophosphate
PRM1	Protamine 1
P/S	Penicillin/Streptomycin
PS	Phosphatidylserine
P™S	Pluriton™ Supplement
PTX3	Pentraxin 3

Q

qRT-PCR	quantitative RealTime-PCR

R

RA	Retinoic Acid
RET	Ret Tyrosine Kinase Transmembrane Reseptor
RNA	Ribonucleic Acid
RNase	Ribonuclease
ROCK	Rho-associated Kinase
rpm	rotations per min
RT-PCR	Reverse Transcriptase-PCR

S

SALL4	Sal-Like Protein 4
SCF	Stem Cell Factor
SCO	Sertoli Cell Only Syndrome
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
sec	second
SHF	Scrotal Human Fibroblast
SMA	Smooth Muscle Actin
SNRPN	Small Nuclear Ribonucleoprotein Polypeptide N
SOX2	Sex Determining Region Y (SRY)-Box 2
SRY	Sex Determining Region of Chromosome Y
SSC	Spermatogonial Stem Cell
SSEA-4	Stage-specific Embryonic Antigen 4
STAR	Steroidogenic Acute Regulatory Protein
STO	SIM mouse embryo-derived thioguanine and oubain resistan

STRA8	Stimulated by Retinoic Acid Gene 8
SYCP3	Synaptonemal complex protein 3

T

TA	Talarozole
Tab.	Table
TALEN	Transcription-Activator Like Effector Nuclease
TBP	TATA-Binding Protein
TESE	Testicular Sperm Extraction
TGF-ß	Transforming Growth Factor-β
TP2	Transition Protein 2
TSPAN8	Testraspanin 8
Tw20	Tween 20

U

U	Unit(s), enzyme activity

V

V	Volt
VASA	aliases: DDX4
VIM	Vimentin

W

WT	Wild Type

X

x g	Multiple of acceleration of gravity
X-Gal	5- Brom-4-chlor-3-indolyl-β-D-galactopyranoside

Y

Y-27632	Inhibitor of Rho-associated Kinase (ROCK)

Z

ZFN	Zinc-Finger Nucleases

1 Introduction

1.1 Causes and current treatment opportunities of male infertility

Many couples are involuntarily childless because one or both partners suffer from infertility, which is defined as the inability of a couple to conceive a pregnancy after 12 month of intercourse without the use of any contraception (Gnoth et al., 2005). Between 10-15% of couples are faced with fertility problems- in half of these cases due to men- and most of them make demands on assisted reproduction treatments (ART), pioneered by Robert Edwards in 1978, in order to father an own child (Boivin et al., 2007).

Infertile men are mostly diagnosed with oligoasthenoteratozoospermia (OAT) or azoospermia. While patients with OAT possess sperm in a reduced number (oligospermia), with a decreased motility (asthenozoospermia) or morphological displastics (teratozoospermia) in their ejaculate, patients diagnosed with azoospermia exhibit no sperm at all in the semen. Here urologists discriminate between the inability of sperm to reach the ejaculate (obstructive azoospermia; OA) and the failure of sperm production at all due to an impaired or blocked spermatogenesis (non-obstructive azoospermia; NOA). The underlying causes, which result in OAT or azoospermia, determine the possible therapeutical approaches, with which childless couples might be able to achieve a pregnancy from their own genetic material. The study of Tüttelmann et al. (2011) provides an insight into the major causes of azoospermia. They viewed data derived from counselling over 30 years of azoospermic patients and determined frequencies of diagnoses in this cohort (Fig. 1). These data revealed that 31% of azoospermic men had general andrological diagnoses including varicocele and urogenital infections. The infertility was also due to genetic causes. 21% of the patients suffered from chromosomal aberrations like the Klinefelter syndrome, Y-chromosomal microdeletions or other genetic defects. Malignancies of testicular or non-testicular cancer caused infertility in 15% of the patients. Other patients were infertile because of obstructions and endocrine or other chronic diseases like diabetes. A considerable fraction of 12% was classified as idiopathic infertile and the underlying cause of the azoospermia was unclear.

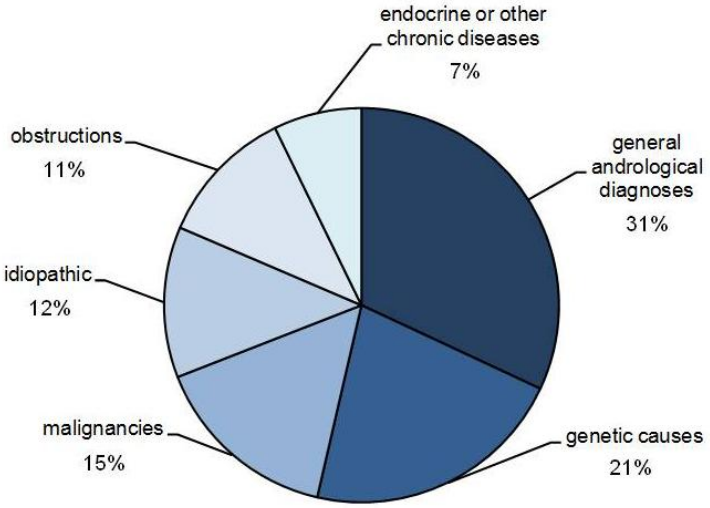


Fig. 1: Frequencies of diagnoses in azoospermic patients. Data derive from Tüttelmann et al., 2011 and reflect diagnoses of 1583 azoospermic patients, who were retrospectively examined regarding the underlying cause of their azoospermia. The analyses involved medical history, physical examination as well as semen and hormone analysis of each patient.

The treatment options are dependent on the diagnosis of male infertility. In some cases hormone treatments or other medications are sufficient to improve the semen quality and pregnancy can be achieved naturally. If healthy sperm can be isolated from the ejaculate, it can be used for ART. However, in most cases the underlying cause of infertility like sperm abnormalities of genetic origin cannot be removed. Provided that the patient produces healthy sperm at least in a small extent, minimal invasive techniques for sperm retrieval like MESA (Microscopic Epididymal Sperm Aspiration), PESA (Percutaneous Epididymal Sperm Aspiration) or TESE (Testicular Sperm Extraction) can be used to obtain small amounts of sperm from the patient's testes or epididymides, which can be further used to fertilize oocytes using ART (Hsiao et al., 2011). Dependent on the quality of isolated sperm, the fertilization can be achieved *in vivo* by placing the sperm into the woman's uterus to relieve fertilization (Intrauterine Insemination; IUI), *in vitro* by fusing sperm and oocyte naturally (*in vitro* Fertilization; IVF) or by using a needle (Intracytoplasmic Sperm Injection; ICSI) (Fig. 2, top). But these existing reproductive therapies are not suitable to help complete sterile men, who do not produce any functional sperm. Here several experimental and promising approaches in the research might help to restore or manipulate men's fertility (Fig. 2, bottom). These different treatment options have all one commonality and try to take advantage of the potentials derived from cell pluripotency. Researchers worldwide make great efforts to use pluripotent stem cells for the derivation of male germ cells, which are summarized in the following introductory part.

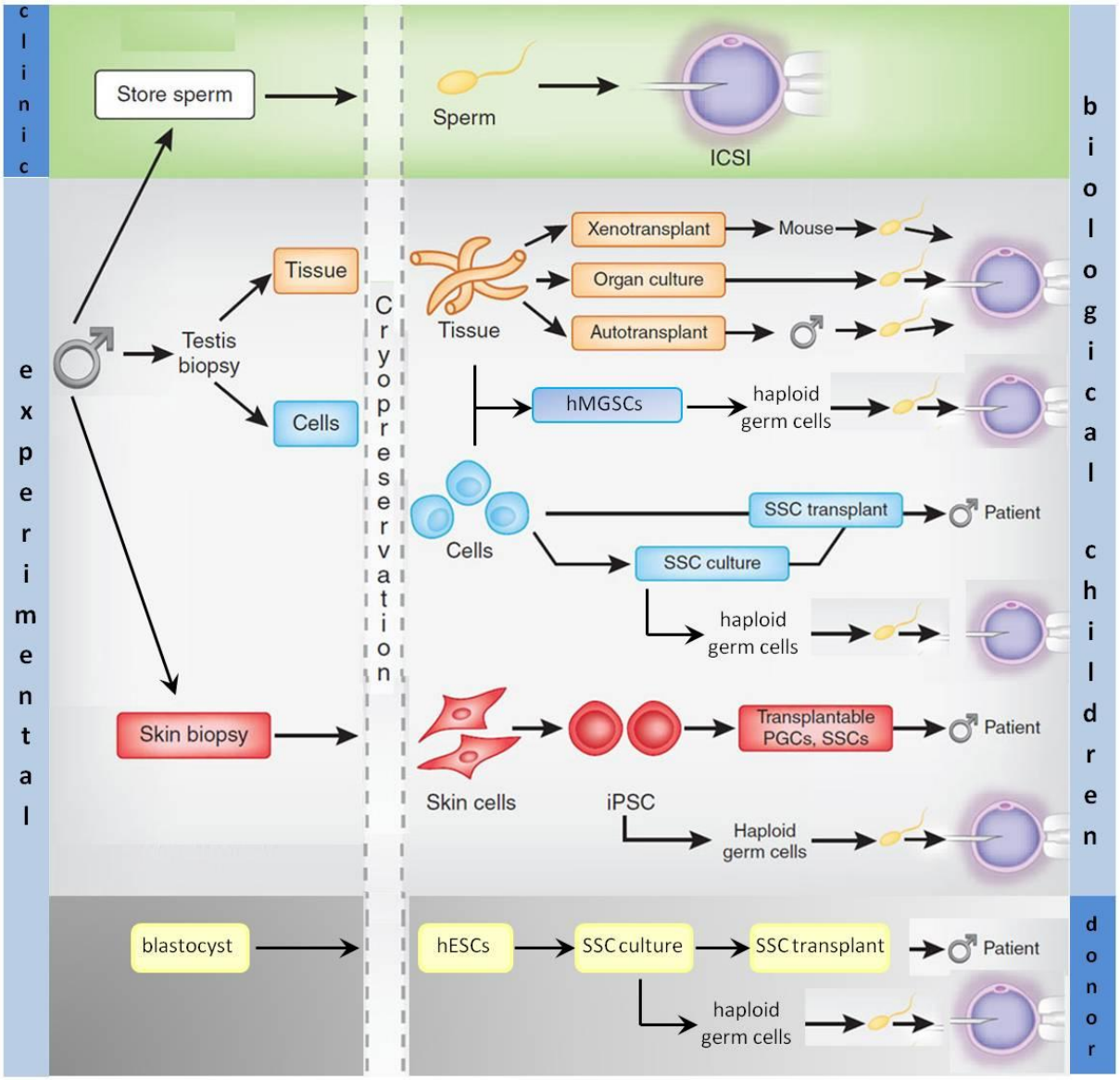


Fig. 2: Clinical and experimental approaches for treatment of male infertility. (Top) Independent from the underlying cause of their infertility, men, who produce any healthy sperm, can currently make use of assisted reproduction treatments. **(Bottom)** Researchers are working on several experimental approaches to treat male infertility, including the use of patient-derived testicular tissue for xenotransplantations, organ cultures or autotransplantations (*orange boxes*) and patient-derived SSCs for transplantations. *In vitro* differentiation of haploid germ cells might be possible with patient-specific SSCs and hMGSCs (*blue boxes*). Patient-derived iPSCs are beneficial for infertile men without any spermatogenetic activity to obtain PGC or SSC transplantation or functional sperm by *in vitro* differentiation (*red boxes*). Theoretically, donor-derived hESCs offer the opportunity for *in vitro* differentiation of SSCs or haploid germ cells (*yellow boxes*). ICSI: Intracytoplasmic Sperm Injection; hMGSCs: human Multipotent Germline Stem Cells; SSC: Spermatogonial Stem Cell; iPSC: induced Pluripotent Stem Cell; PGCs: Primordial Germ Cells; hESCs: human Embryonic Stem Cells. Modified, Clark et al., 2011.

1.2 A journey through years of research: from pluripotent stem cells to male germ cells

In recent years many researchers were dedicated to the derivation of male germ cells and applied several approaches to generate different stages of male gametes. The use of pluripotent stem cells is a promising method to recapitulate spermatogenesis *in vitro* (for review: Nolte and Engel, 2013; Hou et al., 2014). Their ability of self-renewal without genetic changes and their potential to differentiate into any cell type including germ cells makes pluripotent stem cells especially suitable for *in vitro* spermatogenesis (Martin, 1981; West et al., 2006).

Scientists developed several strategies for the establishment of robust and reproducible culture systems for the differentiation of mature and functional haploid male germ cells derived from mouse and human pluripotent stem cells. Cultivation of these cells under differentiating conditions including induction with retinoic acid (RA) and growth factors was sufficient to obtain cells at premeiotic and meiotic stages (Drusenheimer et al., 2007; Eguizabal et al., 2009, 2011). The embryoid body (EB) differentiation strategy is also a frequently used method for male germ cell differentiation. Cultivation of pluripotent stem cells in hanging drops without antidifferentiation factors leads to spontaneously three dimensional EB formation, which reflects cell differentiation during early mammalian embryogenesis (Keller, 1995; Kurosawa, 2007). EB formation derived from different types of stem cells gave rise to the generation of various stages of male germ cells (Clark et al., 2004; Tilgner et al., 2008; Aflatoonian et al., 2009; Imamura et al., 2010). Other groups proposed modified germ cell differentiation protocols via EB formation and additional treatment with RA (Geijsen et al., 2004; Lee et al., 2006; West et al., 2006; Zhu et al., 2012; Cai et al., 2013), hormones (Lee et al., 2006) or growth factors (Toyooka et al., 2003).

The differentiation of human germ cell precursors and meiotic cells could also be achieved by co-culture of pluripotent stem cells with other cell types such as mouse embryonic fibroblasts (MEFs) or human fetal gonadal stromal cells known to support the primary culture of germ cells (West et al., 2008; Park et al. 2009). Furthermore, advancement of germ cell progression through meiosis and the formation of haploid germ cells and even motile tailed sperm could be achieved by the overexpression of germ cell-related genes in stem cells during *in vitro* differentiation (Kee et al., 2009; Yu et al., 2009; Medrano et al., 2012). The use of reporter genes for enrichment of premeiotic cells and detection of haploid male germ cells represents a further significant method for male germ cell differentiation (Nayernia et al., 2006). Some studies using such selection strategies in combination with RA induction even resulted in the generation of haploid gametes, whose fertility was proven by the birth of viable offspring after intracytoplasmic sperm injection (ICSI) (Nayernia et al., 2004; Nolte et al., 2010).

These different approaches for *in vitro* spermatogenesis could be successfully applied with various types of mouse and human pluripotent stem cells known so far. Mostly embryonic stem cells (ESCs) derived from the inner cell mass of a pre-implanted blastocyst were used for the efficient generation of haploid gametes. Using an EB approach with growth factor treatment Toyooka and colleagues (2003) were the first, who succeeded in the derivation of germ cell precursors, the primordial germ cells (PGCs), from mouse ESCs. The expression of an endogenous homolog of Vasa, a cytoplasmic protein expressed from premeiotic stages onwards (Toyooka et al., 2000), was used for purification of PGCs. After co-culture with gonadal cells, the ESC derived PGCs were able to form haploid germ cells in transplanted host testis. Just one year later haploid male germ cells were obtained via EB formation with RA induction, whose fertility was proven by fertilization of oocytes and further blastocyst development (Geijsen et al., 2004). Nayernia and colleagues (2006) reported a further breakthrough in the research of *in vitro* spermatogenesis and presented a double selection strategy based on reporter genes for the isolation of premeiotic germ cells and detection of mature sperm, which was finally able to give rise to viable offspring after ICSI. The prematurely death of the offspring due to global imprinting defects revealed that the establishment of correct imprinting during *in vitro* spermatogenesis is one of the major problems, which have to be solved. These imprinting defects could be overcome by a two step differentiation approach (Hayashi et al., 2011). After *in vitro* generation of primordial germ cell-like cells (PGCLCs) from mouse ESCs and completion of spermatogenesis *in vivo*, transplanted mice gave rise to fertile offspring with correct imprinting.

During the same time period PGCs as well as premeiotic, meiotic and even haploid male germ cells could also be derived from human ESCs using approaches of EB formation (Clark et al., 2004; Tilgner et al, 2008; Aflatoonian et al., 2009), co-culture (West et al., 2008) as well as overexpression of germ cell-related genes (Medrano et al., 2012).

The generation of mouse and human induced pluripotent stem cells (iPSCs) derived from somatic cells was a further milestone in the establishment of *in vitro* culture systems for the differentiation of male germ cells (Takahashi et al., 2006; Meissner et al., 2007; Takahashi et al., 2007). Both mouse (Imamura et al., 2010; Zhu et al., 2012; Cai et al., 2013) and human iPSCs (Park et al., 2009; Panula et al., 2011; Medrano et al., 2012) were successfully differentiated into various stages of male germ cells. Especially with regard to the derivation of human germ cells, iPSCs provide a promising cell type for *in vitro* spermatogenesis of personalized gametes without ethical concerns. Human iPSC-derived haploid male germ cells were obtained within ten weeks (Eguizabal et al., 2011) and even within ten days (Easley et al., 2012) by using complex differentiation culture media, which forced the progression through meiosis. The possibility to validate the functionality of *in vitro* derived human gametes in the human system is limited. However, methylation studies confirmed

also improper imprinting patterns of these male germ cells assuming that these *in vitro* derived gametes could not lead to healthy offspring (Park et al., 2009; Eguizabal et al., 2011; Easley et al., 2012). So the acquisition of correct epigenetic marks in sperm emerged to be a great problem in *in vitro* spermatogenesis approaches. Therefore, researchers invest great hope in the differentiation of male germ cells derived from natural spermatogonial stem cells (SSCs), which ensure the maintenance of spermatogenesis and fertility throughout male life. Because SSCs isolated from the testes of different species were used in several approaches for *in vitro* differentiation of male gametes (Feng et al., 2002; Riboldi et al., 2012; Minaee Zanganeh et al., 2013), the following introductory part should provide some more details of SSCs and their potential to give rise to sperm *in vitro*.

1.3 The potential of spermatogonial stem cells in *in vitro* spermatogenesis approaches

SSCs are unipotent adult tissue stem cells, which are located along the basement membrane of the highly structured seminiferous tubules of the testis and give rise to spermatogenesis. SSCs are characterized by the ability to balance between their self-renewal to maintain a life-long pool of SSCs and their differentiation into mature sperm throughout the postpubertal life of men. Based on these features SSCs represent a life long storage of male fertility during reproductive life carrying the father`s genetic material to subsequent generations (Oatley and Brinster, 2008; Orwig and Hermann, 2011).

Several experimental approaches have addressed the isolation of SSCs and their expansion in culture using cell surface markers and defined culture conditions, which is proven to be difficult because of the absence of specific markers for the identification and characterization of SSCs (Xu et al., 2011; Kossack et al., 2013). Therefore, cell transplantation assays are indispensable for the final functional proof of isolated SSCs until now (Brinster et al., 1994). So far numerous research groups have reported the isolation and at least short-term culture of SSCs from different animal species such as rodents including mice (Kanatsu-Shinohara et al., 2003), rats (Hamra et al., 2005) and hamsters (Kanatsu-Shinohara et al., 2008b) as well as bovine (Aponte et al., 2008), buffalos (Kala et al., 2012) and pigs (Luo et al., 2006; Kuijk et al., 2009). The establishment of long-term SSC cultures remains challenging and becomes apparent in the less number of reports, which claimed extended cultivation of SSCs derived from mouse, rat or hamster (Kanatsu-Shinohara et al., 2003; Kubota et al., 2004b; Ryu et al., 2005; Kanatsu-Shinohara et al., 2008b). Based on these insights of SSC long-term cultures, several groups have tried to develop strategies for the isolation and culture of human SSCs (hSSCs), which is of particular interest in regard to therapeutical approaches to treat male infertility due to a disrupted spermatogenesis caused by various reasons (1.1; Fig. 2). Since

autotransplantations of isolated SSCs from a wide range of species were successfully performed (Brinster and Zimmermann, 1994; Schlatt et al., 2002; Honaramooz et al., 2003a, b; Izadyar et al., 2003b), *in vitro* expanded hSSCs isolated from human testicular biopsies might be able to repopulate the testis after transplantation and lead to restored spermatogenesis in men. Such a clinical application requires established and reliable protocols for the *in vitro* propagation of hSSCs isolated from small testicular biopsies, which contain a too small amount of SSCs to repopulate a human testis after autotransplantation. The fact that SSCs represents just 0.03% of all male germ cells illustrates the difficulty to isolate this small cell fraction and its expansion in culture (Tegelenbosch and de Rooij, 1993).

To date, the different attempts to establish long-term cultures of hSSCs have been unsuccessful although different isolation methods and culture conditions have been used (He et al., 2010; Kokkinaki et al., 2011; Liu et al., 2011; Mirzapour et al., 2012). Some research groups were able to expand the culture of hSSCs up to four month. Using a culture method of putative hSSCs isolated from human fetal testes with media inspired by the formulation used for cultivation of mSSCs and a feeder layer consisting of human embryonic stem cell derived fibroblast-like cells was suitable to maintain isolated hSSCs for two month in culture (Chen et al., 2009). Sadri-Ardekani and colleagues (2009) were even able to propagate germline stem cell clusters derived form adult human testes for four month using medium inspired by Kanatsu-Shinohara et al. (2003) and human placental laminin-coated dishes. The functionality of these *in vitro* cultured putative hSSCs could be proven by xenotransplantation to mice. This progress shows that long-term culture and *in vitro* propagation of hSSCs is achievable, nevertheless it also illustrates the importance of the less popular basic research to identify factors, which might determine the status of proliferating and senescent hSSCs, and helps to figure out culture and growth requirements for the long-term culture of hSSCs (Yoo et al., 2010).

The discovery to derive pluripotent cells from testicular cells was a considerable breakthrough in stem cell research and created new opportunities in the research field of reproductive biology. First Kanatsu-Shinohara and colleagues (2004) succeeded in the establishment of ES cell-like cells from SSCs derived from neonatal mouse testis, which conformed several pluripotency-related features including the ability to form teratomas and germline chimeras, and were termed multipotent germline stem cells (mGSCs). The fascinating part of this study was the finding that pluripotency of somatic cells can be induced by a physiological method: when removed from their natural environment and cultured under standard ESC conditions, neonatal testis cells spontaneously produce ESC-like cells *in vitro* indicating that germ cells retain their pluripotency character. Two years later Guan et al. (2006) were able to expand this approach and presented the derivation of pluripotent cells

from SSCs isolated from the adult mouse testis, therefore called multipotent adult germline stem cells (maGSCs). Here, germline stem cells were enriched using the premeiotic reporter gene Stra8-EGFP for fluorescence activated cell sorting (FACSorting), whose SSC character was proven by transplantation assays showing regeneration of spermatogenesis in germ cell depleted mice. After propagation of EGFP positive cells representing a mouse SSC population in culture, these cells were cultured under different conditions revealing that SSCs responded to standard ESC culture conditions and displayed ESC properties. The suitability of maGSCs derivation from adult mouse testis was further validated by several groups (Seandel et al., 2007; Izadyar et al., 2008; Kanatsu-Shinohara et al., 2008a; Ko et al., 2009).

Furthermore, Nolte and colleagues (2010) proved that maGSCs can also be used for the derivation of functional haploid male germ cells. Using the double selection strategy published by Nayernia et al. (2006) the researchers established stable premeiotic germ cells derived from maGSCs. By culturing these cells without antidifferentiation factors and additional RA treatment, the onset of meiosis was induced and haploid male germ cells were detected by the expression of the postmeiotic reportergene Prm1-DsRed. The fertility of haploid cells was proven by the birth of viable offspring with maGSC origin after ICSI. Although up to now this is the only report of male germ cells derived from maGSCs, this finding is of great importance if it is possible to implement it into reliable protocols for the derivation of human male germ cells. If adult human testicular cells could be reprogrammed to pluripotent stem cells, then termed human multipotent germline stem cells (hMGSCs), just by culture, they could be used for the derivation of male germ cells instead of human ESCs or iPSCs. Consequently, the use of hMGSCs would avoid the ethical concerns using ESCs as well as the usage of exogenous pluripotent factors needed for the generation of iPSCs from adult somatic cells at the same time. Thus, the risk of cell transformation and tumor formation in the patient originating from the use of iPSCs could be also obviated. Moreover, the use of patient-derived testicular material for the generation of germ cells greatly reduces the likelihood of an immunological response to the transplanted cells and further implies the production of personalized gametes to obtain biological identical children.

Inspired by the idea to develop an alternative therapeutic strategy to treat male infertility, many researchers are dedicated to establish stable hSSC lines in culture followed by reprogramming into a pluripotent state. Several groups reported the phenomenon of testis-derived ESC-like cells (Conrad et al., 2008, retracted in 2014; Golestaneh et al., 2009; Kossack et al., 2009; Mizrak et al., 2010), but the pluripotency of these cells was called into question based on the results of subsequent analyses (Ko et al., 2010; Tapia et al., 2011; Chikhovskaya et al., 2012). The so called human ESC-like cells do not fulfil the criteria for cellular pluripotency published by the National Institutes of Health. Different reviewers complained that in none of the studies these cells revealed a detectable expression of

pluripotency markers or the ability to form highly proliferating teratomas. Moreover, whole testicular cell suspensions were used instead of established and characterized hSSC cell lines and global gene expression analyses could demonstrate similarities to a fibroblast cell line suggesting rather a mesenchymal or fibroblast origin of the human ESC-like cells (Ko et al., 2010; Tapia et al., 2011). These facts corroborate their non-pluripotent character and initiated a still ongoing debate and efforts for novel strategies for the derivation of pluripotent cells from human testicular cells. But up to now no research group was able to publish a reliable and convincing protocol for the establishment of proliferating hSSC cell lines in culture and their reprogramming into a pluripotent cell line without genetic modifications. Here, the *in vitro* imitation of the SSCs' *in vivo* environment is the most challenging part and implies the investigation of the impact of the testicular environment on the SSCs, which is further described in the following introductory part.

1.4 Co-culture- a promising method for SSC culture and derivation of male germ cells

SSCs receive numerous signals from the testicular environment, which is described as the spermatogonial stem cell niche (SSC niche) and plays an important role in regulating testicular homeostasis and the specific properties of SSCs. In 1978 Schofield introduced a concept of a so called stem cell niche, which represents a specialized microenvironment consisting of several components, which provide structural features as well as an adequate growth factor milieu for tissue-specific stem cells (Schofield, 1978). According to this model a niche provides generally all essential factors to sustain a balance between a proliferating stem cell pool and the onset of the differentiation to their final cell fate. Consequently, the removal of components from the stem cell niche would disrupt its structure accompanied by the loss of the identity and the regulated proliferating and differentiating status of the residing stem cells (Voog and Jones, 2010). Therefore, the identification and characterization of niche properties and their emanating signals would facilitate the establishment of long-term cultures of stem cells in general.

In regard to the culturing of SSCs many studies concentrate on investigating the biology of the complex SSC niche. The sertoli cells within the seminiferous tubules, the basement membrane and the interstitial cells are the structural constituents of this niche in the testis. The fact that sertoli cells are the only somatic cell type within the tubules illustrates their special role as one of the most important components in the SSC niche. As a part of the epithelium on the inner surface of the basement membrane they have physical contact with the self-renewing SSCs, which reside along the basement membrane of the tubules connected via adhesion molecules (Tung et al., 1984). Additionally, sertoli cells reach to the

lumen of the tubule and escort the differentiating germ cells released to the centre thereby providing various nutrients. The connections of sertoli cells via tight junctions form the blood-testis barrier and create an immune-privileged environment for differentiating germ cells crossing the tight junctions at the onset of meiosis. Due to this sub-compartmentalization of the tubules into basal and adluminal compartments, SSCs and developing germ cells can also be exposed to different factors. The interstitial tissue between the seminiferous tubules consists of peritubular cells covering the outer surface of the basement membrane, leydig cells, which are mostly arranged in clusters, blood vessels maintaining the vascular network as well as immune cells (for review: de Rooij, 2009). These structural components of the SSC niche provide a defined milieu for the maintenance of a balanced self-renewal of SSCs and differentiation of germ cells by providing physical support and secreting growth factors (Fig. 3).

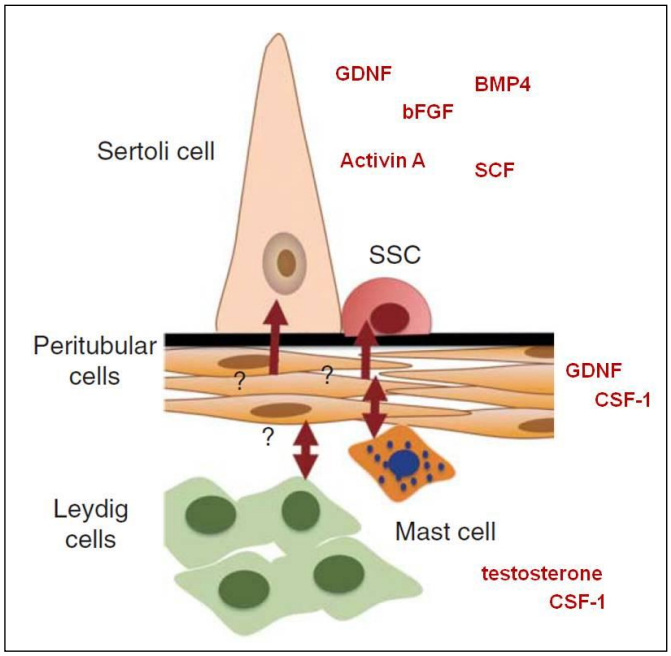


Fig. 3: Model of environmental signals within the spermatogonial stem cell niche. The SSC niche consists of sertoli cells, the basement membrane and interstitial tissue with blood vessels, mast-, leydig- and peritubular cells and provides several directly or indirectly cues for the SSCs residing at the basement membrane indicated by arrows. Additionally, some fundamental growth factors released within the SSC niche are indicated. The marked signalling as well as the indicated growth factors are not exhaustive. GDNF: Glial cell line-Derived Neurotrophic Factor; bFGF; basic Fibroblast Growth Factor; BMP4: Bone Morphogenetic Protein 4; SCF: Stem Cell Factor; CSF-1: Colony Stimulating Factor 1. Modified, Mayerhofer, 2013.

In general, the released factors induce directly or indirectly either the proliferation or the differentiation of SSCs. Sertoli cells have an outstanding role in producing growth factors within the tubules: while GDNF (Glial cell line-Derived Neurotrophic Factor) and bFGF (basic Fibroblast Growth Factor) stimulate the self-renewal of SSCs (Tadokoro et al., 2002; Oatley et al., 2007; Simon et al., 2007), differentiation of SSCs is initiated by the secretion of activin A, BMP4 (Bone Morphogenetic Protein 4) and SCF (Stem Cell Factor) (Ohta et al.,

2000; Nagano et al., 2003a). The impact of the cells residing in the interstitial tissue is surprising because of their distance to the SSCs within the tubules preventing a direct contact. Nevertheless, several studies revealed a cross communication between sertoli and interstitial cells including leydig- and peritubular cells, which allows the interstitial cells to contribute to the SSC niche via the sertoli cells (Skinner et al, 1991). Thus, sertoli cells orchestrate the signals of the other SSC niche components. While sertoli cells receive testosterone secreted by leydig cells to support spermatogenesis (Skinner et al, 1991), their GDNF production is stimulated by FSH (Follicle-Stimulating Hormone), which is highly concentrated near the blood vessels (Tadokoro et al., 2002). Moreover, CSF-1 (Colony Stimulating Factor 1) was identified as an extrinsic stimulator of SSC self-renewal and proliferation produced by leydig and peritubular cells (Ryan et al., 2001, Oatley et al., 2009). However, recent work shed some new light on the presumptive role of peritubular cells within the SSC niche and demonstrated that human peritubular cells constitutively produce and release GDNF, thus indicating an additional role of these cells in survival and maintenance of SSCs (Spinnler et al., 2010). Based on these results and the fact that GDNF is suggested to be a key player within the niche responsible for SSC maintenance by regulating their self-renewal (Meng et al., 2000; Naughton et al., 2006), the peritubular cells may have a direct impact on SSCs by their GDNF production. However, these are just a few of the various compounds, which are part of the growth factor milieu within the SSC niche. Further are discussed later in the context of the results of the presented thesis (4.2.1).

This continuously increasing insight in the regulating machinery within the SSC niche provide the opportunity for researchers to create culture conditions, which resemble the SSC niche and promote the homing of isolated SSCs *in vitro*. The idea is to mimic the SSC niche by using specific culture media supplemented with nutrients, growth factors and hormones, which are normally provided by the testicular cells, and co-culturing with testicular stromal cells. Because of the already described extraordinary role of sertoli cells within the SSC niche, it is not surprising that the majority of groups performed co-culture experiments of isolated SSCs with sertoli cells. However, co-cultures using different established sertoli cell lines as a feeder layer for SSCs revealed contradictory results, although the cells did not differ in their growth factor secretion pattern: some sertoli cell lines were not sufficient to maintain SSCs in culture (van der Wee et al., 2001; Nagano et al., 2003a), while others seemed to have a beneficial effect (van der Wee et al., 2001). In contrast, the use of sertoli cells derived from primary cell cultures seems to have a significant effect on SSC maintenance and proliferation *in vitro*. Baazm and colleagues (2013) prepared adult and embryonic sertoli cells as feeder layers for co-culture with isolated mouse SSCs and showed that these sertoli cells- in combination with medium supplemented with growth factors- have a supportive effect on the maintenance of SSCs, whose identity was further proven by

regeneration of spermatogenesis after transplantation in germ cell depleted mice. Moreover, co-cultures with primary sertoli cells using medium without additional growth factors were also sufficient to promote SSC survival and colony formation *in vitro* (Koruji et al., 2009; Mohamadi et al., 2012). The positive effect of primary sertoli cells might be due to their natural secreted mix of nutritional and immune protective factors (Huleihel and Lunenfeld, 2004; Baazm et al., 2013).

Apart from the *in vitro* cultivation of isolated SSCs without losing their potential to repopulate a testis in a transplantation assay, further studies indicate that co-culture systems are also suitable for the *in vitro* differentiation of SSCs to male germ cells of different species (Rassoulzadegan et al., 1993; Sousa et al., 2002; Izadyar et al., 2003a; Gohbara et al., 2010). Especially sertoli cells, mostly derived from primary cultures, can stimulate the onset of meiosis *in vitro*. By testing different co-cultures with sertoli cells and media supplemented with hormones and vitamins, Minaee Zanganeh and colleagues (2013) examined the supportive role of sertoli cells for the production of spermatid-like cells derived from isolated mouse SSCs. Also putative human SSCs enriched from testicular biopsies revealed *in vitro* differentiation of haploid cells after co-culture with patient-derived sertoli cells (Riboldi et al., 2012).

Certainly because of the outstanding role of sertoli cells, the investigation of co-culture systems not only based on these cells was unfortunately neglected during the last years. But especially the latest finding of GDNF production by human peritubular cells (Spinnler et al., 2010) suggest an underestimated role of these and possibly other testicular cells for *in vitro* co-culture systems and encourage research groups to check also co-culture systems based on primary testicular cells. Using such a system, recently Mäkelä and colleagues (2014) demonstrated a “spontaneous reconstruction of testicular cellular microenvironments” (Mäkelä et al., 2014) based on the culture of primary and mitotically active mouse seminiferous tubule cells consisting of spermatogonia as well as sertoli- and peritubular cells. Based on these results co-culture systems seem to be a suitable and promising method for the cultivation of SSCs and their further improvement will definitely include more testicular cells than just sertoli cells.

1.5 Objectives of the presented PhD thesis

1. Establishment of proliferating human SSC cell lines from infertile men and their *in vitro* differentiation into haploid male germ cells

Several studies already proved that ESCs are suitable for the *in vitro* derivation of male gametes using different strategies. Since it was shown that mouse SSCs can also be reprogrammed into a pluripotent state and successfully used for *in vitro* spermatogenesis experiments, many researchers failed to establish a similar approach for the reprogramming of human SSCs. Therefore, one aim of this thesis was to generate proliferating human SSC cell lines using testicular biopsies from infertile men, which should be further used for direct differentiation to haploid male germ cells. Here it should be also examined if the failure in sperm production associated to different causes in the patients could be overcome during *in vitro* differentiation of haploid cells from the isolated human SSCs.

1.1 Generation of hiPSCs from infertile men for further *in vitro* differentiation of human SSCs

Numerous studies also revealed great difficulties in the establishment of long-term cultures of human SSCs. Therefore, in a second approach human pluripotent stem cells should be used for *in vitro* differentiation of human SSCs. Firstly, human iPS cells had to be generated for this approach and established from somatic cells derived from infertile men.

2. Establishment of a co-culture system for the generation of putative SSCs from mouse ESCs

The co-culture of species-derived SSCs using testicular stromal cells was shown to be a suitable culture system for the cultivation of functional SSCs as well as their differentiation into male gametes. In the presented thesis it was analyzed if a co-culture system has also the potential to support the generation of SSC-like cells from mouse ESCs. For this approach a multi-species co-culture system using mouse ESCs and human testicular stromal cells should be tested and characterized.

3. Role of *Stra8* during *in vitro* spermatogenesis of mouse ESCs

A strategy for the *in vitro* differentiation of male gametes derived from mouse ESCs was established in this Institute (Nayernia et al., 2006; Nolte et al., 2010). Nevertheless, the prematurely death of the offspring derived from the *in vitro* produced haploid cells draws the attention on their improper imprinting pattern. Since the premeiotic gene *Stra8* (Stimulated by Retinoic Acid Gene 8) is known to be essential for the induction of meiosis (Baltus et al.,

2006; Anderson et al., 2008) and is suggested to interact with chromatin remodelling proteins, which are regulators of imprinting control regions (Pantakani, unpublished data), it should be tested here, if a Stra8 overexpression during *in vitro* spermatogenesis can enhance the progression of meiosis and support correct imprinting.

2 Materials and methods

2.1 Material

2.1.1 Chemicals, biochemicals and cytokines

Chemical, biochemical, cytokine	Manufacturer
2-Mercaptoethanol	Gibco, Eggenstein
Acetic Acid	Merck, Darmstadt
Activin A	Sigma-Aldrich, Deisenhofen
Agar	Carl Roth, Karlsruhe
Agarose	Carl Roth, Karlsruhe
Ampicillin	Carl Roth, Karlsruhe
Ampuwa	Fresenius, Bad Homburg
Barricidal	Interchem Hygiene, Zürich, Switzerland
Biofreeze	Biochrom AG, Berlin
Boracid	MP Biomedicals, Ohio, USA
Bovine Serum Albumine (BSA)	Serva, Heidelberg
BrdU (5-Bromo-2′-deoxyuridine)	Sigma-Aldrich, Deisenhofen
Busulfan	Sigma-Aldrich, Deisenhofen
Cacodylic Acid	Serva, Heidelberg
Chloroform	J.T. Baker, Griesheim
Citrinin	Sigma-Aldrich, Deisenhofen
Colcemid	Biochrom AG, Berlin
Collagenase, from *Clostridium histolyticum*	Sigma-Aldrich, Deisenhofen
cOmplete Protease Inhibitor Cocktail Tablet	Roche, Mannheim
Coomassie Brilliant Blue R250	Sigma-Aldrich, Deisenhofen
CryoStem™ Freezing Mediuim	Stemgent, Cambridge, USA
DAPI	Sigma-Aldrich, Deisenhofen
Developer	AGFA HealthCare, Bonn
Dimethylformamide	Carl Roth GmbH, Karlsruhe
Dimethylsulfoxide (DMSO)	Carl Roth GmbH, Karlsruhe
	Sigma-Aldrich, Deisenhofen
DNaseI, from bovine pancreas	Sigma-Aldrich, Deisenhofen
dNTPs	Invitrogen, Karlsruhe
Eosin	Sigma-Aldrich, Deisenhofen
	Carl Roth, Karlsruhe

Ethanol	J.T. Baker, Griesheim
Ethidium Bromide	Inno-Train Diagnostik, Kronberg am Taunus
Ethylenediaminetetraacetic Acid (EDTA)	Carl Roth, Karlsruhe
Eukitt	Fluka, Deisenhofen
Fetal Calf Serum (FCS)	PAN, Aidenbach
Formaldehyde	Invitrogen, Karlsruhe
Formamide	Sigma-Aldrich, Deisenhofen
Gelatine	Sigma-Aldrich, Deisenhofen
Geniticin (G418)	Gibco, Eggenstein
Giemsa	Merck, Darmstadt
Glutaralaldeyde	Carl Roth, Karlsruhe
Glycerol	Carl Roth, Karlsruhe
Glycine	Carl Roth, Karlsruhe
Hank's Balanced Salt Solution (HBSS)	Gibco, Eggenstein
hbFGF	Invitrogen, Karlsruhe
	Peprotech, Rocky Hill, USA
Hematoxylin	Merck, Darmstadt
	Carl Roth, Karlsruhe
Hematoxylin QS	Biozol, Eching
hGDNF	Gibco, Eggenstein
	Miltenyi Biotec, Bergisch Gladbach
Hyaluronidase, from bovine testes	Sigma-Aldrich, Deisenhofen
Hybond™-C Extra	GE Healthcare, Freiburg
Hydrochloric acid (HCl)	Merck, Darmstadt
Isopropanol	J.T. Baker, Griesheim
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Carl Roth, Karlsruhe
Kanamycin	Carl Roth, Karlsruhe
L-Glutamine	Gibco, Eggenstein
LIF	Millipore, Billerica, USA
Lipofectamine® RNAiMAX Transfection Reagent	Invitrogen, Karlsruhe
Loading Buffer (10x)	Invitrogen, Karlsruhe
Magnesium Chloride ($MgCl_2$)	Merck, Darmstadt
Methanol	J.T. Baker, Griesheim
Milk powder	Carl Roth, Karlsruhe
Mitomycin C	Sigma, Deisenhofen
Natriumchloride (NaCl)	Carl Roth, Karlsruhe

Non-Essential Amino Acids (NEAA, 100x)	Gibco, Eggenstein
Paraformaldehyde	Sigma, Deisenhofen
Paraffin	Carl Roth, Karlsruhe
Penicillin	Carl Roth, Karlsruhe
Peptone	Carl Roth, Karlsruhe
Phenol	Biomol, Hamburg
Phenylmethylsulfonfluorid (PMSF)	Sigma, Deisenhofen
Phosphate Buffered Saline (PBS)	PAN, Aidenbach
Picric acid	Fluka, Deisenhofen
Plasmocure	InvivoGen, San Diego, USA
Potassium Chloride (KCl)	Carl Roth, Karlsruhe
Proteinase K	Carl Roth, Karlsruhe
Puromycin	Sigma, Deisenhofen
Rapid Fixer	AGFA HealthCare, Bonn
Restriction endonucleases	Invitrogen, Karlsruhe
	New England Biolabs, Frankfurt am Main
Retinoic Acid	Sigma, Deisenhofen
Reverse Transcriptase	Invitrogen, Karlsruhe
	Bioline, Luckenwalde
RNase A	Sigma, Deisenhofen
Roti®Mount Aqua	Carl Roth, Karlsruhe
Roti® Nanoquandt	Carl Roth, Karlsruhe
Saccharose	Sigma, Deisenhofen
S.O.C Medium	Invitrogen, Karlsruhe
Sodium Acetate (NaAc)	Merck, Darmstadt
Sodium Chloride (NaCl)	AppliChem, Darmstadt
Sodium Citrate	Carl Roth, Karlsruhe
Sodium Dodecyl Sulfate (SDS)	Serva, Heidelberg
Sodium Pyruvate	Gibco, Eggenstein
StemPro® Accutase	Gibco, Eggenstein
Stop solution	Sigma, Deisenhofen
Streptomycin	PAN, Aidenbach
Talarozole (R115866)	Active Biochemicals, HongKong, China
Tris	AppliChem, Darmstadt
Triton X-100	Serva, Heidelberg
Trypan Blue Solution (0.4 %)	Sigma, Deisenhofen
Trypsin/EDTA	Invitrogen, Karlsruhe

Trypton	Carl Roth, Karlsruhe
Tween® 20	Carl Roth, Karlsruhe
Vectashield with DAPI	Vector Laboratories, Burlingame, USA
X-Gal	AppliChem, Darmstadt
Xyline	Carl Roth, Karlsruhe
Y-27632 (ROCK-Inhibitor)	Miltenyi Biotec, Bergisch Gladbach
Yeast extract	Carl Roth, Karlsruhe

2.1.2 General equipment

Equipment	Manufacturer
Cover slides	Thermo Scientific, Schwerte
Cuvette, 10 mm	Hartenstein, Würzburg
E-cups	Sarstedt, Nümbrecht
Gloves	Rösner-Mautby Meditrade, Kiefersfelden
PCR cups	Invitrogen, Karlsruhe
Petri dishes	Sarstedt, Nümbrecht
Pipette tips	Eppendorf, Hamburg
qRT-PCR plates	4titude, Berlin
qRT-PCR Adhesive Clear Seals	4titude, Berlin
Serological pipettes	
2 ml, 5 ml and 10 ml	Sarstedt, Nümbrecht
25 ml	Greiner Bio-one, Frickenhausen
Superfrost® Plus slides	Thermo Scientific, Schwerte
Transfer pipettes	Sarstedt, Nümbrecht
Tubes, 13 mL	Sarstedt, Nümbrecht
Tubes, 15 mL	Greiner Bio-one, Frickenhausen
Tubes, 50 mL	Greiner Bio-one, Frickenhausen

2.1.3 Equipment for Cell Culture

Equipment	Manufacturer
Cell culture flasks	
25 cm^2, 75 cm^2 with filter	Sarstedt, Nümbrecht
75 cm^2 without filter	Greiner CELLSTAR®, Frickenhausen
300 cm^2	Techno Plastic Products, Switzerland
Cell culture plates	

96-well, 24-well	Greiner Bio-one, Frickenhausen
12-well	Greiner CELLSTAR®, Frickenhausen
6-well	Sarstedt, Nümbrecht
5 cm, 10 cm	Greiner Bio-one, Frickenhausen
Cell scraper	Sarstedt, Nümbrecht
Cell strainer (40 µm)	BD Biosciences, Erembodegem, Belgium
Centrifuge: Biofuge™ Primo	Heraeus, Hanau
Counting chambers, bright-lined (Neubauer)	Sarstedt, Nümbrecht
Cryovials	Greiner Bio-one, Frickenhausen
CultureSlides (2-well, 4-well, 8-well)	BD Biosciences, Erembodegem, Belgium
Disposable syringes	BD Biosciences, Erembodegem, Belgium
Filter (Ø 0.20 µm, Ø 0.45 µm)	Th. Geyer, Renningen
Incubator (normoxia, hypoxia)	Sanyo, Munich
Inverted Routine Microscope *Primovert*	Zeiss, Göttingen
MACS® MS Columns	Miltenyi Biotec, Bergisch Gladbach
MACS™ Separator	Miltenyi Biotec, Bergisch Gladbach
Media	
DMEM	Gibco, Eggenstein
DMEM/F12	Gibco, Eggenstein
EMEM	ATCC, Manassas, USA
Knockout™ DMEM	Gibco, Eggenstein
KnockOut™ Serum Replacement	Gibco, Eggenstein
M2	Sigma-Aldrich, Deisenhofen
MEM	Gibco, Eggenstein
Opti-MEM® I + GlutaMAX TM I	Gibco, Eggenstein
Opti-MEM® Reduced Serum Medium	Gibco, Eggenstein
StemPro®-34 SFM Complete Medium	Gibco, Eggenstein
Mr. Frosty™ Freezing Container	Thermo Fisher Scientific, Hennef
Pasteur pipettes	Greiner Bio-One GmbH, Frickenhausen
Sterile bench: HERAsafe®	Sarstedt, Nümbrecht

2.1.4 Technical equipment

Equipment	Manufacturer
7900HT Fast Real-Time PCR System	Applied Biosystems Deutschland
Accu Jet	Brand, Wertheim
Autoclave	Webeco, Bad Schwartau
Automatic Processor Curix 60	AGFA HealthCare, Bonn
Biophotometer	Eppendorf, Hamburg
Centrifuges	
Centrifuge 5415 D	Eppendorf, Hamburg
Heraeus Fresco 21 microcentrifuge	Heraeus, Hanau
Heraeus Megafuge 16R	Heraeus, Hanau
Heraeus Megafuge X3R	Heraeus, Hanau
Heraeus Pico microcentrifuge	Heraeus, Hanau
Genetic analyzer ABI3500 XL	Applied Biosystems, Darmstadt
Incubators	
for bacteria (37°C)	Memmert, Schwabach
for paraffin (60°C)	MWG Biotech, Ebersbach
Magnetic stirrer	IKA, Staufen
Microscopes	
Confocal laser scanning microscope	Olympus, Hamburg
Fluorescence microscope BX60	Olympus, Hamburg
Inverse microscope IX71	Olympus, Hamburg
Nanodrop 2000	Thermo Scientific, Schwerte
Oven	Memmert, Schwabach
pH Meter	Sartorius, Göttingen
Pipettes	Gilson
Semi-Dry Blotter	Biometra GmbH, Göttingen
Sonifier	Branson, Dietzenbach
SpeedVac concentrator	Schuett, Göttingen
SynergyMx plate reader	BioTek, Friedrichshall
Thermocycler	
Advanced Primus 96	Peqlab, Erlangen
Thermal cycler 2720	Applied Biosystems Darmstadt
Thermomixer	Eppendorf, Hamburg
UV Solo TS Imaging System	Biometra, Göttingen
Vortexer-Genie® 2	Scientific Industries
Waterbath	Julabo, Seelbach

2.1.5 Sterilization

Solutions and buffers were sterilized for 20 min in the vapour autoclave (Webeco; Bad Schwartau; Germany) at 120°C and 10^5 Pa. Heat sensitive solutions were sterile filtrated using filtration units of 0.2-0.45 µm pore size. Laboratory equipment was either autoclaved as mentioned above or heat sterilized for 8-12 h at 180°C (Memmert; Schwabach, Germany).

2.1.6 Buffers, solutions and reagents

Buffer / Solution / Reagent	Composition
Activin A	10 µg/ml in 0.1% BSA/PBS
Ampicillin Stock Solution	100 mg/ml Ampicillin in ddH_2O
Blocking Solution (Immunoflourescence staining)	0,2% BSA 0.1% Tween20 in PBS
Blocking Solution (Western Blotting)	100 mM Tris-HCl, pH 7.5 0.1 % Tween20 5% milk powder in ddH_2O
Bouin's Solution	Picric acid : Formaldehyde : Acetic acid (15:5:1)
Collagenase, from *Clostridium histolyticum*	1 mg/ml in HBSS
DNaseI, from bovine pancreas	1 U/µl in 0.15 M NaCl
dNTP Solution	10 µl 100 mM dATP 10 µl 100 mM dCTP 10 µl 100 mM dGTP 10 µl 100 mM dTTP 60 µl Ampuwa

Fixative Solution (Chromosomal Analysis)	Methanol : Acetic Acid (3 : 1)
Fixative Solution (EM Analysis)	1% Paraformaldehyde 3% Glutaralaldeyde in 0.1 M Cacodylic Acid
Giemsa Solution	2 ml Giemsa filtered in 75 ml Phosphate Buffer
hbFGF	10 µg/ml in 0.1% BSA/PBS
hGDNF	10 µg/ml in 0.1% BSA/PBS
Hyaluronidase, from bovine testes	50 mg/ml in PBS
Hypotonic Solution (Chromosomal Analysis)	2 g / 500 ml Sodium Citrate 2,96 g / 500 ml Potassium Chloride
IPTG	100 mM in ddH_2O
Kanamycin Stock Solution	25 mg/ml Kanamycin in ddH_2O
Lysis Buffer (for cell culture)	100 mM Tris-HCl, pH 8.5 5 mM EDTA 0.2% SDS 200 mM NaCl 100 µg/ml Proteinase K
Lysis Buffer (for tissue)	50 ml Tris-HCl, pH 8.0 100 mM EDTA 0.5% SDS
MACS Buffer	1:20 MACS BSA Stock Solution in autoMACS Rinsing Solution

mEGF	10 µg/ml in 0.1% BSA/PBS
Phosphate Buffer	5.92 g $(Na_2HPO_4)_2$ H_2O 4.5 g KH_2PO_4 in 1000 ml ddH_2O, pH 6.8
Protein Lysis Buffer (Western Blotting)	10 mM Tris-HCl, pH 8.0 1 mM EDTA 2.5% SDS 1 mM PMSF (in isopropanol) cOmplete Protease Inhibitor Cocktail Tablets (Roche); 1 tablet / 10 ml in ddH_2O
Puromycin	10 mg/ml in ddH_2O
Talarozole (R115866; Cyp26 inhibitor)	1 mM in DMSO
TBE (Tris-Borate-EDTA, 5x)	450 mM Tris-HCl 450 mM Boric Acid 10 mM EDTA, pH 8.5
Transfer Buffer (Western Blotting, 10x)	25 mM Tris-HCl, pH 8.3 150 mM Glycin 20% Methanol
Washing Solution (EM Analysis)	0.1 M Cacodylic acid 0.1 M Saccharose in ddH_2O
Washing Solution (Western Blotting)	100 mM Tris-HCl, pH 7.5 0.1 % Tween20 2% milk powder in ddH_2O

X-Gal Solution	2% X-Gal in Dimethylformamide
Y-27632	10 mM in DMSO

2.1.7 Ready-to-use reaction systems

Reaction System	Manufacturer
BigDye® Terminator Sequencing Kit	Invitrogen, Karlsruhe
ECL Prime Western Blotting Detection Reagent	GE Healthcare, Munich
Leukocyte Alkaline Phosphatase Kit	Sigma-Aldrich, Deisenhofen
Mini / Midi Plasmid Kit	Qiagen, Hilden
NucleoSpin® RNA II	Macherey & Nagel, Düren
PE Annexin V Apoptosis Detection Kit I	BD Biosciences, Heidelberg
QIAquick PCR Purification Kit	Qiagen, Hilden
Stemgent® mRNA Reprogramming System	Stemgent, Cambridge, USA
VECTASTAIN Universal Quick Kit, R.T.U.	Vector Laboratories, Burlingame, USA
Wizard® SV Gel and PCR Clean-Up System	Promega, Wisconsin, USA

2.1.8 Plasmids and Vectors

Plasmid / Vector	Source
Integrin β1-tev-BHCys	Addgene
pEBTetD	Bach et al., 2007
pGEM®-T Easy Vector System	Promega, Wisconsin, USA
phEF1α-mStra8	Dr. Jessica Nolte-Kaitschick, Institute of Human Genetics, Göttingen

2.1.9 Bacteria

Bacteria	Manufacturer
Escherichia coli DH5α	Invitrogen, Karlsruhe

2.1.9.1 Media and plates for bacteria culture

Medium / Plate	Composition
LB Medium	10 g / 1l Trypton 10 g / 1l NaCl 5 g / 1l Yeast Extract
LB/Ampicillin	0.1% mg/ml Ampicillin in LB Medium
LB/Kanamycin	0.05% mg/ml Kanamycin in LB Medium
LB/OJA	0.4% X-Gal 0.1% mg/ml Ampicillin 100 µM IPTG in LB Medium
LB Plates	10 g / 1l Trypton 10 g / 1l NaCl 5 g / 1l Yeast Extract 15 g / 1l Agar

2.1.10 Mouse strains

The used mice were obtained from the animal house of the Institute of Human Genetics (Göttingen) or the Max-Planck-Institute for Experimental Medicine (Göttingen). Mice were kept at a 12 hours light/dark cycle at 22°C and 55 ± 5% relative humidity and fed with animal food provided by the company Ssniff (Soest). Mice were sacrificed either by CO_2 asphyxiation or cranial dislocation and all experiments were conducted according to the European and German protection of animals act.

2.1.11 Eukaryotic cell lines

Cell line	Description	Source	Culture medium
NIH-3T3	Mouse Embryonic Fibroblasts	ATCC®	FB
BJ	Human Fibroblasts	Stemgent®	BJ *or* FB
CRL-2691	Human Fibroblasts	ATCC®	FB
ES-RI	129SV mouse ES cell line	Nagy et al., 1993	ESC
hiPSCs (iSHFs)	human induced Pluripotent Stem Cells	generated in presented thesis (3.2)	hiPSC
HTF	Human Testicular Feeder	generated in presented thesis (3.3.1)	FB
iPS WT	mouse wild type induced Pluripotent Stem Cells	Wernig et al., 2007	ESC
MEF	Murine Embryonic Fibroblasts	routinely generated in the laboratory (2.2.4.3.3)	FB
NuFF	Neonatal Human Foreskin Fibroblasts	GlobalStem®	NuFF
SHF	Scrotal Human Fibroblasts	generated in presented thesis (3.2.1)	FB
SSC 12	Transgenic ES-RI cell line, stable transfected with Stra8-EGFP and Prm1-DsRed	Nayernia et al., 2006	ESC + 100 µg/ml G418
SSC 12/11; SSC 12/13	Stra8 overexpressing cell lines; SSC 12, stable transfected with phEF1α-puro-mStra8 for stable Stra8 overexpression	generated in presented thesis (3.4.3)	ESC + 100 µg/ml G418 + 0.75 µg puromycin

2.1.11.1 Media for cell culture

Medium	Composition
BJ medium	EMEM 10% FCS 1 mM P/S
ESC medium	DMEM 20% FCS 0.1 mM NEAA 1 mM Sodium Pyruvate 0.2 mM 2-ME 2 mM L-Glutamine 1 mM P/S 1000 U/ml LIF
Fibroblast medium (FB medium)	DMEM 10% FCS 2 mM L-Glutamine 1 mM Penicillin/Streptomycin
hiPSC-M3	DMEM/F12 15% KO™-SR 5x NEAA 5x GlutaMAX™ 1.25% P/S 25 µM 2-ME 10 ng/ml hbFGF
hiPSC medium (hiPSC-M4)	DMEM/F12 15% KO™-SR 5x NEAA 5x GlutaMAX™ 1.25% P/S 25 µM 2-ME

	5 ng/ml hbFGF
KSOM medium	80 ml KO™-DMEM
	20 ml KO™-SR
	0.1 mM NEAA
	1 mM Sodium Pyruvate
	0.2 mM 2-ME
	2 mM L-Glutamine
	1 mM P/S
	1000 U/ml LIF
NuFF medium	DMEM
	10% FCS
	1x GlutaMAX
	1 mM Penicillin/Streptomycin
StemPro+3 medium	StemPro®-34 + supplement
(Smorag et al., 2012)	1% FCS
	0.1 mM NEAA
	2 mM L-Glutamine
	1 mM Sodium Pyruvate
	1 mM Penicillin/Streptomycin
	100 µM 2-ME
	1000 U/ml LIF
	50 µg/ml Vitamin C
	10 ng/ml hbFGF
	20 ng/ml mEGF
	10 ng/ml hGDNF

2.1.11.2 Freezing media

Freezing medium	Composition
ESC freezing medium	20% DMSO 30% culture medium 50% FCS
Fibroblast freezing medium	20% DMSO 30% culture medium 50% FCS
hiPSC freezing medium	CryoStem™ Freezing Medium *or* 90% KO™-SR 10% DMSO 10 µM Y-27632

2.1.12 Antibodies

2.1.12.1 Primary antibodies

antigen	company	code	species	clonality	dilution
Actin/Sma	Sigma-Aldrich, Deisenhofen	A2547	mouse	monoclonal	1:1000
α-Tubulin	Sigma-Aldrich, Deisenhofen	T5168	mouse	monoclonal	1:10.000
BrdU	Abcam, Cambridge, UK	ab6326	rat	monoclonal	1:100
Dazl	Abcam, Cambridge, UK	ab34139	rabbit	polyclonal	1:300
Ddx4	Abcam, Cambridge, UK	ab13840	rabbit	polyclonal	1:100
IgG	Sigma-Aldrich, Deisenhofen	I8140	rabbit	-	according to 1st ab
α6-Integrin, biotin conj.	Abcam, Cambridge, UK	ab28080	rat	monoclonal	1:50
α6-Integrin	Santa Cruz Biotechnology, Heidelberg	sc-6597	goat	polyclonal	1:15 to 1:25
Oct4	Millipore, Billerica, USA	MAB4419	mouse	monoclonal	1:200 to 1:1000
Plzf	Santa Cruz Biotechnology, Heidelberg	sc-28319	mouse	monoclonal	1:20 to 1:50
Sall4	Abcam, Cambridge, UK	ab29112	rabbit	polyclonal	1:100
Vim	Santa Cruz Biotechnology, Heidelberg	sc-5565	rabbit	polyclonal	1:50
StemLight™ Pluripotency Surface Marker Antibody Kit (Cell Signaling, Danvers, MA, USA)					

2.1.12.2 Secondary antibodies

antibody	company	code
Anti-Biotin MicroBeads	Miltenyi Biotec, Bergisch Gladbach	130-090-485
Anti-mouse IgG, HRP-linked Antibody	Cell Signaling, Danvers, MA, USA	7076
Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling, Danvers, MA, USA	7074
Anti-Rat IgG (whole molecule)–FITC antibody produced in goat	Sigma-Aldrich, Deisenhofen	F6258
Cy3 Streptavidin	BioLegend, San Diego, USA	405215
F(ab')2-Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 488 conjugate	Invitrogen, Karlsruhe	A11017
F(ab')2-Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 488 conjugate	Invitrogen, Karlsruhe	A11070
Goat anti-Mouse IgG (H+L) Secondary Antibody, Cy3 conjugate	Invitrogen, Karlsruhe	A10521
Goat anti-Rabbit IgG (H+L) Secondary Antibody, Cy3 conjugate	Invitrogen, Karlsruhe	A10520
Monoclonal Anti-Biotin–Cy3™ antibody produced in mouse	Sigma-Aldrich, Deisenhofen	C5585

2.1.13 Synthetic oligonucleotides

All synthetic oligonucleotides were purchased from Eurofins MWG Operon, Ebersberg. Primer stocks at a final concentration of 100 pmol/µl dissolved in Ampuwa were stored at -20°C. Primers were used for PCR experiments at a concentration of 10 pmol/µl.

2.1.13.1 Synthetic Oligonucleotides for RT-PCR experiments

name	direction	primer sequence 5' → 3'	fragment size (bp)	annealing (C°)	cycles
mAcr	for	TCC TGA AGG CAA GAT TGA CAC	209	63	33
	rev	GAA TCA AGT GCA AGG CGT TAG			
mDazl	for	GAT CGA ACT GGT GTG TCG AAG	307	33	63
	rev	CTG GTG AAC TTG GAT AAG GAG			
mHPRT	for	CGT CGT GAT TAG CGA TGA TG	322	51	35
	rev	TAT GTC CCC CGT TGA CTG AT			
mOvol1	for	CTG CCA CAT CTG CCA GAA GTC	378	63	33
	rev	GTC TTG CGC AGC AGT GGG CTG			
mPrm1	for	CCC ACA AAA TTC CAC CTG CTC	377	60	33
	rev	CAG GTG GCA TTG TTC CTT AGC			

mStra8	for	TCA CAG CCT CAA AGT GGC AGG	440	64	33
	rev	GCA ACA GAG TGG AGG AGG AGT			
mSycp3	for	GTT GCA GCA GTG GGA ACT GG	390	61	33
	rev	CTA AAG GCA TGC CTC TTA GC			
hTBP	for	AGC CTG CCA CCT TAC GCT CAG	161	60	35
	rev	TGC TGC CTT TGT TGC TCT TCC			
mTP2	for	CGG CCT CAA AGT CAC ACC AGT	255	59	40
	rev	AGT CCG TTT CCG CCT CCT GAC			
mVasa	for	AGC ACA GCC TCT AGT TAA AGT	327	58	30
	rev	ATA TCA GGT TTC ACA CTT ATG T			

2.1.13.2 Synthetic Oligonucleotides for qRT-PCR experiments

name	direction	primer sequence 5' → 3'	fragment size (bp)	annealing (C°)	cycles
mAcr	for	TCC TGA AGG CAA GAT TGA CAC	209	55	40
	rev	GAA TCA AGT GCA AGG CGT TAG			
mFkbp6	for	ACC TGT CCT TTG TGT ACC TG	247	55	40
	rev	CTG TCC ACA TAG TCC CTG TAG			
mGpr125	for	AAC CGT AGA TAC CCA CCA C	233	55	40
	rev	GTA CAA CTC TGT CCC AGT CAG			
mGpx4	for	TGA GTG TGG TTT ACG AAT CCT G	177	55	40
	rev	TGG ACT TTC ATC CAT TTC CAC			
mHPRT	for	AGC CCC AAA ATG GTT AAG GTT GC	222	55	40
	rev	TTG CAG ATT CAA CTT GCG CTC AT			
mMov10l1	for	ATG CTA CAT CTG GAG AGC AC	245	55	40
	rev	GTG CTT TAT CCC AGT TAG CC			
mPrm1	for	CCC ACA AAA TTC CAC CTG CTC	377	55	40
	rev	CAG GTG GCA TTG TTC CTT AGC			
mStra8	for	GAG AAA AAG GCC AGA CTC CTG	229	55	40
	rev	TAG ACA TAT GCT GGG CCT CAC			

2.1.13.3 Synthetic Oligonucleotides for genotyping PCR experiments

name	direction	primer sequence 5' → 3'	fragment size (bp)	annealing (C°)	cycles
Stra8-EGFP	for	CGT AGT GTG CCA AAC TGA TGT GG	779	60	35
	rev	CAG GGT CAG CTT GCC GTA GGT GG			
Prm1-DsRed	for	TCC TGA TGC CAA AGC CCT GCC	539	61	33
	rev	CAC TTG AAG CCC TCG GGG AAG			
phEF1α-puro-mStra8	for	AGG GAG CTC AAA ATG GAG GAC	1050	61	33
	rev	CGG CAG AGA CAA TAG GAA GTG			
pEBTetD-mStra8	for	GTG AAC CGT CAG ATC GCC TG	556	60	35
	rev	GGG GAC TGT CCT GAA GAA AAC			
Sycp3-DsRed, transgene	for	CAGGCTGTTCAGACTGGAATGTG		62°C -1°C 60°C	5 32
	rev	TTGAAGCGCATGAACTCCTTGATGACG	1121, transgene		
	rev	GGACACAGGCTATTTGAGTTAG	856, wild type		
Stra8-EGFP, transgene	for	GATCGAGTGCGGAGAATTAGGGTG		62°C -1°C 60°C	5 32
	rev	ACTCTCGGGCGGCCTTCGTTGTGGGTTT	448, transgene		
	rev	CCTTTGGCTCGCCTCAGGTTTGCA	584, wild type		

2.1.14 Molecular Weight Standards

Application	Standard and Manufacturer
DNA molecular weight standards	GeneRuler 1 kb DNA Ladder (250 to 10,000 bp; Thermo Scientific, Illinois, USA) GeneRuler 100 bp DNA Ladder (100 to 1000 bp; Thermo Scientific, Illinois, USA) DNA molecular weight standards were diluted with stop solution according to manufacturer`s protocol.
Protein molecular weight standards	SeeBlue® Plus2 Pre-stained Protein Standard (Invitrogen, Kalrsruhe)

2.1.15 Databases

Analysis	Database
Analysis of nucleotide sequences	Ensembl v32 http://www.ensembl.org National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov
Sequence alignments	Basic Local Alignment Search Tool (BLAST) provided by NCBI http://blast.ncbi.nlm.nih.gov/Blast.cgi
Analysis of restriction sites	DNA restriction mapper tool NEB Cutter 2.0 http://tools.neb.com/NEBcutter2/index.php
Designing of plasmid maps	http://www.bioinformatics.org/savvy/
Designing of primers	Primer3 http://bioinfo.ut.ee/primer3-0.4.0/

2.2 Methods

2.2.1 Molecular biology methods

2.2.1.1 Isolation of nucleic acids

2.2.1.1.1 Isolation of genomic DNA from tissue (Laird et al., 1991)

For isolation of genomic DNA (gDNA) from tissue, the tissue was mixed with 700 µl tissue lysis buffer (2.1.6) and 40 µl proteinase K (10 µg/µl) and incubated at 55°C on a thermo mixer overnight. After centrifugation at 13.000 x rpm for 10 min, the supernatant was transferred into a new e-cup, mixed with 700 µl phenol-chloroform (1:1) and centrifuged at 13.000 x rpm for 10 min. The upper aqueous phase was transferred into a new e-cup and mixed with 700 µl chloroform. After centrifugation at 13.000 x rpm for 10 min, the upper aqueous phase was transferred into a new e-cup, mixed with 700 µl isopropanol and centrifuged at 13.000 x rpm for 15 min. The supernatant was discarded and the DNA pellet was incubated with 1 ml 70% EtOH for 10 min at room temperature. The mixture was centrifuged at 13.000 x rpm for 5 min and the supernatant was discarded. The DNA pellet was dissolved in 100 µl Ampuwa and incubated for 10-20 min at 60°C on a thermo block with opened lid and further stored at 4°C.

2.2.1.1.2 Isolation of genomic DNA from cultured cells

For isolation of genomic DNA from cultured cells, cells were seeded on gelatine coated 24-well plates. When the cells reached 100% confluence, the cells were washed once with PBS and cultured with 500 µl cell lysis buffer (2.1.6) and 5 µl proteinase K (10 µg/µl) at 37°C and 5% CO_2 overnight. The next day the cell suspension was transferred into a sterile e-cup and mixed with 500 µl isopropanol. After centrifugation at 13.000 x rpm for 15 min the supernatant was discarded and the DNA pellet was incubated with 1 ml EtOH for 10 min at room temperature, followed by a centrifugation at 13.000 x rpm for 5 min. The supernatant was carefully discarded and the DNA pellet was resuspended in 40-80 µl Ampuwa. After incubation for 10-20 min at 60°C on a thermo block with opened lid, the isolated DNA was stored at 4°C.

2.2.1.1.3 Mini-preparation of plasmid DNA

The isolation of plasmid DNA from bacteria in an analytic scale was performed with the ready-to-use reaction system for Mini Plasmid Preparation (Qiagen). After inoculation of 5 ml LB medium containing the appropriate antibiotic with a single colony or a sample from a glycerol stock, the culture was incubated for 12-16 h at 37°C with shaking at 150 rpm. For preparation of a glycerol stock 700 µl of the overnight culture were mixed with 300 µl sterile glycerol and immediately stored at -80°C. The rest of the overnight culture was centrifuged at 4000 x g for 10 min at 4°C and the pellet was resuspended in150 µl buffer P1. After addition of 150 µl buffer P2, the solution was mixed by inverting, incubated for 5 min at room temperature and neutralized with 150 µl neutralization buffer P3. The solution was mixed vigorously before centrifugation at 13.000 x g for 15 min at room temperature. The supernatant was transferred to a new e-cup. DNA was precipitated with 1 volume of isopropanol and centrifuged at 13.000 x g for 20 min at room temperature. The pellet was washed with 1 ml 70% EtOH, resuspended in 30 µl Ampuwa and incubated for 15 min with shaking at 55°C. The isolated DNA was stored at -20°C.

2.2.1.1.4 Endotoxin-free midi-preparation of plasmid DNA

The isolation of plasmid DNA from bacteria in a large scale was performed with the ready-to-use reaction system for Midi Plasmid Preparation (Qiagen). After inoculation of 100 ml LB medium containing the appropriate antibiotic with a single colony or a sample from a glycerol stock, the culture was incubated for 12-16 h at 37°C with shaking at 150 rpm. After centrifugation for 15 min at 4000 x g and 4°C, the cell pellet was resuspended in 4 ml buffer P1, further mixed with 4 ml buffer P2 thoroughly by inverting up to 6 times and incubated at room temperature for 5 min. Then 4 ml buffer P3 were added and the suspension was mixed

thoroughly by inverting up to 6 times. After incubation on ice for 10 min and centrifugation for 10 min at 4000 x g and 4°C, the supernatant was applied to an equilibrated QIAGEN column. Subsequently the QIAGEN column was washed two times with 10 ml buffer QC. The DNA was eluted with 4 ml buffer QF and further precipitated by adding 6 ml isopropanol. After thoroughly mixing, the suspension was centrifuged for 60 min at 4000 x g and 4°C and the supernatant was carefully discarded. The DNA pellet was washed once with 2 ml 70% EtOH and centrifuged for 10 min at 4000 x g and 4°C. The airdried pellet was resolved in an appropriate volume of Ampuwa and stored at -20°C.

2.2.1.1.5 Isolation of total RNA from cultured cells

The ready to use reaction kit “NucleoSpin® RNA II” (Macherey & Nagel) was used for total RNA isolation from cultured cells. Fresh or at -80°C stored cell pellets were mixed with lysis buffer provided by the kit, which immediately inactivates RNases. The further RNA isolation was performed according to manufacturer’s protocol. RNA was eluted with supplied RNAse free H_2O, which was preheated at 37°C before adding to the column for RNA elution. After incubation for 1 min at room temperature the column was centrifuged. The elution was put back on the column for a second centrifugation. The finally eluted RNA was stored at -80°C.

2.2.1.1.6 Determination of nucleic acid concentration

Nucleic acid concentrations and purities were determined by measuring the absorption at 260 nm using the Thermo Scientific NanoDrop Spectrophotometer. Protein and salt contaminations were estimated with the absorbance ratios of $A_{260/280}$ and $A_{260/230}$, respectively.

2.2.1.2 Polymerase Chain Reactions

2.2.1.2.1 Amplification of DNA

The Polymerase Chain Reaction (PCR) represents an *in vitro* amplification of specific DNA fragments (Saiki et al., 1988). A typical PCR program for the exponential multiplication of selected DNA region consists of the denaturation of DNA strands, the annealing of specific primers and the synthesis of DNA. The PLATINUM® Taq DNA Polymerase (Invitrogen, Karlsruhe) as well as the IMMOLASE™DNA Polymerase (Bioline, Luckenwalde) with the supplied reagents were used for DNA amplification.

The following reagents were pipetted into a PCR reaction tube:

components	final concentration
DNA	10-50 ng
primer forward	10 pmol
primer reverse	10 pmol
dNTP mix	0.2 mM
Mg_2Cl	1.5 mM
PCR buffer	1x
polymerase	1 U
Ampuwa	ad 25-50 µl

The PCR reaction was performed in a thermocycler with the following parameters:

step	temperature	time	repeats
predenaturation	94-95°C	5 min	1
denaturation	94-95°C	30 sec	2.1.13
annealing	2.1.13	45 sec	
elongation	72°C	30 sec	
final elongation	72°C	5 min	1
storage	8°C	∞	1

The annealing temperature and cycle numbers were individually adapted to the used primer pairs. 2.1.13 provides an overview of the used primers and their annealing temperature and cycle numbers. Further analysis of synthesised DNA fragments was performed using agarose gel electrophoresis of DNA (2.2.1.3).

2.2.1.2.2 Reverse transcription

Prior to transcription of mRNA into cDNA using a reverse transcriptase, a DNAse digestion (Sigma-Aldrich, Deisenhofen) was performed in order to remove remaining DNA. 5 µg RNA in a total volume of 8 µl Ampuwa were treated with 1 µl DNaseI and carefully mixed with 1 µl 10x buffer by pipetting. After 15 min at room temperature, the reaction was stopped by the addition of 1 µl stop solution and further incubation for 10 min at 70°C. Subsequently the cDNA synthesis was directly performed using SuperScript® II Reverse Transcriptase (Invitrogen, Karlsruhe) or Tetro Reverse Transcriptase (Bioline, Luckenwalde), respectively, or the DNaseI digested RNA was stored at -80°C.

For the use of SuperScript® II Reverse Transcriptase, DNaseI digested RNA was mixed with 1 µl oligo(dT)-primer and 1 µl 10 mM dNTPs and incubated for 5 min at 65°C. Further 4 µl 5x First-Strand buffer and 2 µl 0.1 M DTT were added and the reagents were carefully mixed by pipetting. After an incubation of 2 min at 42°C, 1 µl SuperScript® II Reverse Transcriptase were added and carefully mixed. The mixture was incubated for 50 min at 42°C for the final reverse transcription, which was subsequently inactivated at 70°C for 15 min.

For cDNA synthesis using Tetro Reverse Transcriptase, DNaseI digested RNA was mixed with 1 µl oligo(dT)-primer and incubated for 5 min at 70°C. Afterwards 1 µl 10 mM dNTPs, 1 µl Tetro Reverse Transcriptase, 2.75 µl Ampuwa and 4 µl 5x buffer were added. The sample was mixed carefully by pipetting and incubated at 42°C for 30 min. The reaction was stopped by incubation at 95°C for 5 min.

The successful cDNA amplification was verified by PCR analysis using specific primers for the ubiquitously expressed housekeeper gene mouse HPRT (Hypoxanthine-guanine phosphoribosyltransferase) or human TBP (TATA-Binding Protein). The cDNA was stored at -20°C.

2.2.1.2.3 Quantitative Real-Time PCR

A quantitative Real-Time PCR (qRT-PCR) analysis is used to quantify the relative mRNA expression levels of a certain gene of interest by using fluorescent labelled dNTPs for PCR reactions. The fluorescent intensity of labelled double stranded DNA is measured after each PCR cycle using the ABI Prism 7900T Sequence Detection System. A threshold value of PCR cycles during the exponential phase is determined in a Ct value, which determines the PCR cycle with the best PCR conditions and is used for further quantification of relative mRNA expression. This evaluation was performed with the SDS 2.2.1 software (Applied Biosystems) using the ΔΔ-Ct method. The mRNA expression of the mouse housekeeping gene HPRT was used for normalization.

qRT-PCR experiments were carried out in 10 µl reactions with 1 µl of 1:20 in Ampuwa diluted cDNA, 1 µl of each primer (3 µM; 0), 2 µl Ampuwa and 5 µl KAPA™ SYBR® FAST (Peqlab) or SYBR® Green PCR Master Mix (Qiagen) on 7900HT Fast Real-Time PCR System using the following PCR parameters:

step	temperature	time	repeats
	50°C	2 min	1
predenaturation	95°C	15 min	1
denaturation	95°C	15 sec	40
annealing	55°C	30 sec	
elongation	72°C	30 sec	
dissociation curve	95°C	15 sec	1
	60°C	15 sec	1
	95°C	15 sec	1

All reactions were performed in triplicate on one 384-well plate and at least repeated twice as technical replicates.

2.2.1.2.4 Sequencing analysis (Sanger et al., 1977)

Based on the chain-terminating Sanger sequencing this analysis was performed in order to determine the nucleotide sequence of a DNA fragment of interest.

For the sequencing analysis using the BigDye® Terminator Sequencing Kit (Invitrogen, Karlsruhe) 1 µl DNA, 1 µl forward or reverse primer, 1 µl BigDye® Terminator Mix, 2 µl BigDye® Terminator buffer and 5 µl Ampuwa were pipetted into a PCR reaction tube and a PCR reaction was performed in a thermocycler with the following parameters:

step	temperature	time	repeats
predenaturation	95°C	1 min	1
denaturation	95°C	30 sec	30
annealing	60°C	2.30 min	
final elongation	60°C	5 min	1
storage	8°C	∞	1

After the PCR reaction each sample was mixed with 10 µl Ampuwa. The final sequencing analysis using ABI3500 XL was performed in house sequencing service (Institute of Human Genetics, Göttingen).

2.2.1.3 Agarose gel electrophoresis of DNA

Standard agarose gel electrophoresis is used for the separation of DNA fragments after PCR reactions, restriction digestions or ligations. The DNA fragments are separated within an electric field according to their length.

Depending on the expected separated DNA fragments, a 0.5-2% agarose gel was prepared. The appropriate amount of agarose was dissolved in 100 ml of 0.5x Tris-Borate-EDTA (TBE) buffer (2.1.6) by boiling in the microwave. In order to visualize later the DNA under UV light, ethidium bromide was added before the mixture was poured into a horizontal gel chamber. After polymerization, samples were loaded to the gel next to a DNA length standard (2.1.14), which is needed for the determination of DNA fragment lengths. The application of an electric field (70-120 V) yielded in the separation of DNA fragments, which were visualized using the UVsolo TS Imaging System (Biometra, Göttingen).

2.2.1.3.1 Purification of DNA fragments from agarose gels

The purification of DNA fragments from agarose gels was performed using the ready to use reaction systems QIAquick PCR Purification Kit (Qiagen, Hilden) or Wizard® SV Gel and PCR Clean-Up System (Promega, Wisconsin, USA). After separation of nucleic acids to identify DNA-fragments of interest, the DNA fragment was excised from the gel with a sterile scalpel on an UVtranslluminator and transferred to an e-cup and weighed. The appropriate buffer was added according to the weight of the excised fragment. The further purification was performed according to manufacturer's protocol. The final eluted DNA was stored at -20°C.

2.2.1.4 Cloning techniques

2.2.1.4.1 Restriction of plasmid DNA

Enzymatic cleavage of plasmid DNA was performed with restriction endonucleases derived from different companies including Invitrogen and New England BioLabs. Generally, 1 µg DNA were incubated with 5-10 units of enzyme in 1x appropriate buffer and 1 µg BSA at 37°C for at least 2 h, preferentially over night, in a total volume of 10-100 µl. A digestion with two different enzymes was performed using a buffer compatible for both. The digestion of DNA was checked by agarose gel electrophoresis of DNA (2.2.1.3).

2.2.1.4.2 Ligation of PCR products

For ligation of a DNA fragment- the insert- into a target vector both were prepared using appropriate restriction enzymes. The insert was amplified using a PCR reaction with primers providing the essential restriction sites or it was cut out from another vector. The target vector was linearized by digestion using the appropriate restriction enzymes. In a total reaction volume of 10 µl, 1 µl linearized vector, 2.5 µl insert DNA, 5 µl ligase buffer and 0.5 µl Ampuwa were mixed and incubated at 65°C for 5 min. After 5 min on ice, 1 µl ligase (Promega, Wisconsin, USA) was added and the reaction was incubated at 16°C overnight.

2.2.1.4.3 Subcloning of PCR products using TA cloning

The pGEM®-T Easy Vector System (Promega, Wisconsin, USA) provide a convenient system for the TA cloning of PCR products, which are amplified using polymerases providing a terminal transferase activity. Due to this activity a single deoxyadenosine (A) is added to the 3' end of the PCR product, which is recognized by the complimentary 3´ deoxythymidine (T) residues of the linearized pGEM®-T Easy Vector and results in an efficient ligation of insert and vector.

The ligation reaction was performed in a total volume of 10 µl containing an insert to vector ratio of 3:1. For this reaction 50 ng pGEM®-T Easy, 150 ng insert DNA, 1 µl T4 DNA ligase 10x buffer and 1 µl T4 ligase were mixed with an appropriate amount of Ampuwa and incubated at 16°C overnight.

2.2.1.4.4 Transformation of competent cells with plasmid DNA (modified to Hanahan, 1983)

For transformation of competent bacteria 50 µl *E.coli* DH5α competent cells and plasmid DNA were placed on ice for 10 min. The cells were carefully mixed by pipetting. Further 5 µl plasmid DNA were added and the mixture was incubated for 15-20 min on ice. After a heat shock at 42°C for 1 min, the mixture was placed on ice for 5 min. Further 950 µl SOC outgrowth medium were added and the bacteria were slightly shaking incubated for 1 h at 37°C on a thermo mixer. The transformed bacteria were seeded on a LB agar plate providing the appropriate antibiotic for bacteria selection and incubated at 37°C overnight.

2.2.2 Protein manipulation methods

2.2.2.1 Protein isolation from eukaryotic cells

For protein isolation cells were harvested from 6-well plates. After washing once with PBS, according to the size of the cell pellet 200-500 µl protein lysis buffer (2.1.6) were added. The

cell suspension was incubated on ice for 1 h. After a brief sonification, the mixture was centrifuged for 20 min at 13.000 rpm and 4°C to pellet cell debris. The supernatant containing the proteins was transferred to a new e-cup and stored at -20°C, while it was kept at -80°C for long term storage.

2.2.2.2 Measurement of protein concentration (Bradford, 1976)

Protein concentrations were determined according to the method of Bradford (1976), which bases on the usage of the Coomassie Brilliant Blue dye and its property to bind unspecifically to proteins in acidic solution. This is accompanied by a shift of its absorption maximum from 465 nm to 595 nm. Standard protein concentrations using bovine serum albumine are used to generate a calibration curve in order to calculate the measured protein concentrations.

For measurement of protein concentration 20 ml Roti® Nanoquant (Carl Roth, Karlsruhe) were mixed with 80 ml ddH_2O. After proteins were diluted 1:100, triplicates of 50 µl of this dilution were mixed with 200 µl diluted Roti® Nanoquant. After incubation for 5 min at room temperature, the absorptions were measured using the SynergyMx plate reader (BioTek, Friedrichshall) and calculated with the appropriate Gene5 software.

2.2.2.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (Laemmli, 1970)

Separation of proteins was performed using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), which bases on the use of gels and buffers with an operating pH of 7.0 yielding in an increase of stability of proteins and gels and consequently in a better result of electrophoresis.

Protein samples were prepared with 0.4 volume of 4x NuPAGE® LDS sample preparation buffer (Invitrogen, Karlsruhe) in a total volume of 30 µl, incubated at 70°C for 10 min and subsequently placed on ice for 5 min. After centrifugation for 10 min at 13.000 rpm and room temperature, samples were loaded onto a gradient gel (NuPAGE™ 4-12% Bis-Tris Gel, Invitrogen, Karlsruhe) next to a prestained molecular weight protein standard (2.1.14) to determine the size of separated proteins. Depending on the molecular weight of the proteins, electrophoresis was performed in 1x NuPAGE® MOPS SDS Running Buffer (Invitrogen, Karlsruhe) for 15 min at 80 V and further at 120-150 V.

2.2.2.4 Western Blotting (Gershoni and Palade, 1983)

Transfer of proteins from a SDS-PAGE to a nitrocellulose membrane (Hybond™-C Extra, GE Healthcare, Freiburg) was performed with the semi-dry technique. One membrane and six

sheets of Whatman GB003 filter paper (Schleicher & Schull, Dassel) were cut to the size of the polyacrylamide gel. The membrane was activated by rinsing in 100% methanol for 5-10 sec and equilibrated in transfer buffer (2.1.6) for 10 min. Whatman GB003 filter papers were placed in transfer buffer. The components were placed on the anode of the semi-dry electro blotter (Biometra, Göttingen) according to a sandwich model: three sheets of Whatman paper, membrane, polyacrylamide gel and three sheets of Whatman paper. The protein transfer was performed at 150 mA for 15 min and further at 250 mA for 1-2 h.

2.2.2.5 Staining of polyacrylamide gels

After semi-dry blotting, the protein transfer efficiency onto the nitrocellulose membrane was checked by staining of the polyacrylamide gel with Coomassie-Brilliant Blue R-250 for 10 min at room temperature. After this incubation the gel was destained in ddH_2O until the desired staining of proteins was visible.

2.2.2.6 Protein detection on nitrocellulose membranes using antibodies

After protein transfer onto a nitrocellulose membrane, unspecific binding sites were blocked by incubating the membrane with blocking solution containing 5% milk powder (2.1.6) for at least 1 h at room temperature followed by incubation with the primary antibody (2.1.12.1) diluted in washing buffer (2.1.6) overnight at 4°C. Next day the membrane was extensively washed three times for 20 min at 4°C with washing buffer to remove unbound primary antibody and further incubated with the appropriate secondary antibody conjugated with horseradish peroxidase (HRP; 2.1.12.2) diluted in washing buffer for approximately 2 h at room temperature. The membrane was washed three times for 20 min at 4°C with washing buffer and 5 min in PBS. The ECL Prime Western Blotting Detection Reagent (GE Healthcare, Freiburg) was used for the detection of chemi-luminescent signals. The detection solution was prepared according to manufacturer's protocol protected from light. The membrane was placed on a plastic film, covered with prepared detection solution, enclosed in the plastic film and placed in a film cassette. After an incubation of 3 min protected from light, a X-ray film was placed on the membrane in a dark room and the film cassette was closed. Depending on the intensity of the signal, the incubation varied from seconds from minutes followed by the development using the Automatic Processor Curix 60 (AGFA HealthCare, Bonn).

After development, the bound antibodies could be removed by an incubation of the membrane with 0.1 M glycine solution for 1 h at room temperature and the membrane could be used for a further protein detection using antibodies.

2.2.3 Histological methods

2.2.3.1 Fixation of cells on slides

For histological analyses cells were grown on BD Falcon™ CultureSlides providing two, four or eight chambers. Before seeding of cells, slides were coated with gelatine (2.2.4.1.1) or MEF feeder layer (2.2.4.2.1) depending on the cultured cell type.

Before further histological analysis, cells were washed three times with PBS and fixed with 4% PFA for 10 min at 4°C. Cells were carefully washed three times with PBS and further treated according to the intended analysis.

2.2.3.2 Fixation of organs and tissues

Organs prepared from sacrificed mice were fixed in 4% PFA under slightly shaking at 4°C for 2-3 days or in Bouin's solution (2.1.6) without shaking at room temperature for 2-3 days.

Organs fixed in PFA were washed in 70% EtOH overnight at room temperature. Next day the organs were dehydrated using an ethanol series (70% EtOH for 15 min, 80% EtOH for 20 min, 90% EtOH for 30 min, 96% EtOH for 30 min and 100% EtOH for 20 min) at room temperature and were kept slightly shaking in isopropanol overnight at room temperature. Next day organs were incubated in isopropanol:xylene mixtures for each 30 min (75:25, 50:50 and 25:75 isopropanol:xylene) and kept in 100% xylene overnight at room temperature. Next day organs were incubated two times in paraffin at 60°C for 3 h and kept in paraffin at 60°C overnight.

Organs fixed in Bouin's solution were washed under slightly shaking two times in 70% EtOH for 1 h at room temperature. Afterwards, organs were incubated in 80% EtOH containing 1% Ammonia solution under slightly shaking. The solution was changed several times until it was not stained anymore by remaining Bouin's solution. The organs were further incubated in 80% EtOH containing 1% Ammonia solution under slightly shaking overnight at room temperature. Next day organs were incubated two times in 90% EtOH for 1 h at room temperature and kept slightly shaking in 100% EtOH overnight at room temperature. Next day organs were incubated slightly shaking in 100% xylene for 2-2.5 h at room temperature and kept in xylene:paraffin (1:1) overnight at 60°C. Next day the organs were incubated in pure paraffin at 60°C, which was changed several times, and kept in pure paraffin overnight at 60°C.

Finally, paraffin treated organs were embedded in paraffin blocks using the Shandon HistoCentre 2.

2.2.3.3 Preparation of paraffin sections

For further histological analysis the tissue was cut into 3-6 µm sections using the Leica RM 2035 BioCut Microtome. Sections were placed on prewarmed water (58°C) and afterwards on slides, which were kept at 30°C on a heating plate for at least 30 min and further stored at room temperature.

Before further histological analysis sections were incubated three times in 100% xylene for 5 min under slightly shaking in order to remove paraffin, watered using an ethanol series (100% EtOH, 90% EtOH, 80% EtOH and 70% EtOH) at room temperature each for 2 min and rehydrated in ddH_2O for at least 3 min.

2.2.3.4 Hematoxylin-Eosin staining of tissue sections

For Hematoxylin-Eosin (H&E) counterstaining sections were rinsed in ddH_2O for several times and incubated in concentrated Hematoxylin (Carl Roth, Karlsruhe) for 2-3 min. After washing in ddH_2O for 2 min, sections were rinsed with running tap water for at least 10 min. The slides were placed shortly in 0.3% HCl/ 70%EtOH to take out excess Hematoxylin and immediately put in ammonia H_2O (0.25% NH_3) for several times. After rinsing with running tap water, sections were incubated in Eosin (Carl Roth) with freshly added 0.3% $C_2H_4O_2$ (Acetic Acid) for 4-5 min and washed in ddH_2O for several times. Sections were dehydrated through several changes of ethanol, each for 1 min (70%, 80%, 90% and 100%), cleared in xylene for 10 min and mounted with Eukitt® quick-hardening mounting medium (Fluka).

2.2.3.5 Immunohistochemical analysis using VECTASTAIN Universal Quick Kit, R.T.U.

For immunohistochemical analysis sections were shortly washed in 0.1% Tween20/ PBS, boiled in 1% Tween20/ 10 mM EDTA (pH 8) buffer for 6 min and cooled on ice for 10 min. Afterwards sections were washed in 0.1% Tween20/ PBS and further incubated in 30% H_2O_2 and 100% methanol (ratio: 1:10) for 15 min at room temperature to remove any endogenous peroxidase. The following staining was performed with VECTASTAIN Universal Quick Kit, R.T.U. (Vector Laboratories, USA). After blocking with prediluted horse serum provided by the kit for 10 min, the sections were incubated with primary antibody diluted in prediluted horse serum and 0.1% Tween20/ PBS (ratio: 1:1) in a humidity chamber overnight at 4 °C. The next day the sections were incubated for 60-90 min at room temperature, followed by a washing step in 0.1% Tween20/ PBS and incubation with prediluted biotinylated panspecific universal secondary antibody provided by the kit for 10 min at room temperature. After washing in 0.1% Tween20/ PBS, the sections were incubated with ready-to-use streptavidin/peroxidase preformed complex for 5 min at room temperature. The sections

were washed in 0.1% Tween20/ PBS and incubated with DAB (Roche) diluted 1:10 in provided buffer for 2 min until the colour of sections turned to brown. After washing in 0.1% Tween20/ PBS, the sections were counterstained with Hematoxylin QS (Biozol, Eching) and mounted with Roti®Mount Aqua (Carl Roth, Karlsruhe).

2.2.3.6 Immunofluorescence staining

Before immunoflourescence staining, cells were fixed (2.2.3.1) and sections were watered and rehydrated (2.2.3.3), respectively, and further washed three times with PBS. Unspecific binding sites were blocked by incubation with blocking solution (2.1.6) for at least 1 h at room temperature. Cells or sections were incubated with the appropriate primary antibody (2.1.12.1) diluted in PBS in a humidity chamber overnight at 4°C. Next day, cells or sections were washed three times with PBS and incubated with the appropriate secondary antibody (2.1.12.2) diluted in PBS in a humidity chamber for 1.5-2 h at room temperature. Afterwards, cells and sections were washed three times with PBS and shortly air-dried. Cells or sections were mounted with DAPI, sealed with nail polish and stored at 4°C.

2.2.3.7 Electron Microscopy

Analyses using Electron Microscopy (EM) were performed by Prof. Dr. Andreas Meinhardt from the Department of Anatomy & Cell Biology of the Justus Liebig University in Giessen. The cells were fixed as described in (2.2.3.7.1) and send to Giessen for further preparation and EM analysis.

2.2.3.7.1 Fixation of cells for EM analysis

After trypsin/EDTA treatment and centrifugation (2.2.4.3.4), cells were washed once in PBS and incubated in fixation solution (2.1.6) for 8-12 h at 4°C on a rolling platform. Afterwards, cells were washed three times in washing solution (2.1.6) at 4°C and additionally washed two times in PBS. Cells were finally stored in PBS and send to Giessen for EM analysis.

2.2.4 Cell culture methods

2.2.4.1 Coating of culture vessels

2.2.4.1.1 Preparation of gelatine coated culture vessels

Depending on the size of used culture vessels, they were coated with 1-10 ml sterile 0.1% gelatine and incubated for at least 30 min at 37°C and 5% CO_2. Before use the gelatine solution was aspirated and the cells were seeded on the culture vessel.

2.2.4.1.2 Preparation of matrigel coated plates

BD Matrigel™ Basement Membrane Matrix (BD Bioscience, Heidelberg) was used for preparation of matrigel coated plates. Chilled serological pipettes and culture plates were used during working with matrigel. After thawing on ice, aliquots were prepared at 4°C and further stored at -20°C. Before use, one aliquot was thawed on ice and diluted 1:100 in ice cold DMEM/F12. One 6-well vessel was covered with 1 ml diluted matrigel and kept at room temperature for at least 2 h, but preferentially overnight. Before seeding cells, the matrigel solution was carefully removed from the 6-well vessels.

2.2.4.2 Mitomycin C treatments

2.2.4.2.1 Mitomycin C treatment of murine embryonic fibroblasts

Murine embryonic fibroblasts (MEFs, 2.2.4.3.2) were treated with 10 µg/ml mitomycin C diluted from a 50x stock solution in FB medium. After 3 h incubation at 37°C and 5% CO_2, the cells were washed twice with PBS and detached from the culture vessels by trypsin treatment. Because mitomycin C treated cells can not adhere to culture vessels, the cells have to be fixed to the culture vessels by gelatine. Therefore the mitotically inactivated embryonic fibroblasts were seeded on gelatine coated vessels.

2.2.4.2.2 Mitomycin C treatment of neonatal human foreskin fibroblasts

Neonatal Human Foreskin Fibroblast (NuFF) cells were cultured in NuFF medium and passaged to confluence on a total of three 300 cm^2 culture vessels. Cells were treated with 10 µg/ml mitomycin C diluted in NuFF medium. After 3 h incubation at 37°C and 5 % CO_2, the cells were washed twice with PBS and detached from the culture vessels by trypsin treatment. After centrifugation at 1000 x g for 5 min, cells were cryopreserved in 1 ml FB freezing medium at a cell density of 8 x 10^6 cells per stock and stored at -80°C. Cells were thawed according the protocol for thawing of eukaryotic cells (2.2.4.3.5). Because mitomycin C treated cells can not adhere to culture vessels, cells have to be fixed to the culture vessels by gelatine. Therefore the mitotically inactivated NuFF cells were seeded on gelatine coated vessels before their further use.

2.2.4.3 Culture of eukaryotic cells

2.2.4.3.1 Culture of murine embryonic stem cells

In order to keep embryonic stem cells (ESCs) in their undifferentiated state, they were continuously cultured on mitotically inactivated mouse fibroblasts (MEF feeder layer;

2.2.4.2.1) and in ESC medium containing the cytokine LIF (Leukemia Inhibitory Factor; 2.1.11.1).

2.2.4.3.2 Culture of murine embryonic fibroblasts

Feeder cells are indispensable for the culture of ESCs. These are mitotically inactivated murine embryonic fibroblasts (MEFs), which are homogenously seeded on gelatine treated culture vessels and cultured in FB medium (2.1.11.1). A treatment with mitomycin C leads to the non dividing status of these cells (Martin and Evans, 1975).

2.2.4.3.3 Isolation of murine embryonic fibroblasts

Mouse embryos were isolated from gestating mice at stage 13.5-15.5 days post coitum (dpc) and washed in PBS. After opening of the abdomen and removal of organs, the heads were separated and the bodies were shredded. The cell suspension was transferred in a glass bulb with glass pellets and 20 ml Trypsin/EDTA and 1 ml DNAse were added. After 30 min incubation at 37°C with mixing, the cell suspension was centrifuged by 270 x g for 10 min. The supernatant was discarded and then resuspended in FB medium. The cells were seeded in appropriate culture vessels. After approximately 24 h of cultivation the medium was changed to remove not adherent cells. After further cultivation of approximately 3 days, the cells were passaged in a ratio 1:3. The cells were then cryopreserved after 3-5 days in passage No. 1.

2.2.4.3.4 Passaging of eukaryotic cells

Depending on the cell line and cell density, the cells were cultured for 2 to 4 days until they reached confluence for passaging. The cells were washed with PBS and further treated with 1-5 ml trypsin/EDTA, depending on the size of the culture vessel. After 3-5 min at 37°C and 5% CO_2, all cells were detached from the culture vessel, which was controlled unter the light microscope. An equal volume of appropriate culture medium was added to inactivate the trypsin/EDTA treatment. The cell suspension was centrifuged at 1000 x g for 5 min. After the supernatant was discarded, the cell pellet was resuspended in appropriate culture medium (2.1.11.1) and seeded on an appropriate number of culture vessels. After approximately 24 h of cultivation at 37°C and 5% CO_2, the medium was changed to remove not adherent cells.

2.2.4.3.5 Cryopreservation and thawing of eukaryotic cells

Cells were cryopreserved from a confluent culture vessel. After washing with PBS, the cells were treated with 1-5 ml trypsin/EDTA, depending on the size of the culture vessel, and

cultured for 3-5 min at 37°C and 5% CO_2. Under the light microscope, it was controlled that cells were detached from the culture vessel. The effect of the trypsin/EDTA treatment was inactivated by an equal volume of appropriate culture medium and the cell suspension was centrifuged at 1000 x g for 5 min. After the supernatant was discarded, the cell pellet was resuspended in 500 µl appropriate culture medium (2.1.11.1) and subsequently mixed with 500 µl appropriate freezing medium. The mixture was transferred into well labelled cryovials, which were immediately frozen at -80°C. After 24 h at -80°C, the cryovials were placed at -152°C for long term storage.

Because the DMSO contained in the freezing medium is toxic for cells at room temperature, the procedure of thawing has to be done quickly. The cryovial was placed in a water bath at 37°C until the cells were almost completely thawed. The cells were mixed with a bigger volume of appropriate culture medium in order to dilute the DMSO and then centrifuged at 1000 x g for 5 min. After removal of the supernatant, the cells were resuspended in appropriate culture medium and seeded on a culture vessel. After approximately 24 h of cultivation at 37°C and 5% CO_2, the medium was changed to remove not adherent cells.

2.2.4.3.6 Culture of Human Testicular Feeder

Human Testicular Feeder (HTF) cells were derived from MACSortings (2.2.4.7.2) with human testicular biopsies from infertile men. During this procedure the flow through was captured in an e-cup, which was stored on ice, if HTF cells had to be established. The cell suspension was centrifuged at 1000 x rpm for 5 min, resuspended in 2 ml FB medium, seeded on one 6-well vessel coated with 0.1% gelatine and cultured at 37°C and 5% CO_2. During further culture, cells were expanded, routinely passaged (2.2.4.3.4) and cryopreserved using FB freezing medium (2.2.4.3.5).

2.2.4.3.7 Digestion of human testicular material

Testicular biopsies were obtained simultaneously during surgery for testicular sperm extraction (TESE) for the diagnosis of male fertility from patients as previously described (Jezek et al., 1998; Schulze et al., 1999; Feig et al., 2007). TESE was performed by Prof. Schulze at the Department of Andrology, University Hospital Hamburg-Eppendorf in Hamburg. Ethic Committee Approval to Prof. Schulze was obtained (OB/X/2000) and the study was performed according to the ethical guide lines of the current Declaration of Helsinki.

Testicular tissue was kept in medium at ~32°C during the transport to the Institute of Human Genetics in Göttingen. Upon arrival testicular biopsies underwent a two-step enzymatic digestion procedure. Testicular biopsy was placed in culture plates containing HBSS and

was washed in HBSS by using forceps. Further it was transferred into culture plates containing HBSS supplemented with 1 mg/ml collagenase and 2 U/ml DNaseI. Tissue was mechanically dissociated into small pieces by using forceps, transferred into a 15 ml falcon tube and digested for 15 min at 37°C. During the incubation time testicular pieces were occasionally mixed by shaking. After centrifugation at 1000 x rpm for 5 min, the supernatant was carefully removed and the pellet was resuspended in HBSS supplemented with 1 mg/ml collagenase, 2 U/ml DNaseI and 0.5 mg/ml hyaluronidase. The suspension was carefully mixed six times by pipetting using a glass pasteur pipette and digested for 10 min at 37°C. During the incubation time the tissue was occasionally mixed by shaking. After addition of 1 ml FCS to inactivate enzymes, the tissue solution was extensively mixed by pipetting using a glass pasteur pipette and transferred with a 10 ml serological pipettes on a nylon mesh cell strainer (40 µm pore size) to remove cell debris. The cell suspension was captured in a falcon tube, centrifuged at 1000 rpm for 5 min and the supernatant was discarded. The cell pellet was washed once in PBS and centrifuged at 1000 rpm for 5 min. For isolation of human spermatogonial stem cells these cells underwent a MACSorting (2.2.4.7.2).

2.2.4.3.8 Culture of Scrotal Human Fibroblasts

Scrotal Human Fibroblasts (SHF) were derived from scrotal skin from infertile men. Scrotum biopsies were obtained simultaneously during surgery for testicular sperm extraction (TESE) for the diagnosis of male fertility from patients at the Department of Andrology, University Hospital Hamburg-Eppendorf in Hamburg, embedded in medium containing vials and send by mail to the Institute of Human Genetics in Göttingen for further study. Ethic Committee Approval to Prof. Schulze was obtained (OB/X/2000) and the study was performed accordingl to the ethical guide lines of the current Declaration of Helsinki. On arrival scrotal skin samples were cultured on 0.1% gelatin-coated culture dishes in FB medium at 37°C and 5% CO_2. During culture, the cells expanded from scrotal skin and started to proliferate. These so called SHF cells were expanded, routinely passaged (2.2.4.3.4) and cryopreserved using FB freezing medium (2.2.4.3.5).

2.2.4.3.9 Co-culture of mouse ESCs with HTF cells

Mouse ESCs were cultured under standard ESC culture conditions at 37°C and 5% CO_2. Using FB medium co-culture experiments were performed with 100.000 mESCs seeded on mitotically active HTF cells, which reached nearly 80% confluence. First medium change was performed 3 days at the earliest after starting the co-culture, subsequently every second to third day. After 10-12 days in co-culture cells were passaged in a ratio of 1:2-1:3 on 0.1%

gelatine-coated culture dishes and further cultured for at least 8-10 days. These cells were used for further analysis.

2.2.4.3.10 Cultivation and passaging of hiPSCs

Generally, established hiPSCs were cultured in hiPSC medium (2.1.11.1) on 6-well vessels providing a MEF feeder layer (2.2.4.2.1) at 37°C, 5% CO_2 and 5% O_2 (hypoxia). Every second day cells were washed once with PBS and covered with equilibrated hiPSC medium.
hiPSCs had to be passaged when colonies filled in the size observed through a 10x objective of the light microscope, colonies physically contacted each other and/or the MEF feeder layer was older than 7 days. Depending on the confluence, hiPSCs cultured on one 6-well vessel were passaged in a ratio of 1:2 – 1:4.
For passaging, hiPSCs were washed once with PBS and covered with 1 ml accutase for enzymatic cell detachment. Cell dissociation was observed under the light microscope. When the colonies started to dissociate from the culture well and the edges of colonies were detached, the accutase was carefully removed from the frame of the culture well and cells were immediately covered with equilibrated hiPSC medium. hiPSC colonies were collected by scratching the surface of the 6-well vessel with a serological pipette and simultaneously rinsing with medium. Remaining colonies were collected with a cell scraper. Collected cells were pelleted by gently centrifugation (300 x g, 5 min) and carefully resuspended in an appropriate volume of equilibrated hiPSC medium supplemented with 10 µM Y-27632 (2.1.6) without making a single cell suspension. According to the passaging ratio hiPSCs were seeded on one ore more 6-well vessels coated with MEF feeder layer and the 6-well plate was placed in the incubator at 37°C, 5% CO_2 and 5% O_2, where it was gently rocked from side to side and front and back to distribute the hiPSC aggregates across the well. The first medium change was performed 48 h after passaging procedure.

2.2.4.3.11 Cryopreservation and thawing of hiPSCs

The procedure for cryopreservation and thawing of hiPSCs had to be established in the presented thesis. The establishment was carried out by testing different ways of enzymatic cell detachment, cell collection, centrifugation, compositions of freezing media and freezing procedures, which is summarized in Tab. 7 in the corresponding result part (3.2.3).
The survival of hiPSC colonies cryopreserved by the tested procedures was evaluated by counting the number of emerged colonies after replating on MEF feeder layer. The presence of Rho associated kinase (ROCK) inhibitor Y-27632 is known to increase hiPSC colony formation (Watanabe et al., 2007) and to improve the recovery and further growth of cryopreserved hiPSCs by exhibiting anti-apoptotic activity (Claassen et al., 2009). Because

of these findings, thawed hiPSCs were always cultured in appropriate hiPSC medium supplemented with Y-27632 (10 µM) for 48 h after replating on MEF. Additionally, the impact of pre-incubation of hiPSCs with Y-27632 before cell detachment was tested. Cell detachment was performed with or without using cell dissociation. Enzymatic cell detachment was performed using collagenase or accutase, while cell dissociation was observed under the light microscope. Finally, hiPSC colonies were collected by using a needle, scratching with a serological pipette and cell scraper or rinsing with medium followed by scratching with a cell scraper. Collected cells were pelleted by gently centrifugation (200-500 x g, 3-5 min) and carefully resuspended in appropriated freezing medium. To asses the best freezing medium, hiPSCs were frozen in freezing media according to Wagner and Welch (2010), containing KO™-SR and DMSO (90:10) with or without Y-27632 as well as in commercial cryopreservation media (CryoStem™ Freezing Medium; Biofreeze and hiPSC medium (90:10) with Y-27632). For cryopreservation vials were frozen directly in -80°C or cooled -1°C/min (using Mr. Frosty™ Freezing Container) in -80°C. After 2-3 days stocks were transferred to -152°C for long term storage. Cryopreserved stocks were recovered by thawing frozen hiPSCs in a 37°C water bath until the vials' content was almost, but not completely thawed, and diluted with at least 4 ml hiPSC medium in a 15 ml falcon tube. After centrifugation (200-500 x g, 3-5 min), the cell pellet was gently resuspended in appropriate hiPSC medium supplemented with Y-27632 without making a single cell suspension and finally repleated on MEF feeder layer. Different freezing/thawing methods led to the emergence of hiPSC colonies after cryopreservation. Nevertheless, enzymatic cell detachment using accutase revealed better efficiencies of colony formation. The survival rate of hiPSCs could be increased by using Y-27632 in freezing medium containing KO™-SR and DMSO (90:10). However, the best survival rate of cryopreserved hiPSCs was achieved using commercial CryoStem™ Freezing Medium, in which cells were frozen gradually -1°C/min in -80°C. In general, formation of small clusters with a compact morphology could be observed earliest three days after replating on MEF feeder layer. Final hiPSC colony formation with clear borders could take up to 14 days.

After testing these different conditions, hiPSCs were cryopreserved using freezing medium containing KO™-SR and DMSO (90:10) supplemented with 10 µm Y-27632 as well as using CryoStem™ Freezing Medium. For the purpose of cryopreservation, hiPSCs were pre-incubated with equilibrated hiPSC medium supplemented with 10 µM Y-27632 for 1 h at 37°C, 5% CO_2 and 5% O_2. Then hiPSCs were treated with accutase and pelleted according to the protocol for passaging of hiPSCs (2.2.4.3.10). After centrifugation the pellet was resuspended in either 1 ml ice cold CryoStem™ Freezing Medium and the cryopreservation vial was directly cooled -1°C/min in -80°C using Mr. Frosty™ Freezing Container or in 1 ml freezing medium containing KO™-SR and DMSO (90:10) supplemented with 10 µM Y-27632

and the cryopreservation vial was frozen directly in -80°C. After 2-3 days stocks were transferred to -152°C for long term storage.

For thawing of cryopreserved hiPSCs, the cryovial was placed in a water bath at 37°C until the cells were almost completely thawed. The cells were mixed with a bigger volume of hiPSC medium in order to dilute the freezing medium and then centrifuged at 300 x g for 5 min. After removal of the supernatant, the cells were carefully resuspended in an appropriate volume of equilibrated hiPSC medium supplemented with 10 µM Y-27632 and seeded on one 6-well vessel prepared with MEF feeder layer. The 6-well plate was placed in the incubator at 37°C, 5% CO_2 and 5% O_2, where it was gently rocked from side to side and front and back to distribute the hiPSC aggregates across the well. The first medium change was performed 48 h after thawing procedure.

2.2.4.4 Transfection

2.2.4.4.1 Preparation of constructs for transfection

Constructs used for transfection were linearized in order to support integration. First 100 µg DNA were digested in a total volume of 100 µl with 80-100 U of suitable digestion enzyme at 37°C overnight. To confirm complete DNA digestion 2-3 µl of the reaction volume were resolved by agarose gel electrophoresis.

Purification of digested DNA was performed by phenol-chloroform extraction. The reaction volume was filled up to 400 µl with Ampuwa, mixed with 400 µl phenol-chloroform (1:1) and centrifugation at 12.000 x rpm for 15 min. The upper aqueous phase was transferred into a new e-cup, mixed with 400 µl chloroform by inverting for 3 min and centrifuged at 12.000 x rpm for 15 min. Further work was performed under the sterile bench. After centrifugation the upper aqueous phase was transferred into a new e-cup, mixed with 350 µl isopropanol and centrifuged at 12000 x rpm for 15 min. The supernatant was carefully discarded and the DNA pellet was washed twice with 70% EtOH, followed by resuspension in 100 µl sterile PBS and stored at 4°C. After at least 24 h at 4°C, 2 µl of linearized and purified DNA were analysed by agarose gel electrophoresis 0.8%.

2.2.4.4.2 Electroporation

For electroporation exponentially growing cells were detached from the culture vessels by trypsin/EDTA treatment. The cell pellet was washed once with PBS by centrifugation at 1000 x g for 5 min. Afterwards the cells were resuspended in 600 µl PBS and mixed with 100 µg linearized and purified plasmid DNA in an electroporation cuvette. After 5 min on ice, the electroporation was performed by 240 V and 500 µF at room temperature. The

electroporated cells were kept on ice for 20 min and then seeded on appropriate culture dishes prepared with feeder layer.

2.2.4.5 Culture of single clones

2.2.4.5.1 Selection, picking and culture of single clones

The constructs used for electroporation contained a neomycin or puromycin resistance gene cassette for identification of transfected cells by positive selection for the resistance of geneticin (G418) or puromycin. After electroporation the cells were cultured in selection medium with increased antibiotic concentration (G418: 350 µg/ml; 1.25 µg/ml puromycin:) for 4 days. Further cultivation was performed with appropriate medium with decreased antibiotic concentration (G418: 100 µg/ml; 0.75 µg/ml puromycin). After 8-10 days of selection the resistant clones had a sufficient size to be isolated and separated. The positive clones were picked mechanically under the light microscope, which was disinfected and put under the opened sterile bench. During the working procedure a surgical mask was worn in order to minimize the risk of contamination. The cells were washed with PBS and covered with fresh PBS. The single clones were separated from the feeder layer with a 20 µl Gilson pipette and an appropriate pipette tip and transferred in a 96-well-plate. Within this plate the clones were treated with trypsin/EDTA, separated into single cells by resuspending and seeded on 24-well-plates prepared with feeder layer. The picked clones were cultured in appropriate medium with decreased antibiotic concentration, which was changed every 24 h. After 4 to 7 days the cells reached 100% confluence and replica plating was performed.

2.2.4.5.2 Replica plating

When the cells reached 100% confluence, they were treated with trypsin/EDTA, seeded on two gelatine coated 24-well plates and cultured till 100% confluence. From one plate, cells' DNA or RNA was isolated for genotyping by PCR or expression analyses by qRT-PCR, respectively. On the second gelatine coated plate, seeded cells were treated with freezing medium and stored at -80°C. The positive clones identified by genotyping PCR or expression analysis were thawed and further cultured.

2.2.4.6 Cell induction with retinoic acid

Retinoic acid (RA) is a metabolite and high effective form of vitamin A and affects proliferation and differentiation of several cell types. Induction of cells was performed with RA at a concentration of 10^{-6} M, which was added directly from a stock solution of 10^{-3} M to fresh

culture medium, after the cells were washed with PBS. During induction the medium supplemented with RA was changed every 24-48 h. The duration of RA treatment was dependent on the experiment.

2.2.4.7 Cell sortings

2.2.4.7.1 Fluorescence Activated Cell Sorting

Cells can be sorted according to their size, granularity and different fluorescence by flow cytometry using Fluorescence Activated Cell Sorting (FACSorting). Here the sorting of cells was carried out according to their expression of EGFP. EGFP expressing cells can be excited at 510 nm with an argon-ion laser, which emits blue-green light at 488 nm.

The cells were washed with PBS, treated with trypsin/EDTA, centrifuged at 1000 x g for 5 min and once more washed with PBS, followed by the cell sorting by flow cytometry. A non-EGFP expressing cell line was used as a negative control. The FACSorting was performed in the Cell Sorting Facility of the University Medical Center, Göttingen (Prof. Dr. Gerald Wulf). After sorting EGFP positive cells were pelletized by centrifugation and once washed with PBS. The cells were resuspended in appropriate volume of culture medium and seeded on prepared culture vessels.

2.2.4.7.2 Magnetic Activated Cell Sorting

Magnetic Activated Cell Sortings (MACSortings) were performed using the MACS® Cell Separation provided by Miltenyi Biotech GmbH. MACSorting was carried out using MACS buffer (2.1.6), which was freshly prepared and stored on ice throughout the MACSorting procedure.

Before MACSorting, cells were washed once with PBS and centrifuged at 1000 x rpm for 5 min. Then cells were incubated with the primary antibody (1:50 α6-Integrin, biotin conj.; 2.1.12.1) diluted in MACS buffer for 15 min at 4°C. During the incubation time cells were occasionally mixed by shaking. Afterwards, 2 ml MACS buffer were added to dilute unbound primary antibody and cells were centrifuged at 1000 x rpm for 5 min. The supernatant was carefully discarded and cells were incubated with secondary antibody (1:5 anti-Biotin MicroBeads; 2.1.12.2) diluted in MACS buffer for 20 min at 4°C. During the incubation time cells were occasionally mixed by shaking. Unbound secondary antibody was diluted by the addition of 2 ml MACS buffer. After centrifugation at 1000 x rpm for 5 min, the supernatant was carefully discarded and cells were resuspended in 500 µl MACS buffer. A MACS® MS Column was fixed in a MACS™ Separator providing a strong magnetic field and was equilibrated with 500 µl MACS buffer. The cell suspension was put onto the equilibrated

MACS® MS Column and the flow through was captured in an e-cup, which was stored on ice, if HTF cells had to be established (2.2.4.3.6). Further the MACS® MS Column was washed three times with 500 µl MACS buffer. For elution of retained cells of interest, the MACS® MS Column was removed from the MACS™ separator and cells were eluted with 1000 µl MACS buffer using the provided syringe and captured in an e-cup. Cells were centrifuged at 1000 x rpm for 5 min and the supernatant was carefully discarded. Cells were resuspended in an appropriate volume of StemPro+3 medium (2.1.11.1) and seeded on gelatine (2.2.4.1.1) or MEF feeder layer (2.2.4.2.1) coated culture plates.

2.2.4.8 Cell transplantation in germ cell depleted mice (Ogawa et al., 1997)

The transplantation of cells in germ cell depleted testis of busulfan treated mice is used to test if cells are capable to colonize within the testis and regenerate spermatogenesis to develop functional sperm. For generation of germ cell depleted mice, busulfan was intraperitoneally injected in 6 to 8 weeks old male mice at a concentration of 40 mg/kg body weight (diluted in DMSO). This busulfan treatment destroys all germ cells within the testis. 4 to 6 weeks after treatment one mouse was sacrificed and paraffin sections of the testes were prepared (2.2.3.3). The absence of any germ cells was checked by H&E staining (2.2.3.4). The remaining mice were mated with wild type females for at least 4 weeks in order to prove that there is no endogenous spermatogenesis left, if they begot no offspring. 3 to 4 month after busulfan treatment cells were transplanted into the seminiferous tubules.

Before transplantation the cells underwent a MACSorting (2.2.4.7.2) using an α6-Integrin antibody (2.1.12.1) to remove any HTF. The MACS elution fraction was cultured in StemPro+3 medium (2.1.6). Cells were either directly used for transplantation or cultured on MEF feeder layer at 37°C and 5% CO_2 overnight and the feeder was removed before transplantation. Approximately 30-40 µl of the cell suspension (~1 x 10^8 cells/ml) were transplanted via rete testis into seminiferous tubules of one testis, whereas the non-transplanted testis served as an internal control. Analgesic, anesthetic and antisedan used for transplantation procedure were provided and prepared by workers of the animal house.

At certain time points recipient mice were sacrificed and the testes prepared for further analysis by genotyping PCR or immunohistochemistry. All experiments were performed according to the national regulations of the Care and Use of Labaratory animals.

2.2.4.9 Teratoma assay

The ability of pluripotent cells to form teratomas is generally accepted as one of the reliable proof of pluripotency. The teratoma assay was performed by Prof. Dr. Ralf Dressel, Institute for Cellular and Molecular Immunology in Göttingen.

For teratoma assay, hiPSCs were detached from the culture vessels (2.2.4.3.10) and washed once with PBS. After centrifugation, hiPSCs were resuspended in medium consisting of 1:1 PBS and matrigel (diluted 1:100 in DMEM/F12, 2.2.4.1.2) and stored on ice until transplantation. 1×10^6 – 2×10^6 cells were injected under the skin of immunodeficient NOD/SCID mice. The following weeks the transplanted mice were monitored by Prof. Dressel. In the case of observable incidence of tumors, both the size of tumors had to be recorded and the teratoma had to be histologically processed and analysed. The teratoma assay was stopped, when 3 month after cell injection no teratoma formation was observed.

2.2.4.10 Generation of mouse ES cell lines

Blastocysts were isolated from gestating mice sacrificed at stage 3.5 dpc, whose uteri were flushed with M2 medium. Blastocysts were cultured in groups in KSOM medium (2.1.11.1) on plates coated with MEF feeder layer at 37°C and 5% CO_2. Within ~1 week of culture the outgrowth of the inner cell mass of the blastocysts was observed, which was manually picked, disaggregated by trypsinization and further seeded in ESC culture medium (2.1.11.1) on 24-wells coated with fresh MEF feeder layer. Medium was changed every second day. During further culture cells grew in colonies and revealed a mouse ES cell like morphology. Cells were expanded by routinely passaging (2.2.4.3.4) and cryopreservation (2.2.4.3.5).

2.2.4.11 “Feeder Free“ mRNA/miRNA Reprogramming of human fibroblasts

The generation of integration free human iPSCs was performed with the mRNA reprogramming method, which bases on the repeated delivery of mRNA encoding for pluripotency associated transcription factors. The Stemgent® mRNA Reprogramming System served as a basis, but was modified in several aspects in order to achieve a successful reprogramming of the human target cells. In the following the final working reprogramming approach for “Feeder Free” mRNA/miRNA Reprogramming is described in detail for recapitulation.

2.2.4.11.1 Material preparation

2.2.4.11.1.1 Preparation of media supplements

The media used during the “Feeder Free” mRNA/miRNA Reprogramming had to be supplemented with different reagents supplied by the Stemgent® mRNA Reprogramming System. During preparation of aliquots it was important to thaw the supplements on ice, to work sterile and quickly and to put prepared aliquots directly on dry ice before their final storage at -80°C to minimize protein degradation. Especially, the B18R Recombinant Protein (B18R) is very sensitive and its addition to the medium was fundamental during the mRNA transfection series. Pluriton™ Supplement (P™S) as well as B18R were thawed on ice and each aliquotted in ready-to-use vials of 4 µl volumes and stored at -80°C.

2.2.4.11.1.2 Preparation of basis medium Pluriton™ Medium

Media used during the “Feeder Free” mRNA/miRNA Reprogramming had to be prepared with Pluriton™ Medium, which was supplied by the Stemgent® mRNA Reprogramming System. Pluriton™ Medium was thawed completely within 2 days at 4°C, supplemented with 5 ml penicillin/streptomycin (100x) and thoroughly mixed by pipetting. Aliquots of 40 ml Pluriton™ Medium were prepared and stored at -20°C. Before use aliquots were thawed at 4°C overnight and could be stored at 4°C for up to 2 weeks.

2.2.4.11.1.3 Preparation of NuFF-conditioned Pluriton™ Medium

According to the protocol for the Stemgent® mRNA Reprogramming System, NuFF-conditioned Pluriton™ Medium supports the reprogramming process during the transfection series. In the modified “Feeder Free” mRNA/miRNA Reprogramming protocol this medium was used during the whole transfection period and should be prepared before the start of reprogramming. For the preparation of NuFF-conditioned Pluriton™ Medium 4×10^6 inactivated NuFF cells (2.2.4.2.2) were seeded on a gelatine coated T75 flask in 25 ml of NuFF culture medium and incubated at 37°C and 5% CO_2 overnight. After 24 h adherent cells were washed once with PBS and further cultured in 25 ml Pluriton™ Medium supplemented with 4 ng/ml bFGF at 37°C and 5% CO_2. The NuFF-conditioned Pluriton™ Medium was collected with a sterile pipette after 24 h incubation and replaced with fresh 25 ml Pluriton™ Medium supplemented with 4 ng/ml bFGF. This procedure was repeated daily and the collected NuFF-conditioned Pluriton™ Medium was stored in a sterile 50 ml falcon tube at -20°C. The seeded NuFF cells were used for ten collections. All aliquots with NuFF-conditioned Pluriton™ Medium were thawed at 4°C overnight one day before the final collection, pooled and sterile filtered (0.22 µm pore size). NuFF-conditioned Pluriton™

Medium was further stored in 40 ml aliquots at -20°C and thawed at 4°C overnight before its use. Defrosted NuFF-conditioned Pluriton™ Medium was stable for 3 days at 4°C.

2.2.4.11.1.4 Preparation of NuFF-conditioned Pluriton™ Reprogramming Medium

For the preparation of NuFF-conditioned Pluriton™ Reprogramming Medium 10 ml NuFF-conditioned Pluriton™ Medium (2.2.4.11.1.3) were equilibrated for at least 2 h in a 10 cm dish at 37°C, 5% CO_2 and 5% O_2. Just before use the equilibrated medium was supplemented with 200 ng/ml B18R (= 4 µl B18R; 2.2.4.11.1.1) and bFGF (= 4 µl P™S; 2.2.4.11.1.1) to a final concentration of 20 ng/ml.

2.2.4.11.1.5 Preparation of miRNA cocktail with miRNA cluster 302-367

The following miRNAs (Thermo Scientific Dharmacon®) were used for miRNA transfections:

miRIDIAN Mimic Human hsamiR302a
miRIDIAN Mimic Human hsamiR302b
miRIDIAN Mimic Human hsamiR302c
miRIDIAN Mimic Human hsamiR302d
miRIDIAN Mimic Human hsamiR367

During all working steps it was important to thaw miRNAs on ice, to work sterile and quickly and to put prepared aliquots directly on dry ice before their final storage at -80°C.

For each miRNA a 20 µM stock with RNase free H_2O was prepared under sterile conditions and stored in 10 µl aliquots at -80°C. The master miRNA cocktail was prepared of an equivolume mixture with 4 µM of each individual miRNA: 10 µl of each miRNA were mixed and stored at -80°C. Ready-to-use aliquots were prepared with 3.5 µl master miRNA cocktail and stored at -80°C.

2.2.4.11.1.6 Preparation of mRNA cocktail

The master mRNA cocktail encoding for the pluripotency associated transcription factors was prepared according to the protocol of the Stemgent® mRNA Reprogramming System. In short, all mRNAs were thawed on ice, mixed carefully by pipetting and combined as followed:

Master mRNA cocktail		
Oct4 mRNA	385.1	µl
Sox2 mRNA	119.2	µl
Klf4 mRNA	155.9	µl
cMyc mRNA	147.7	µl
Lin28 mRNA	82.5	µl
nGFP mRNA	110.6	µl
mRNA cocktail mix	1000	µl

Ready-to-use aliquots were prepared with 10 µl master mRNA cocktail and stored at -80°C.

2.2.4.11.2 Procedure of reprogramming

The procedure of the "Feeder Free" mRNA/miRNA Reprogramming is illustrated in Tab. 1. The reprogramming process was performed at 37°C, 5% CO_2 and 5% O_2. All media were equilibrated under these conditions for at least 2 h before their usage.

Tab. 1: Overview oft the procedure of "Feeder Free" mRNA/miRNA Reprogramming.

day	action	medium
d-3	preparation of matrigel coated plates	-
d-2	seeding of target cells	appropriate cell culture medium
d-1	miRNA transfection	NuFF-conditioned Pluriton™ Medium supplemented with bFGF to finally 20 ng/ml
d0	4 h preincubation	NuFF-conditioned Pluriton™ Reprogramming Medium
	mRNA transfection	-
d1	3 h preincubation	NuFF-conditioned Pluriton™ Reprogramming Medium
	mRNA transfection	-
	medium change after 4 h	NuFF-conditioned Pluriton™ Reprogramming Medium
d2 / d3	mRNA transfection	-
	medium change after 4 h	NuFF-conditioned Pluriton™ Reprogramming Medium
d4	mRNA/miRNA co-transfection	-
	medium change after 4 h	NuFF-conditioned Pluriton™ Reprogramming Medium
d5 +	mRNA transfection	-
	medium change after 4 h	NuFF-conditioned Pluriton™ Reprogramming Medium

d: day of reprogramming; h: hours; d5 +: d5 of reprogramming until the emergence of hiPSC colonies.

2.2.4.11.2.1 Seeding of target cells

At day -2 of reprogramming target cells were seeded on matrigel coated 6-well vessels (2.2.4.1.2), which were freshly prepared the day before. For reprogramming of slowly proliferating cell lines such as the used scrotal human fibroblasts (SHFs; 2.2.4.3.8) were seeded in a high cell density of 100.000 cells per 6-well vessel, whereas fast proliferating cell lines were seeded in a cell density of 20.000 cells per 6-well vessel. Target cells were seeded in appropriate culture medium (2.1.11.1) and attached overnight at 37°C, 5% CO_2 and 5% O_2.

2.2.4.11.2.2 miRNA transfection

At day -1 of reprogramming target cells were transfected with miRNA cluster 302-367 (2.2.4.11.1.5). Cell were washed once with PBS and covered with 2 ml per 6-well vessel equilibrated NuFF-conditioned Pluriton™ Medium (2.2.4.11.1.3) supplemented with bFGF (= 4 µl P™S in 10 ml medium) to a final concentration of 20 ng/ml.

The following components were needed for the miRNA transfection of cells per one 6-well vessel:

miRNA transfection per one 6-well vessel

tube 1	Opti-MEM® Reduced Serum Medium	46.5	µl
	miRNA cocktail	3.5	µl
tube 2	Opti-MEM® Reduced Serum Medium	45	µl
	Lipofectamine® RNAiMAX Transfection Reagent	5	µl
		100	µl

All components of each tube were pipetted together and mixed carefully. The content of tube 2 was transferred in tube 1, mixed carefully by pipetting and further incubated for 15 min at room temperature. The 100 µl transfection mixture was mixed carefully by pipetting the solution three times up and down and subsequently distributed on the target cells of one 6-well vessel in a dropwise fashion. The 6-well plate was gently rocked from side to side and front and back to distribute the transfection mix across the well. Cells were further cultured at 37°C, 5% CO_2 and 5% O_2.

2.2.4.11.2.3 mRNA transfection series

In order to maintain a constant level of delivered mRNA in the cells, the mRNA transfection had to be performed always at the same time.

At day 0 of reprogramming target cells were transfected with mRNA (2.2.4.11.1.6). Before mRNA transfection the medium was discarded without washing with PBS and directly replaced with NuFF-conditioned Pluriton™ Reprogramming Medium (2.2.4.11.1.4). The cells were incubated for 4 h at 37°C, 5% CO_2 and 5% O_2 before mRNA transfection. The following components were needed for the mRNA transfection of cells per one 6-well vessel:

mRNA transfection per one 6-well vessel

tube 1	Opti-MEM® Reduced Serum Medium	40	µl
	mRNA cocktail	10	µl
tube 2	Opti-MEM® Reduced Serum Medium	45	µl
	Lipofectamine® RNAiMAX Transfection Reagent	5	µl
		100	µl

The transfection mix was prepared like for miRNA transfection (2.2.4.11.2.2) and distributed on the preincubated target cells in a dropwise fashion. The 6-well plate was gently rocked from side to side and front and back to distribute the transfection mix across the well. Cells were further cultured at 37°C, 5% CO_2 and 5% O_2.

The next day (day 1 of reprogramming) 3 h before mRNA transfection the medium was discarded without washing with PBS and directly replaced with NuFF-conditioned Pluriton™ Reprogramming Medium. The cells were incubated for 3 h at 37°C, 5% CO_2 and 5% O_2 before mRNA transfection, which was performed as described above. Medium was replaced with NuFF-conditioned Pluriton™ Reprogramming Medium 4 h after mRNA transfection and cells were further cultured at 37°C, 5% CO_2 and 5% O_2.

On day 2 and day 3 the preincubation was omitted and the mRNA transfections as well as medium change 4 h after transfection were performed as on day 1 of reprogramming.

A mRNA/miRNA co-transfection was performed on day 4 of reprogramming. The following components were needed for the co-transfection of cells per one 6-well vessel:

mRNA/miRNA co-transfection per one 6-well vessel

tube 1	Opti-MEM® Reduced Serum Medium	36.5	µl
	mRNA cocktail	10	µl
	miRNA cocktail	3.5	µl
tube 2	Opti-MEM® Reduced Serum Medium	45	µl
	Lipofectamine® RNAiMAX Transfection Reagent	5	µl
		100	µl

The transfection mix was prepared as already described and distributed on the cells in a dropwise fashion. The 6-well plate was gently rocked from side to side and front and back to distribute the transfection mix across the well. Cells were further cultured at 37°C, 5% CO_2 and 5% O_2. A medium change with NuFF-conditioned Pluriton™ Reprogramming Medium was performed 4 h after co-transfection and cells were further cultured at 37°C, 5% CO_2 and 5% O_2.

On the following days, cells were further transfected with mRNAs as described above until human iPSC colonies emerged.

2.2.4.11.2.4 Splitting of target cells during the reprogramming process

If target cells exhibited a high proliferation rate during reprogramming, they needed to be split to prevent an over-confluence of cells, which would reduce the reprogramming efficiency because of a decreased mRNA delivery. If splitting of target cells was necessary, it was preferentially performed between day 6 and 8 of the transfection series and cells were replated in a lower cell density.

Cells, which had to be split, were transfected with mRNA and the medium was changed 4 h after transfection as already described (2.2.4.11.2.3). After 2 h at 37°C, 5% CO_2 and 5% O_2, cells were washed once with PBS and covered with 0.5 ml Trypsin/EDTA. After 10 min at 37°C, 5% CO_2 and 5% O_2, cell detachment and dissociation were stopped by the addition of 0.5 ml FB medium. Detached cells were transferred with a serological pipette in a 15 ml falcon tube containing 2 ml NuFF-conditioned Pluriton™ Reprogramming Medium supplemented with 10 µM Y27632 (2.1.6). After centrifugation at 200 x g for 2 min, the cell pellet was washed once with PBS to remove the FB medium completely. The cells were centrifuged at 200 x g for 2 min, resuspended in 4 ml NuFF-conditioned Pluriton™ Reprogramming Medium supplemented with 10 µM Y27632, seeded on two matrigel coated 6-wells (2.2.4.1.2) and further cultured at 37°C, 5% CO_2 and 5% O_2. The next day the transfection series was continued according to the plan (Tab. 1) and passaged cells were further cultured in NuFF-conditioned Pluriton™ Reprogramming Medium supplemented with 10 µM Y27632 till the end of reprogramming.

2.2.4.11.2.5 Picking and passaging of emerged hiPSC colonies

When hiPSC colonies exhibited their characteristic morphology with defined colony edges, they were picked on MEF feeder layer (2.2.4.2.1) in order to expand them and to establish hiPSC cell lines. During the working procedure a surgical mask was worn in order to minimize the risk of contamination. Cells were washed once with PBS and covered with appropriate medium. Observed under the light microscope, which was disinfected and put

under the opened sterile bench, hiPSC colonies were broken into 4-8 fragments and detached manually from the culture plate by using a needle. Here it was important to avoid single cell suspensions. Colony fragments were transferred in appropriate medium on freshly prepared MEF feeder layer using a serological pipette and placed in the incubator at 37°C, 5% CO_2 and 5% O_2, where the plate was gently rocked from side to side and front and back to distribute the hiPSC aggregates across the well. The first medium change was performed 48 h after picking of cells.

First hiPSC colonies were cultured in NuFF-conditioned Pluriton™ Medium (2.2.4.11.1.3) supplemented with bFGF (= 4 µl P™S in 10 ml medium) to a final concentration of 20 ng/ml and hiPSC-M3 mixed in a ratio of 50:50, which was reduced stepwise during further culture in order to reach 100% hiPSC-M3 (2.1.11.1). After culturing in pure hiPSC-M3 this medium was replaced with final hiPSC medium (2.1.11.1) in order to expose hiPSCs to a reduced bFGF concentration of 5 ng/ml, which decreases the risk of spontaneous differentiation of hiPSCs. Furthermore the culture of picked hiPSCs was stepwise propagated on 12-well, followed by the use of 6-well vessels.

2.2.4.12 Chromosomal analysis

For a chromosomal analysis cells have to be arrested in the metaphase of mitotic cell division. Therefore cells were incubated with 190 µl colcemid in 5 ml medium for 4 h at 37°C and 5% CO_2. After washing with PBS, cells were treated with trypsin/EDTA, transferred into a glass tube and centrifuged at 1000 x g for 5 min. The supernatant was discarded except for 1 cm medium above the pellet, which was resuspended in the remaining medium carefully by vortexing. Subsequently 10 ml preheated hypotonic solution (2.1.6) were added slowly. The suspension was incubated for 16 min at room temperature. During the incubation the suspension was mixed carefully by pipetting. After centrifugation at 1000 x g for 5 min, the supernatant was discarded except for 1 cm medium above the pellet and the pellet was resuspended carefully by vortexing. Afterwards 10 ml ice cold fixative solution was added during carefully rotation of the glass tube. After incubation for 5 min at room temperature, the mixture was centrifuged at 1000 x g for 5 min. The fixation was repeated three times without incubation after addition of fixative solution (2.1.6). Finally the supernatant was discarded except for 1 cm medium above the pellet, which was carefully resuspended in the remaining fixative solution. Fixed cells were dropped on degreased slides from a height of nearly 30 centimeters. Slides were air dried completely, stained for 3 min in Giemsa solution and washed under tap water. The chromosomes were analysed with light microscopy and karyotyping was performed using the Leica MCK software.

2.2.4.13 Apoptosis assay using PE Annexin V Apoptosis Detection Kit I

The investigation of cell apoptosis was performed with the PE Annexin V Apoptosis Detection Kit I (BD Biosciences, Heidelberg). This kit bases on the use of the phospholipd binding protein Annexin V, which has a high affinity to the membrane phospholipid phosphatidylserine (PS). PS is translocated to the outer leaflet of the plasma membrane in cells, which undergo apoptosis. Thus the use of Annexin V conjugated with a fluorochrome like Phycoerythrin (PE) allows the measurement of apoptotic cells by flow cytometry. In this kit Annexin V is used in combination with the vital dye 7-Amino-Actinomycin (7-AAD) to distinguish between vital, early and late apoptotic cells.

One cell line was treated with 200 µM citrinin (diluted in DMSO) for 24 h to induce apoptosis and used as a positive control for apoptotic cells as well as for the settings of the flow cytometry system.

First cultured cells were detached from the culture vessels by trypsin/EDTA treatment and washed twice with ice cold PBS by centrifugation at 1000 x g for 5 min. After resuspending at a concentration of 1 x 10^6 cells/ml in 1x Binding Buffer provided by the kit, 100 µl of cell suspension were transferred to a 5 ml FACS tube and stained with 5 µl Annexin V-PE and 5 µl 7-AAD and vortexed gently. Unstained cells, cells stained only with Annexin V-PE as well as cells stained only with 7-AAD were used as references. After incubation for 15 min at room temperature in the dark, 400 µl 1x Binding Buffer was added and the mixture was analyzed by flow cytometry within 1 h. Flow cytometric analysis weas performed by Prof. Ralf Dressel, Institute for Cellular and Molecular Immunology in Göttingen.

2.2.4.14 BrdU based proliferation assay

5-Bromo-2′-deoxyuridine (BrdU) is a thymidine analog, which can be incorporated into DNA during the synthesis phase of cell cycle instead of thymidine (Givan, 1992; Leif et al., 2004). Therefore it can be used as a marker of proliferating cells.

The cells were seeded on chamber slides and cultured with 20 µg/ml BrdU (Sigma; 10 mg/ml stock in Ampuwa) for 48 h at 37°C and 5% CO_2. Then cells were washed three times with PBS and fixated with 4% PFA for 10 min at room temperature. After washing with PBS for three times, cells were incubated with 2 M HCl for 60 min at 37°C for DNA denaturation into single strands allowing antibody access to the BrdU. Cells were washed three times with PBS and incubated with blocking solution containing 0.2% BSA/ 0.1%Tween20 in PBS for 60 min at room temperature. Incubation with anti-BrdU antibody (2.1.12.1) diluted 1:100 in blocking solution was performed in a humidity chamber overnight at 4°C. Next day cells were washed three times with PBS and further incubated with the appropriate secondary antibody (2.1.12.2) diluted 1:200 in blocking solution in a humidity chamber for 2 h at room temperature. After three times washing with PBS, cells

were counterstained with DAPI and mounted with Eukitt® quick-hardening mounting medium (Fluka).

2.2.4.15 Alkaline Phosphatase - staining

Alkaline Phosphatase (AP) - staining can be used to detect specifically not differentiated cells. In contrast to most cells, pluripotent stem cells express AP at a highly increased level, which allows the discrimination between pluripotent and differentiated cells.

AP-Staining was performed with the Leukocyte Alkaline Phosphatase Kit (Sigma-Aldrich, Deisenhofen). Fixative containing citrate, acetone and formaldehyde as well as staining solution containing sodium nitrite, FBB-Alkaline and Naphthol AS-BI Alkaline were prepared according to manufacturer's protocol. After washing with PBS, cells were fixed for 30 sec at room temperature. Cells were washed with ddH_2O for 45-60 sec and incubated with staining solution for 15 min at room temperature. After washing with ddH_2O for 2 min, cells were counterstained with Neutral Red solution for 10-20 sec, washed with tap water and air dried.

2.2.5 Methylation- and imprinting studies

The analysis of the methylation status of imprinted genes was performed in collaboration with Prof. Ulrich Zechner (Institute of Human Genetics, University of Mainz). For this study genomic DNA samples were isolated from the samples of interest (2.2.1.1.2) and send by mail to Mainz. Because bisulfite pyrosequencing of DNA samples as well as data evaluation was performed by the Zechner group, here the principle of this analysis is briefly described (Tost and Gut, 2007).

DNA methylation represents a form of epigenetic modifications, which occur at the 5´position of cytosine in the context of the dinucleotide CpG. Here the analysis of DNA methylation was performed using the bisulfite treatment of genomic DNA as a chemical reaction to translate this epigenetic information into sequential information, which is further analyzed by pyrosequencing. This represents a sequencing-by-synthesis method, which utilizes the release of pyrophosphate (PP_i) molecules during the stepwise incorporation of unmodified deoxynucleoside triphosphates (dNTPs) and the further conversion into bioluminometric signals.

In order to analyse the DNA methylation status, first a sodium bisulfite conversion of genomic DNA is necessary to distinguish between unmethylated and methylated cytosines within the sequence. For this targeted exchange of bases, single-stranded DNA is treated with sodium bisulfite and unmethylated cytosine (C) becomes deaminated to uracil, while methylated cytosine (^mC) remains unchanged. In a subsequent PCR reaction methylated and non-methylated DNA can be discriminated, because the uracils are amplified as thymines,

while ^{m}Cs are further unaltered amplified as cytosines. So after this PCR amplification an exchange of CG basepairs to TA basepairs at non-methylated DNA positions has taken place. Further a comparison of DNA sequences, with and without bisulfite treatment can provide information about the cytosine methylation patterns. The DNA sequences are determined by pyrosequencing, whose template derives from PCR amplification with biotinylated and non-biotinylated primer. The pyrosequencing is performed using the biotinylated single-stranded DNA, four different enzymes including a DNA polymerase, an ATP sulfurylase, the luciferase and an apyrase as well as the substrates D-luciferin and adenosine 5’ phosphosulfate (APS). After annealing of the sequencing primer and addition of one of the four unmodified dNTPs, the added nucleotide is incorporated by the DNA polymerase, if it is complementary to the template DNA. During this incorporation PP_i is released, which is converted into ATP by the ATP sulfurylase using APS. This reaction provides the energy for the luciferase to oxidize D-luciferin, which is generated in an excited state and can be detected by a charge-coupled device camera depicted as a peak in the used program. Before adding the next nucleotide non-incorporated nucleotides are degraded by the apyrase. Afterwards, the process is repeated by the addition of a further dNTP. The nucleotide dATP acts as a natural substrate for luciferase. In pyrosequencing analyses the modified α-S-dATP (2'-Deoxyadenosine-5'-O-(1-Thiotriphosphate)) is used as a nucleotide for primer extension, which is equally incorporated by the polymerase, but is not recognized by the luciferase and therefore does not induce a detectable peak.

3 Results

3.1 Isolation and culture of human spermatogonial stem cells

3.1.1 Human testicular biopsies used for hSSC isolation

Testicular biopsies were obtained simultaneously during surgery for testicular sperm extraction (TESE) for the diagnosis of male fertility from patients and were used for isolation of hSSCs by Magnetic Activated Cell Sorting (MACSorting; 2.2.4.7.2). TESE was performed by Prof. Schulze at the Department of Andrology, University Hospital Hamburg-Eppendorf in Hamburg. Ethic Committee Approval to Prof. Schulze was obtained (OB/X/2000) and the study was performed according to the ethical guide lines of the current Declaration of Helsinki. Tab. 2 gives an overview of samples derived from TESE.

Tab. 2: Overview of human testicular samples used for hSSC isolation experiments.

#	mJ-Score	characteristics of spermatogenesis	elution (cells/ml)	culture			nomenclature	
				layer	medium	condition	hSSC isolation	co-culture
1	10	n.c.	2.2×10^5	gelatine	StemPro+3	normoxia	Gel-1	HTF-1
2	10	n.c.	1.2×10^6	gelatine	StemPro+3	normoxia	Gel-2	HTF-2
3	n.c.	intact spermatogenesis	3.4×10^5	MEF	StemPro+3	normoxia	MEF-1	n.u.
4	n.c.	block in spermatogenesis, but spermatids present	2.9×10^5	MEF	StemPro+3	normoxia	MEF-2	n.u.
5	n.c.	intact spermatogenesis	4.1×10^5	gelatine	StemPro+3	normoxia	Gel-3	n.u.
6	n.c.	intact spermatogenesis	7×10^4	gelatine	StemPro+3	normoxia	Gel-4	n.u.
7	n.c.	ductus aplasia, intact spermatogenesis	1.9×10^5	gelatine	StemPro+3	normoxia	Gel-5	n.u.
8	n.c.	intact spermatogenesis	1.4×10^5	gelatine	StemPro+3	normoxia	Gel-6	n.u.
9	n.c.	intact spermatogenesis, but less germ cells	2.1×10^5	gelatine	StemPro+3	normoxia	Gel-7	n.u.
10	8	no intact spermatogenesis, but normal spermatogonia	1.4×10^5	gelatine	Pluriton+3	hypoxia	Gel-8	HTF-10
11	n.c.	quantitative normal spermatogenesis, but remarkable disorganized germ layer; difficult to estimate because rare case	2.1×10^5	matrigel	StemPro+3	hypoxia	M-3	n.u.
12	n.c.	n.c.	9×10^4	matrigel	StemPro+4	normoxia	M-4	n.u.
13	10	n.c.	5.5×10^4	matrigel	StemPro+4	normoxia	M-5	HTF-3
14	n.c.	n.c.	3.5×10^5	matrigel	StemPro+3	normoxia	M-1	n.u.
15	n.c.	n.c.	2.4×10^5	matrigel	StemPro+3	normoxia	M-2	HTF-15

#: patient number; mJ-Score: modified Johnsen Score (de Kretser & Holstein, 1976); n.c.: spermatogenesis phenotype was not communicated by Prof. Schulze; MEF: MEF feeder layer; StemPro+3 / StemPro+4 / Pluriton+3: media used for culture; media compositions are listed in 3.1.3, Tab. 3; normoxia: culture at 37°C, 5% CO_2; hypoxia: culture at 37°C, 5% CO_2, 5% O_2; Gel: elution fraction cultured on gelatine coated vessels; MEF: elution fraction cultured on MEF feeder layer; M: elution fraction cultured on matrigel coated vessels; HTF: cultivation of flow-through factions as Human Testicular Feeder; n.u.: samples were not used for further experiments.

The table provides information of the spermatogenesis phenotype of the patients, which was classified according to the modified Johnsen Score (de Kretser & Holstein, 1976) and also evaluated by Prof. Schulze (personal communication). Moreover, the calculated cell numbers in the elution fraction after MACSorting and the conditions for further culturing of the cells are listed. Cells were either seeded on MEF feeder layer, on gelatine or matrigel coated vessels as indicated. Cultivation was performed with different media, whose compositions are listed in Tab. 3 (3.1.3), and under normoxia (37°C, 5% CO_2) or hypoxia (37°C, 5% CO_2, 5% O_2). Furthermore Tab. 2 explains the nomenclature of samples, which were used in the presented thesis: cells derived from the elution fraction of MACSorting were named according to the matrix, which was used as growth layer, namely MEF feeder layer (MEF), gelatine (Gel) and matrigel (M). Collected flow-through fractions, which were used in reprogramming (3.2) and co-culture experiments (3.3), were labelled as Human Testicular Feeder (HTF) cell lines.

3.1.2 Isolation of hSSCs by MACSorting

In general SSCs can be isolated from testis tissue mainly by three different approaches: differential plating method, Fluorescence Activated Cell Sortings (FACSortings) and Magnetic Activated Cell Sortings (MACSortings). The sorting efficiency depends on the specificity of the antibody used for enrichment of SSCs. In the presented thesis the hSSC isolation was performed using MACSorting with a specific antibody against α6-Integrin, which is described as a surface marker of murine and human SSCs (Shinohara et al., 1999; 2000; Valli et al., 2014). The MACSorting method was established in the institute for successful isolation of mSSCs (Smorag et al., 2012).

In general MACSorting (Miltenyi Biotech GmbH) is a method for cell separation using MicroBeads for magnetic cell labelling (Fig. 4). These nano-sized, superparamagnetic particles are non-toxic, biodegradable and do not effect the functionality of cells. During MACSorting the cells of interest are selected by a specific antibody recognizing a particular surface protein of these cells. For direct sorting this primary antibody is conjugated to MicroBeads, while for indirect sorting a MicroBeads labelled secondary antibody binds to the primary antibody. After magnetic labelling of the cells, the cell suspension is put onto a column, which is placed in a separator offering a strong magnetic field. This retains magnetic labelled cells in the column, while not-labelled cells are flushed out. If the column is removed from the magnetic field, the labelled cells are eluted as positively selected cells. During the cell separation three fractions are collected: the flow-through, which can be collected as negative fraction depleted of labelled cells, the washing fraction, which should contain only a small amount of the other cell types and the elution fraction, containing the labelled cells. The procedure of MACSorting is described in detail in 2.2.4.7.2.

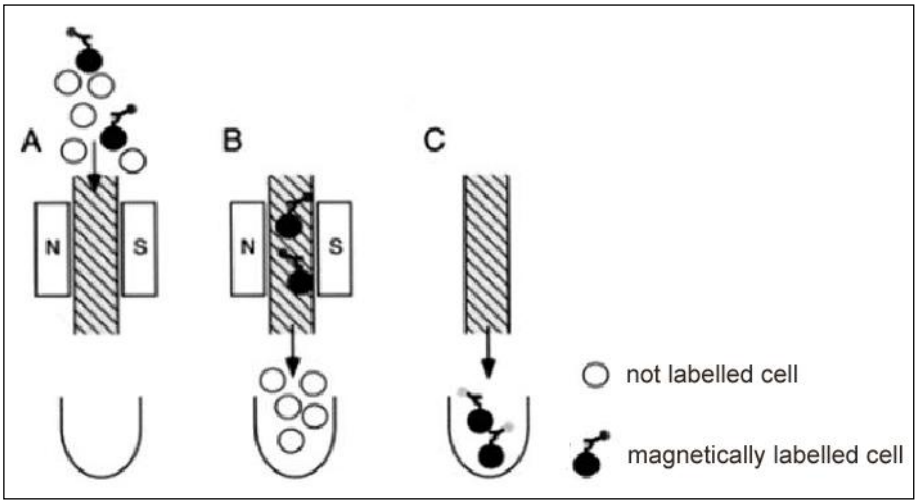


Fig. 4: Principle of Magnetic Activated Cell Sorting. The column providing a strong magnetic field is loaded with cell suspension containing a mixture of not labelled as well as magnetically labelled cells **(A)**. In the presence of the magnetic field labelled cells retain in the column, while not-labelled cells are flushed out **(B)**, which are finally eluted as positively selected cells when removed from the magnetic field **(C)**. modified; Thiel et al., 1998.

In brief, prior to MACSorting the testicular tissue sample (40 - 112 mg) was mechanically disaggregated and prepared as a single cell suspension, which was obtained using a two step enzymatic digestion with collagenase and DNaseI followed by additional treatment with hyaluronidase (2.2.4.3.7). Further the digested testicular cell suspension was incubated within a two step procedure with a specific biotinylated α6-Integrin antibody, which was labelled with MicroBeads binding to biotin in a second step. The elution fractions consisted of ~5.5 x 10^4 -1.2 x 10^6 cells / ml (Tab. 2) and the eluted α6-Integrin positive cells were resuspended in the appropriate culture medium and seeded on a one 6-well culture vessel providing a ~9.5 cm^2 surface area.

3.1.3 Cultivation of hSSCs

To date, a validated protocol for the long term culture of hSSCs has not been published. Nevertheless, several mouse and human studies revealed the importance of growth factors such as FGF (Fibroblast Growth Factor), GDNF (Glial cell line-Derived Neurotrophic Factor), EGF (Epidermal Growth Factor) and LIF (Leukemia Inhibitory Factor) for culturing isolated SSCs to provide the essential stem cell niche factors for the maintenance of SSC self-renewal, proliferation and differentiation (Kanatsu-Shinohara et al., 2003, 2005, 2011; Kubota et al., 2004a, b; He et al., 2008, 2010; Sadri-Ardekani et al., 2009; Lim et al., 2010; for review: Guo et al., 2014). Therefore, these growth factors seem to be indispensable and were included in the different media compositions, which were tested for culturing α6-Integrin positive cells derived from MACSortings (Tab. 3) at normoxia or hypoxia in order to obtain optimal culture conditions.

Tab. 3: Media compositions used for hSSC culture.

	StemPro+3	StemPro+4	Pluriton+3
FCS	1%	1%	-
NEAA	1x	1x	-
L-Glutamine	2 mM	2 mM	-
Sod. Pyr.	1 mM	1 mM	-
P/S	1%	1%	1%
2-ME	100 µM	100 µM	-
LIF	1000 U/ml	1000 U/ml	-
VitC	50 µg/ml	50 µg/ml	-
hbFGF	10 ng/ml	10 ng/ml	10 ng/ml
mEGF	20 ng/ml	20 ng/ml	20 ng/ml
hGDNF	10 ng/ml	10 ng/ml	10 ng/ml
activin A	-	10 ng/ml	-
medium	StemPro®-34 +supplement	StemPro®-34 +supplement	Pluriton™

FCS: Fetal Calf Serum; NEAA: Non-Essential Amino Acids; Sod. Pyr.: Sodium Pyruvate; P/S: Penicillin/Streptomycin; 2-ME: 2-Mercaptoethanol; LIF: Leukemia Inhibitory Factor; VitC: Vitamin C; hbFGF: human basic Fibroblast Growth Factor; mEGF: mouse Epidermal Growth Factor; hGDNF: human Glial cell line-Derived Neurotrophic Factor.

Beside these growth factors StemPro+3 medium, consisting of StemPro®-34 medium with StemPro® supplement, also included fetal calf serum and vitamin C (Smorag et al., 2012). StemPro+4 medium additionally contained activin A, which is known to promote proliferation of spermatogonia as a TGF-β family member (Mather et al., 1990). Pluriton+3 medium was composed of Pluriton™ Medium (2.2.4.11.1.2) from the Stemgent® mRNA Reprogramming System (3.2.2.1) supplemented with the growth factors FGF, GDNF and EGF. Some hSSC cultures were incubated in hypoxic environment. With all tested culture media α6-Integrin positive cells developed into typical SSC clusters with grape-like structures, whereas culturing of cells using StemPro+3 medium at 37°C and 5% CO_2 yielded in the best proliferation of cell clusters. However, clear differences in cell attachment were obtained using MEF feeder layer or other coated culture wells.

3.1.3.1 Culture of putative hSSCs on MEF feeder layer

Feeder layers are known to contribute to maintenance of an undifferentiated cell state of isolated SSCs and might facilitate cell attachment (Nagano et al., 2003a) Therefore after MACSorting α6-Integrin positive cells of MEF-1 and MEF-2 (Tab. 2) were cultured in StemPro+3 on MEF feeder layer at 37°C and 5% CO_2. Small cell clusters could be observed five days after seeding and revealed characteristic morphologies with grape-like structures

described for SSCs (Fig. 5). During further culture cells proliferated visibly into increasing cluster sizes, but detached from MEF feeder layer before they achieved a sufficient cell density for passaging.

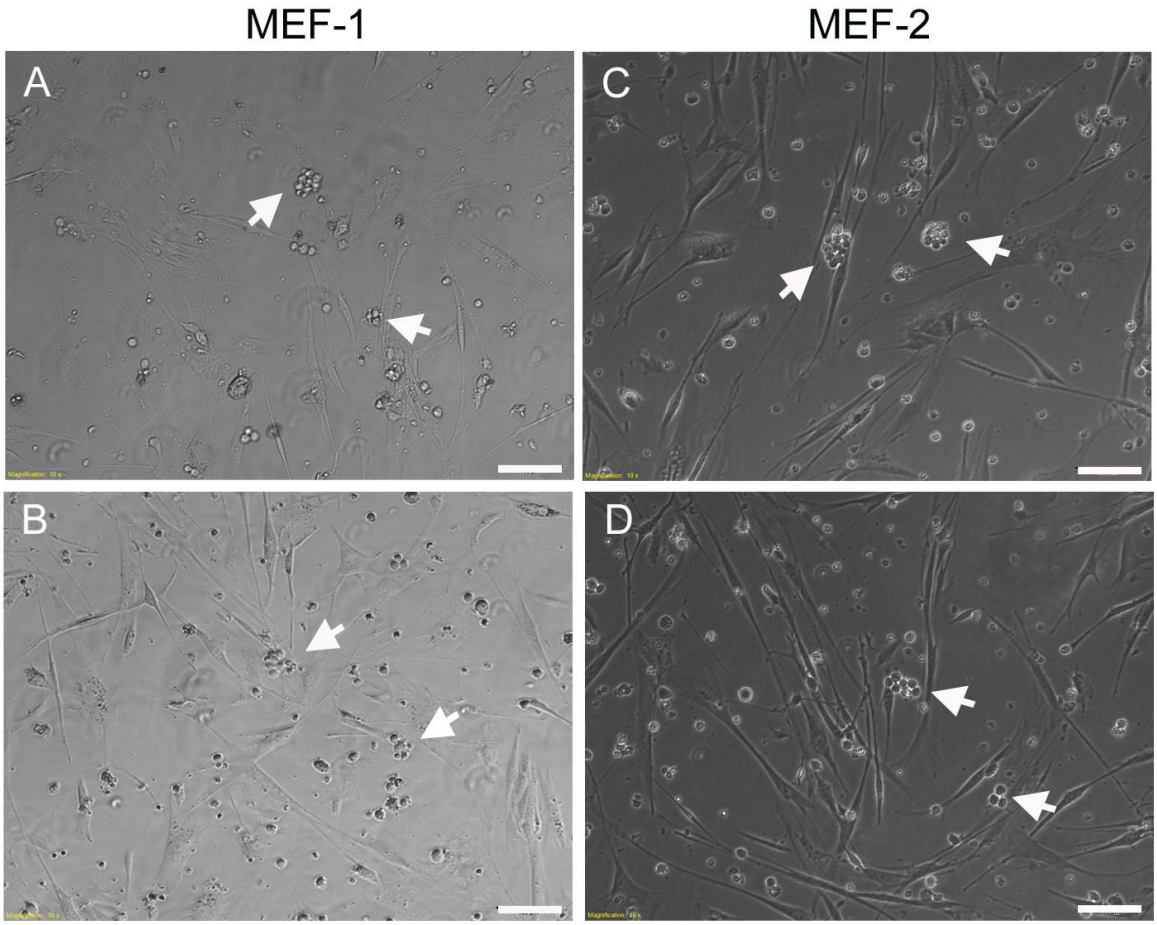


Fig. 5: Brightfield pictures of α6-Integrin positive cell clusters cultured on MEF feeder layer at day 5 after MACSorting. **(A+B)** MEF-1- and **(C+D)** MEF-2-derived cells were both cultured in StemPro+3 on MEF feeder layer at 37°C and 5% CO_2 and revealed SSC characteristic grape-like structures (arrows). Brightfield pictures were taken at day 5 after MACSorting. Scale bars: 100 µm.

3.1.3.2 Culture of putative hSSCs on gelatine coated culture wells

Other protocols propose a feeder-free culture condition using for example gelatine (Kanatsu-Shinohara et al., 2003) or laminin (Sadri-Ardekani et al., 2009) coated culture wells. This coating of plastic surfaces offers a matrix for enhanced cell attachment to the surface and cell proliferation (Goharbakhsh et al., 2013). Therefore α6-Integrin positive cells were cultured in StemPro+3 on 0.1% gelatine coated culture wells at 37°C and 5% CO_2 and revealed a better propagation during culture, which could be repeated for α6-Integrin positive cells derived from different testicular biopsies.

Fig. 6 shows the development of cells derived from MACSorting with testicular biopsies from two different patients (Gel-1 and Gel-2; Tab. 2) within the first 15 days of culture.

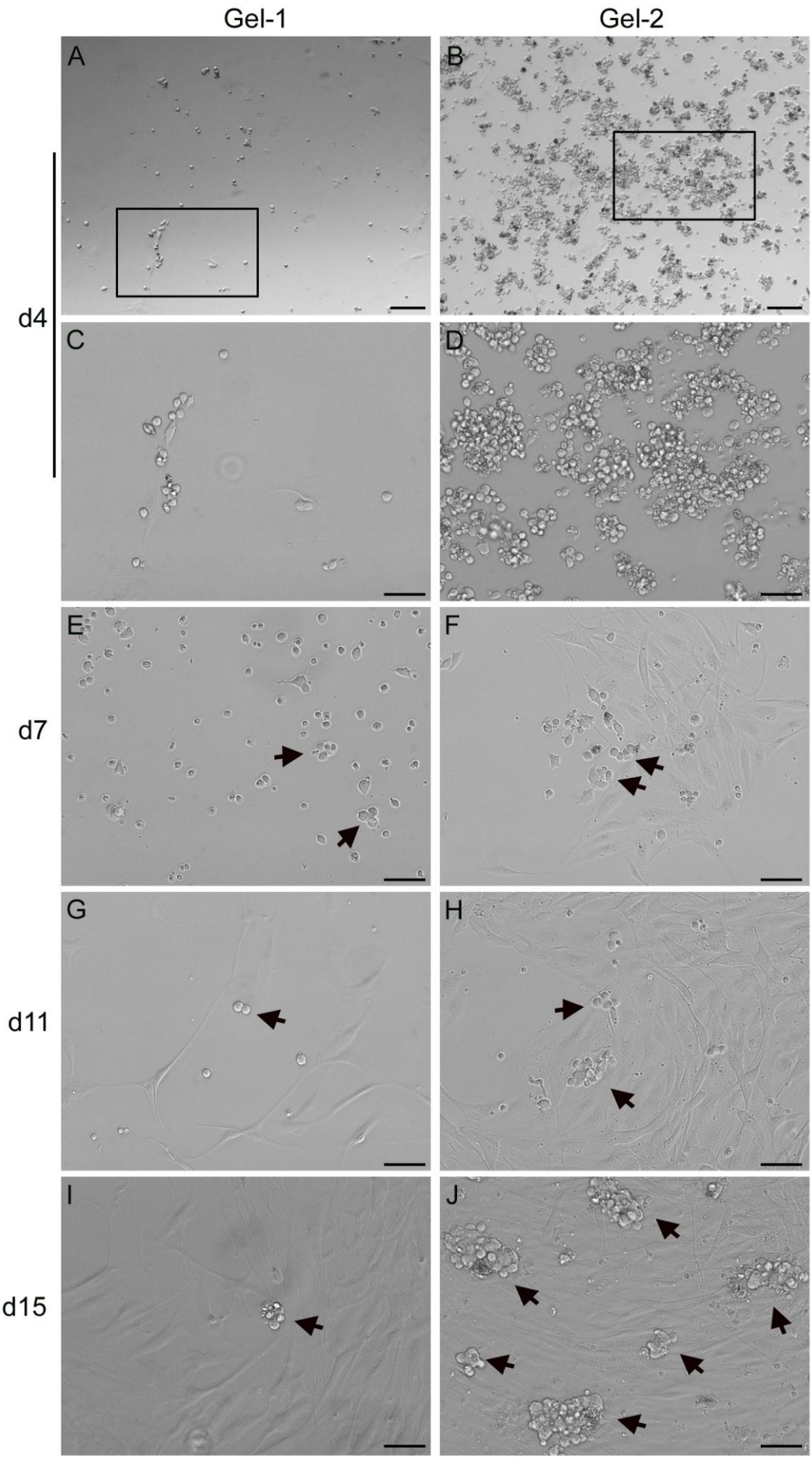

Gel-1
Gel-2
A
B
C
D
E
F
G
H
I
J
d4
d7
d11
d15

Fig. 6: Morphological changes of α6-Integrin positive cell clusters cultured on gelatine coated culture wells after MACSorting. Gel-1- and Gel-2-derived cells were cultured in StemPro+3 medium on gelatine coated culture wells at 37°C and 5% CO_2. **(A+C)** Gel-1 revealed small cell clusters 4 days after MACSorting, **(E, G, I)** which did not proliferate during further culture. **(B+D)** Gel-2-derived cells showed a higher cell density 4 days after MACSorting, **(F+H)** which initially decreased. **(J)** Remaining cell clusters clearly proliferated and revealed an increasing cluster size 15 days after MACSorting. Indicated regions in A and B are shown in a higher magnification in C and D, respectively. Arrows mark SSC-like structures. d: day after MACSorting. Scale bars: A+B: 200 µm; C-J: 100 µm.

Isolated cells adhered to the gelatine coated culture wells. Although the procedure of testis biopsies including mechanical disaggregation, enzymatic digestion and MACSorting was performed in parallel, Gel-1- and Gel-2-derived cells revealed obviously differences in attached cell densities. While Gel-1 cells formed just small clusters consisting of a few cells (Fig. 6 A+C) and did not proliferate as much as needed for passaging (Fig. 6 E, G, I), Gel-2 cells revealed a higher cell density (Fig. 6 B+C), which initially decreased (Fig. 6 F+H), but remaining cell clusters clearly proliferated, which was visible in increasing cluster size at day 15 after MACSorting (Fig. 6 J).

The seeded elution fraction also contained other cells with a morphology comparable to fibroblasts (Fig. 7 A). In spite of several washing steps during MACSorting these cells apparently remained within the column and were flushed out together with the putative hSSCs. Their density increased during culture and provided a natural testicular feeder for α6-Integrin positive cells during culture on gelatine coated wells (Fig. 7 C-F).

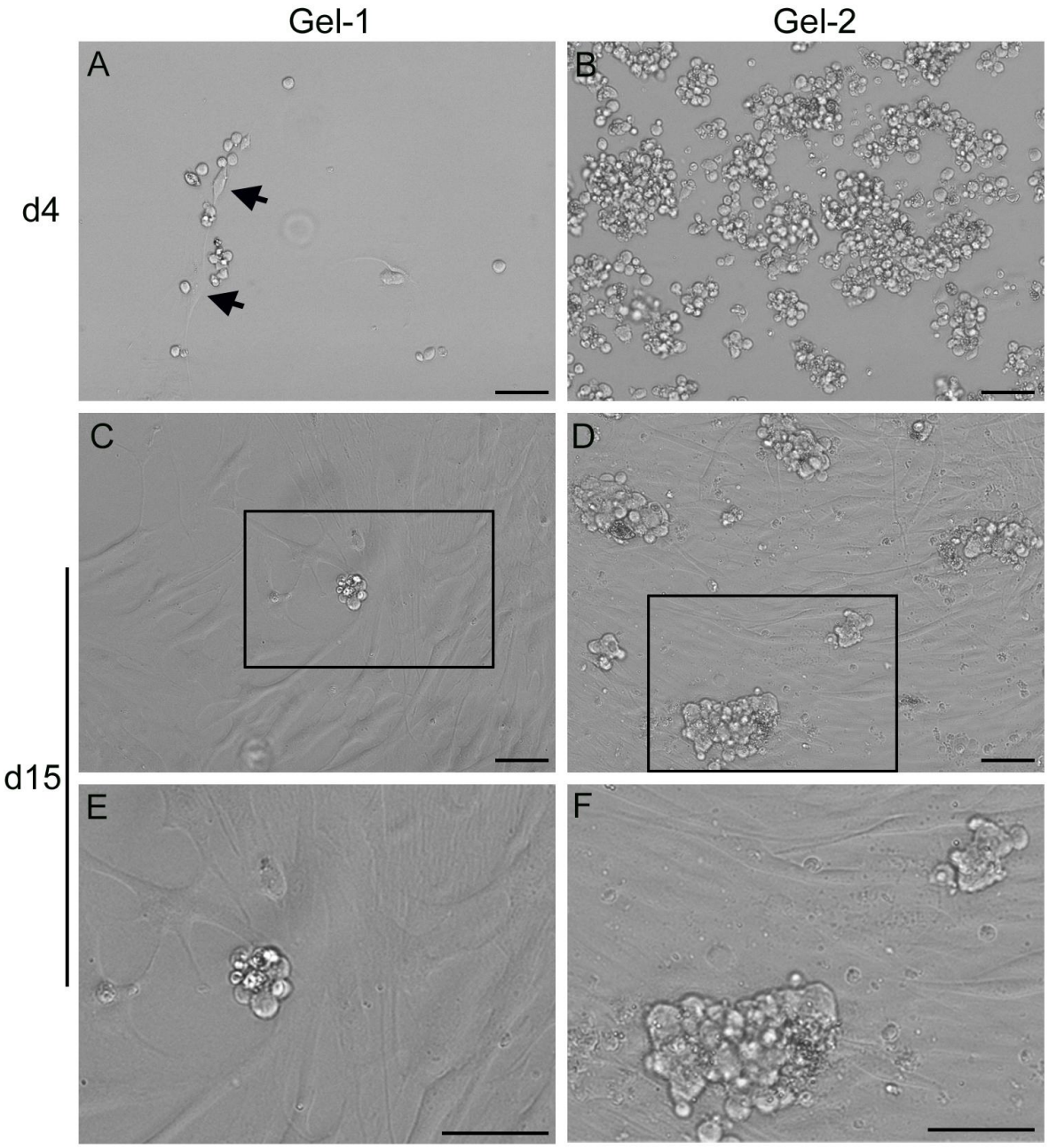


Fig. 7: Development of natural testicular feeder during culture of putative hSSCs on gelatine coated culture wells. (A+B) Gel-1- and Gel-2-derived cells cultured in StemPro+3 medium on gelatine coated culture wells at 37°C and 5% CO_2 at day 4 after MACSorting showing fibroblast-like cells (arrows). **(C+D)** These cells proliferated during culture providing a natural testicular feeder for cell clusters. Indicated regions in C and D are shown in a higher magnification in E and F, respectively. d: day after MACSorting. Scale bars: 100 µm.

Successful enrichment of putative hSSCs and their propagation cultured in StemPro+3 on gelatine coated culture wells at 37°C and 5% CO_2 could be repeated (Gel-9; Tab. 2; Fig. 8).

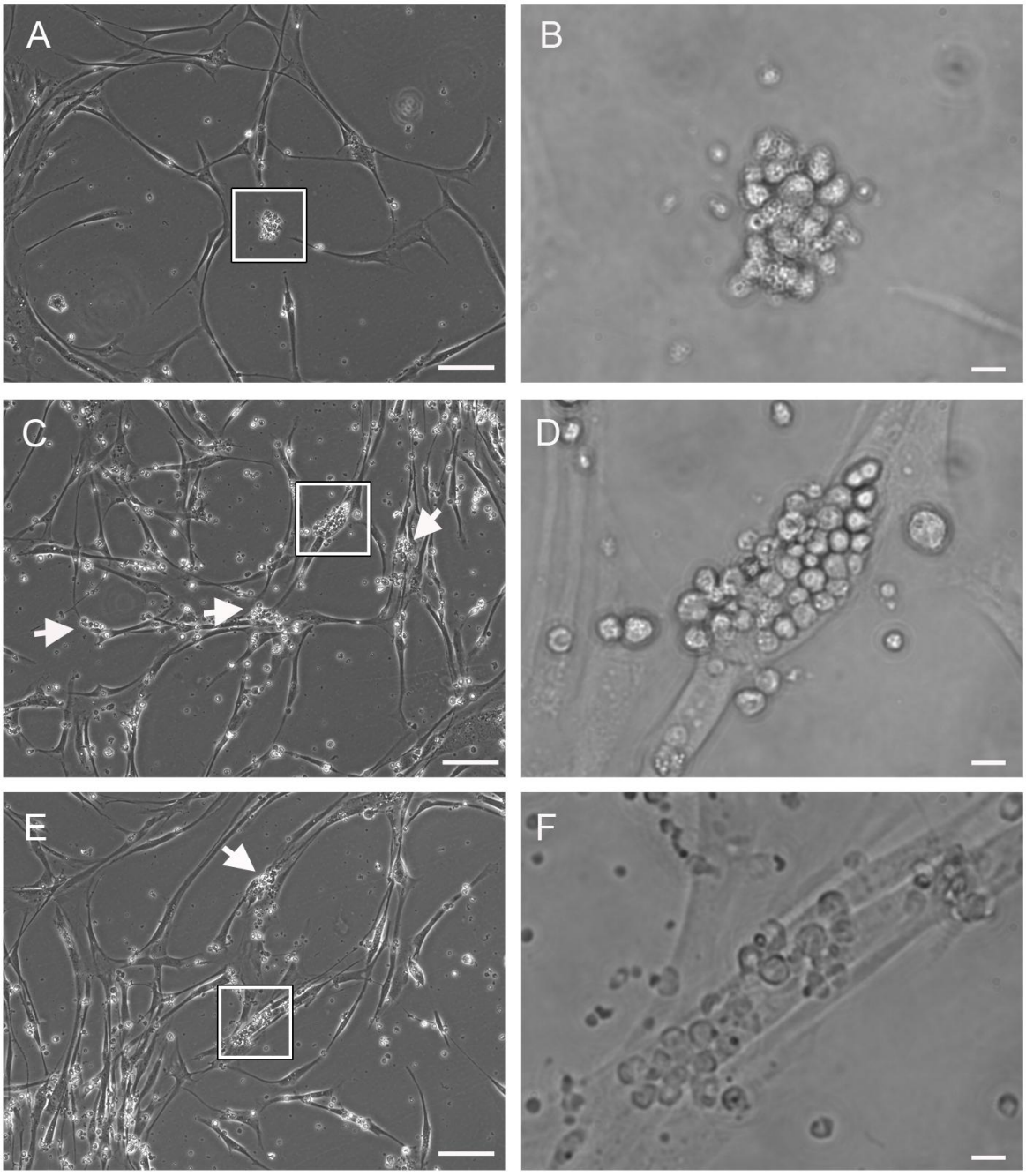


Fig. 8: Brightfield pictures of Gel-9-derived cells cultured on gelatine coated culture wells at day 15 after MACSorting. (A, C, E) Gel-9-derived cells, cultured in StemPro+3 medium on gelatine coated culture wells at 37°C and 5% CO_2, revealed SSC characteristic grape-like structures (arrows). **(B, D, F)** Magnifications of indicated regions in A, C and E, respectively. Scale bars: A, C, E: 100 µm; B, D, F: 10 µm.

3.1.3.3 Culture of hSSCs on matrigel coated culture wells

Matrigel is known as another effective feeder free surface for the cultivation of stem cells and plays an important role in the maintenance of ESC self renewal and pluripotency (Mallon et al., 2006). It is extracted from the Engelbreth-Holm-Swarm mouse sarcoma consisting of many extracellular matrix (ECM) proteins and is often used to mimic the ECM in cell culture. By providing several components like laminin and collagen as well as growth factors such as

FGF, EGF, IGF-1 (Insulin-like Growth Factor 1), TGF-β (Transforming Growth Factor β) and PDGF (Platelet-Derived Growth Factor), it facilitates cell attachment and proliferation. The cultivation of isolated mSSCs as well as embryonic stem cell-like cells derived from human testis on matrigel have already been reported (Mizrak et al., 2010; Choi et al., 2014). Therefore, it was suggested that cultivation of isolated α6-Integrin positive cells on matrigel coated culture wells might yield in an improved enrichment of putative hSSCs, which was achieved so far using gelatine coated surfaces. After MACSorting α6-Integrin positive cells were cultured in StemPro+3 on matrigel coated culture wells at 37°C and 5% CO_2 (M-1 and M-2, Tab. 2) as well as in hypoxic environment (M-3, Tab. 2). At first a high number of cells seemed to attach to the culture surface in both culture conditions. Nevertheless, the cells did not reveal a healthy phenotype and detached from the culture surface during minor movements of the culture plate. Addition of activin A to the medium did not yield in any improvement, but resulted in a high percentage of dead cells and just a less number of adhered cells, which did not proliferate well (M-4 and M-5, Tab. 2). In consideration of these observations the use of matrigel coated culture wells was not a suitable alternative to gelatine coated culture wells for the cultivation of putative hSSCs.

3.1.3.4 Passaging of putative hSSCs

Passaging of putative hSSCs on MEF feeder layer represents one of the most difficult steps during their culture. The cell clusters have to be detached, separated and seeded on new culture wells in order to generate a proliferating cell line. In the presented thesis the efficiency of cell attachment as well as further proliferation on new culture wells of the isolated cells was very low. When cell clusters reached high density (Fig. 9 A), these clusters were detached from the mother plate by using a pipette with 200 µl volume for mechanical dissociation. The detached cells were rinsed with appropriate medium and transferred on freshly prepared MEF feeder layer, which should facilitate cell adherence in the absence of their natural testicular feeder (Fig. 7). Passaged cells were further cultured in StemPro+3 at 37°C and 5% CO_2. Cell clusters were visible, but did not reach proliferation or a cluster density comparable to the mother plate (Fig. 9 B-E). In summary, no proliferating cell line representing hSSCs derived from MACSorting of testicular biopsies could be established in the context of this thesis.

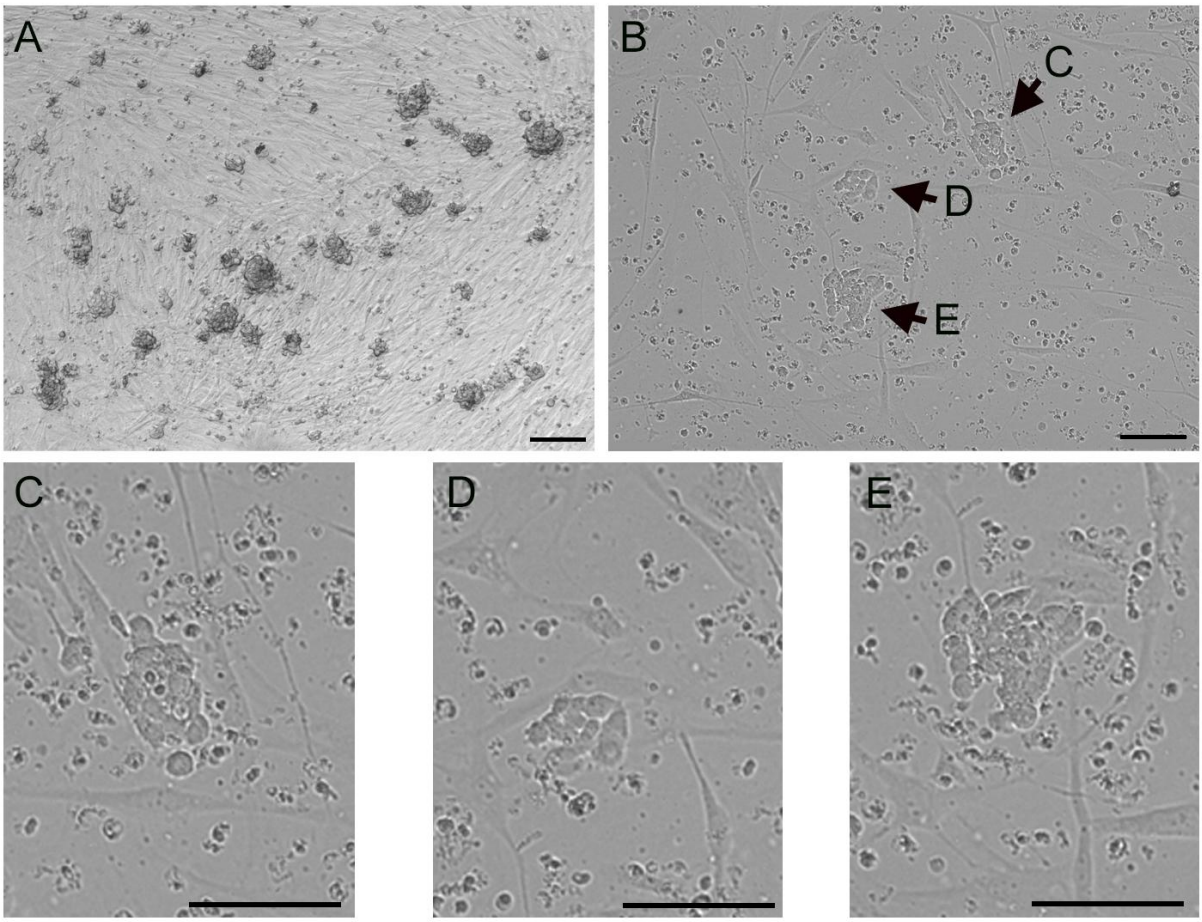


Fig. 9: Gel-2-derived putative hSSCs three days after passaging on MEF feeder layer. (A) Mother plate with putative hSSC clusters, which had to be passaged. **(B)** Just a few cells attached and formed SSC-like clusters three days after passaging on MEF feeder layer cultured in StemPro+3 medium at 37°C and 5% CO_2, but did not reach proliferation and cluster density in comparison to the mother plate. **(C-D)** Arrow marked colonies in B are shown in a higher magnification. Scale bars: 100 µm.

3.1.3.5 Characterization of enriched putative hSSCs

The developing cell clusters could not be established in proliferating cell lines, so that no adequate characterization of the enriched α6-Integrin positive cells could be performed. Nevertheless, some cells (Gel-4, Gel-5, Tab. 2) were directly seeded for immunocytochemical analysis after MACSorting in order to check if the cells isolated from testicular biopsies indeed represented hSSCs. α6-Integrin positive cells were cultured in StemPro+3 medium on gelatine coated cover slips (Gel-4) or chamber slides (Gel-5) at 37°C and 5% CO_2. Immunocytochemical analyses were performed with the already described SSC markers PLZF (Promyelocytic Leukaemia Zinc Finger; He et al., 2010), FGF3 (Fibroblast Growth Factor Receptor 3; Kopylow et al., 2012) and PGP9.5 (Protein Gene Product 9.5; von Kopylow et al., 2010). These analyses were complicated by technical problems, because many cells detached from the culture surface during the procedure of immunostaining, which includes several washing steps. Although those were performed very carefully, after staining

with FGF3 and PGP9.5 no cells were left on the surface, which could be analyzed, while a very small number of PLZF stained cells were left for microscopy (Fig. 10). A sequential image series of a cell cluster was taken in order to record the full depth of the cluster. These images revealed some PLZF stained cells within the cell cluster (Fig. 10). Surrounding immunonegative cells served as an internal negative control.

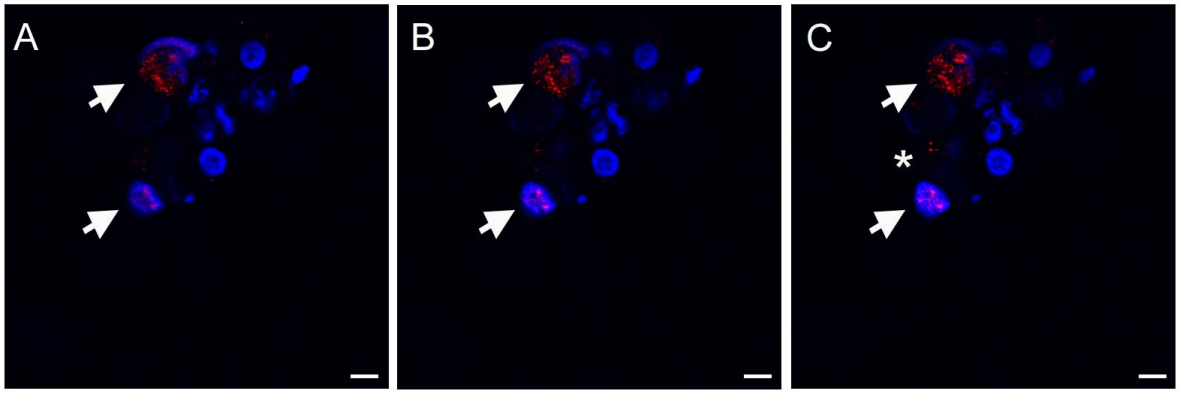


Fig. 10: PLZF staining of putative hSSCs derived from Gel-5. (A-C) Three consecutive images (no. 13-15) of a cell cluster derived from Gel-5, which was analyzed by taking 20 sequential images after immunostaining with a specific PLZF antibody. Arrow marked cells were positively stained for PLZF. Scale bars: 5 µm.

3.2 Generation of hiPSCs from infertile men

3.2.1 Cell lines used for hiPSC generation

Two different types of cells were used for the generation of hiPSCs from infertile men. In addition to HTF cell lines, which were derived from MACSortings with human testicular biopsies from infertile men (2.2.4.3.6; 3.1.1), Scrotal Human Fibroblast (SHF) cell lines derived from scrotal skin samples were used for reprogramming experiments (2.2.4.3.8). Altogether 25 SHF cell lines derived from patients characterized with different spermatogenesis phenotypes were generated and are available in a cryopreserved SHF cell line bank (Tab. 4).

Tab. 4: SHF cell lines established from scrotal skin tissue from men with different spermatogenesis phenotypes.

SHF	spermatogenesis phenotype	proliferation
1	hypergonadotropic NOA with dominant SCO tubuli	~
2	NOA with dominant SCO tubuli; sporadic tubuli with hypospermatogenesis	+
3	spermatogenesis-arrest at the level of primary spermatocytes	+
4	OA; after vasectomy; moderate hypospermatogenesis	+
5	pronounced hypospermatogenesis	+
6	dominant SCO; sporadic detection of spermatogonia	+
7	dominant SCO; sporadic tubuli with hypospermatogenesis	~
8	NOA; mixed atrophy; sporadic tubuli with hypospermatogenesis	+
9	OA; intact spermatogenesis	+
10	OA; predominant intact spermatogenesis	+
11	NOA; dominant SCO; 1 tubule with hypospermatogenesis	+
12	cryptozoospermia; tubuli with hypospermatogenesis	+
13	SCO	~
14	OA; predominant intact spermatogenesis	+
15	dominant SCO; sporadic tubuli with hypospermatogenesis	+
16	OA; intact spermatogenesis	+
17	spermatogenesis-arrest at the level of primary spermatocytes	~
18	widely intact spermatogenesis	~
19	intact spermatogenesis	+
20	dominant SCO; sporadic detection of spermatogonia	+
21	SCO	+
22	SCO	~
23	SCO	+
24	SCO	~
25	dominant SCO; sporadic tubuli sections with spermatogenesis	+

Diagnoses were confirmed by Prof. Schulze, Department of Andrology, University Hospital Hamburg-Eppendorf in Hamburg. The proliferation rate was assessed by observation during cultivation. SHF: Scrotal Human Fibroblast; NOA: Non Obstructive Azoospermia; OA: Obstructive Azoospermia; SCO: Sertoli Cell Only Syndrome; ~: slow proliferation; +: moderate proliferation.

3.2.2 Establishment of mRNA reprogramming

The generation of hiPSCs from infertile men was performed using the mRNA reprogramming method, which offers an efficient, non-integrative strategy for hiPSC derivation by repeated administration of mRNA encoding for the transcription factors hOct4, hKlf4, hSox2, hc-Myc and hLin28 (hOKSML) (Warren et al., 2010; Yakubov et al., 2010). The used synthetic mRNA includes modifications in order to increase ectopic protein expression in transfected cells, to improve their viability as well as to decrease the antiviral responses of the cells to the repeated application of synthetic mRNA (Warren et al., 2010). Based on these findings, the Stemgent® mRNA Reprogramming System provides a self-described validated protocol for reprogramming human fibroblasts, which was planned to be used in the presented thesis. In the course of practical work, the original mRNA reprogramming protocol published by Stemgent had to be modified in order to successfully reprogram fibroblasts of infertile men. These modifications tested within four trials of reprogramming are described below. Here the main focus is on the performed transfections as well as the used hiPSC media for culturing emerged hiPSC colonies. Finally Tab. 5 outlines all different parameters used in the four trials of hiPSC generation by mRNA reprogramming.

Tab. 5: Overview of reprogramming trials for the generation of hiPSCs.

		1st trial		2nd trail		3rd trial		4th trial					
cell preparation	target cells	BJ	HTF-2	BJ	SHF	HTF-2	HTF-10	BJ	SHF-3	SHF-6	SHF-9	SHF-12	SHF-13
	passage	P9	P2	P12	P11	P10	P12	P11	P4	P4	P2	P4	P2
	culture medium	BJ	FB	FB	FB	FB	FB	FB	FB	FB	FB	FB	FB
	cell density	1 x 10^4 1 x 10^5	1 x 10^5	2 x 10^4	7.5 x 10^4 1 x 10^5	1 x 10^5	1 x 10^5	2 x 10^4	1 x 10^5	1 x 10^5	1 x 10^5	1 x 10^5	1 x 10^5
	feeder layer	NuFF	NuFF	Matrigel	Matrigel	Matrigel	Matrigel	Matrigel	Matrigel	Matrigel	Matrigel	Matrigel	Matrigel
transfections	mRNA transfections	d0-d17	d0-d20	d0-d11	d0-d13	d0-d12	d0-d7	d0-d13	d0-d13	d0-d15	d0-d13	d0-d16	d0-d11
	miRNA transfections	-	-	d-1, d4	d-1, d4	d-1, d4, d11	d-1, d4	d-1, d4	d-1, d4	d-1, d4	d-1, d4	d-1, d4, d12	d-1, d4
	nGFP expression	+	+	+	+	+	+	+	+	+	+	+	+
	splitting	+	-	-	-	-	-	-	-	-	-	+	-
hiPSCs	emergence of colonies	+	-	+	+	+	-	+	+	+	+	+	+
	manually picking	≥ d21	-	≥ d14	-	≥ d8	-	≥ d14	≥ d12	≥ d12	≥ d15	-	≥ d12
establishment	hiPSC medium	hiPSC-M1	-	NuFF-C-P; hiPSC-M2	-	NuFF-C-P; hiPSC-M2; hiPSC-M3	-	NuFF-C-P; hiPSC-M3; hiPSC-M4				-	NuFF-C-P; hiPSC-M3; hiPSC-M4
	culture condition	normoxia	normoxia	hypoxia	hypoxia	hypoxia	hypoxia	hypoxia	hypoxia	hypoxia	hypoxia	hypoxia	hypoxia
	comment	loss of hiPSCs before establish-ment	no emergence of colonies	loss of hiPSCs before establish-ment	loss of hiPSCs before manually picking	sponta-neous differentia-tion of hiPSCs	increased apoptosis from d4 onwards; trans-fection series stopped at d8	increased apoptosis from d6 onwards	increased apoptosis from d6 onwards	increased apoptosis from d6 onwards	almost no attached cells at d6	no increased apoptosis	increased apoptosis from d6 onwards

BJ: Stemgent® BJ Human Fibroblasts and culture medium for BJs; HTF: Human Testicular Feeder cell line; SHF: Scrotal Human Fibroblast cell line; P: passage number of cell line used for reprogramming trial; FB: Fibroblast Medium; NuFF: Mitomycin treated Neonatal Human Foreskin Fibroblasts; d: day; ≥d: from day onwards; hiPSC-M: hiPSC medium according to Tab. 6; NuFF-C-P: NuFF-conditioned Pluriton™ Reprogramming Medium.

The different tested hiPSC media are specified in Tab. 6.

Tab. 6: hiPSC media compositions tested during establishment of hiPSC culture.

	hiPSC-M1	hiPSC-M2	hiPSC-M3	hiPSC-M4
FCS	-	15%	-	-
KO™-SR	25%	5%	15%	15%
NEAA	1x	5x	5x	5x
L-Glutamine	2.5 mM	-	-	-
GlutaMAX™	-	5x	5x	5x
P/S	1.25%	1.25%	1.25%	1.25%
2-ME	62.5 µM	25 µM	25 µM	25 µM
hbFGF	20 ng/ml	20 ng/ml	10 ng/ml	5 ng/ml
medium	DMEM/F12	DMEM/F12	DMEM/F12	DMEM/F12

hiPSC-M: hiPSC medium; FCS: Fetal Calf Serum; KO™-SR: Knock Out-Serum Replacement; NEAA: Non-Essential Amino Acids; P/S: Penicillin/Streptomycin; 2-ME: 2-Mercaptoethanol; hbFGF: human basic Fibroblast Growth Factor.

3.2.2.1 Stemgent® mRNA Reprogramming System

The first mRNA reprogramming (1st trial; Tab. 5) was performed according to Stemgent`s protocol (www.stemgent.com; Fig. 11).

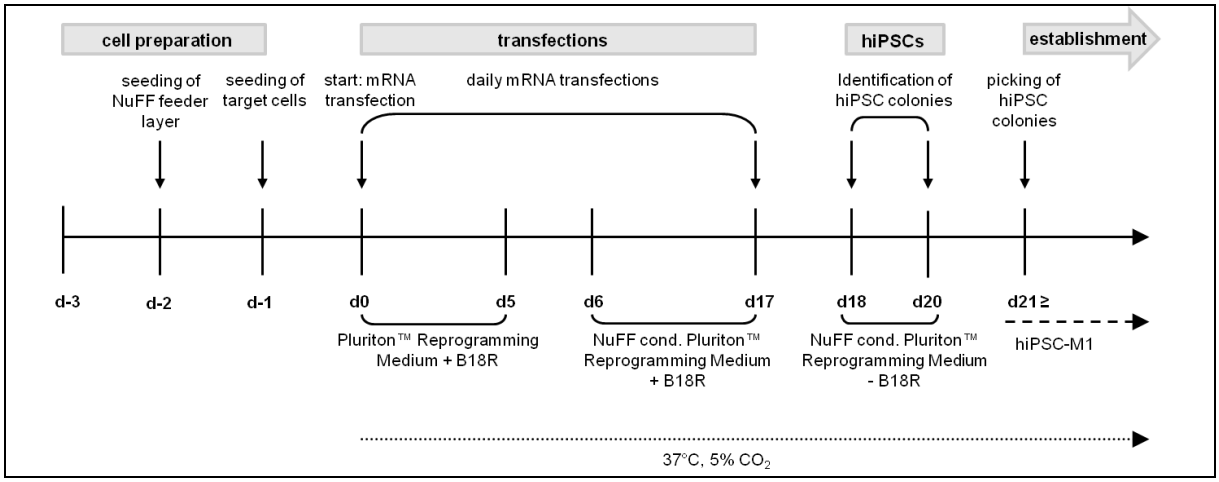


Fig. 11: Schematic overview of the Stemgent® mRNA Reprogramming System. According to Stemgent`s protocol the mRNA reprogramming of human fibroblasts includes cell preparation with seeding of the target cells on NuFF feeder layer, a hOKSML-mRNA transfection series on 18 consecutive days, identification of hiPSCs and further establishment of stable hiPS cell lines. During the reprogramming process cells are cultured in different provided media at 37°C and 5% CO_2. d: day; hiPSC-M1: hiPSC medium 1.

In brief, target cells were seeded on NuFF feeder layer and transfected on six consecutive days with hOKSML-mRNA in provided Pluriton™ Reprogramming Medium consisting of Pluriton™ Medium supplemented with 20 ng/ml bFGF and 200 ng/ml B18R. The protein

B18R, a Vaccinia virus decoy receptor for Type I interferons (Symons et al., 1995), is described to further increase cell viability during RNA transfections in some cell types (Warren et al., 2010). Further transfections until day 17 were performed in NuFF-conditioned Pluriton™ Reprogramming Medium with 200 ng/ml B18R (NuFF-C-P +), generated by culturing NuFF feeder layer with provided Pluriton™ Medium supplemented with 20 ng/ml bFGF. Because at this point of reprogramming the original NuFF feeder layer did not provide all required nutrients for the target cells anymore, this change to NuFF-C-P + was indispensable in the support of cells undergoing reprogramming. First hiPSC colonies might be seen as early as day 8, and should be finally identified between day 18 and day 20. Since day 18 cells were cultured in NuFF-conditioned Pluriton™ Reprogramming Medium supplemented with 20 ng/ml bFGF, but without B18R (NuFF-C-P -). The hiPSC colonies were picked manually since day 21 onto MEF feeder layer and cultured in adequate hiPSC medium. The first mRNA reprogramming experiment was carried out with a human testicular feeder cell line (HTF-2; 1 x 10^5 cells; Fig. 12 C) derived from a MACSorting with a human testicular biopsy from an infertile man with a spermatogenesis classified at modified Johnsen Score 10 (3.1.1). Stemgent® BJ Human Fibroblasts (BJs), established from normal human foreskin and characterized by a long lifespan and high proliferation rate, were used in two different cell densities (1 x 10^4 cells; 1 x 10^5 cells; Fig. 12 A, B) as a reprogramming control as recommended by the Stemgent® mRNA Reprogramming Protocol.

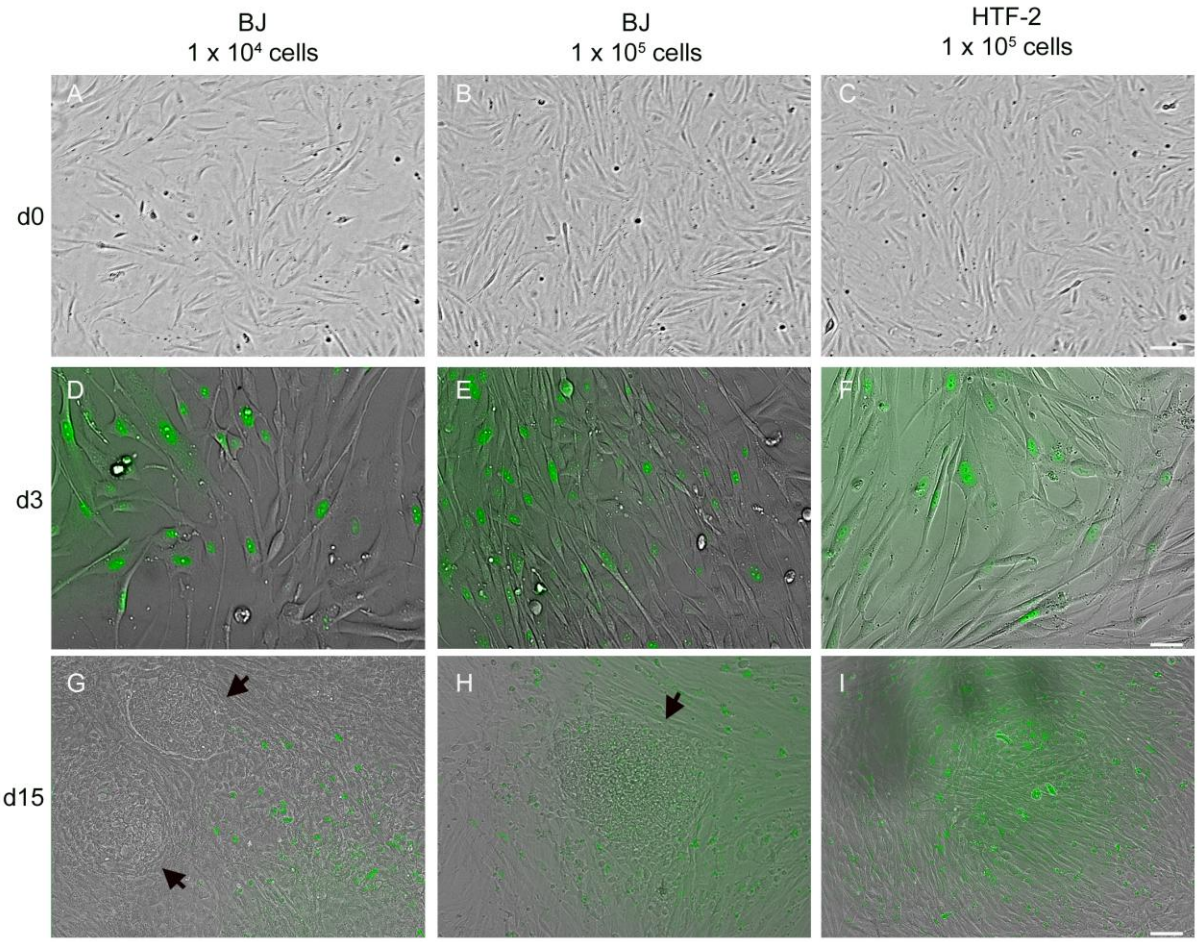


Fig. 12: Morphology changes of BJs and HTF-2 cells during mRNA reprogramming using the Stemgent® mRNA Reprogramming System (1st trial). (A-C) The morphology of target cells BJs and HTF-2, seeded in a density of 1 x 10^4 cells / 1 x 10^5 cells and 1 x 10^5 cells, respectively, was checked on day 0 before the start of hOKSML-mRNA transfection series. **(D-F)** Uniform nGFP expression confirmed successful transfection of BJs and HTF-2 cells on day 3. **(G-I)** BJs revealed morphology changes with final colony formation of iBJs (arrows) and further nGFP expression in surrounding cells. HTF-2 cells still showed uniform nGFP expression without any iPS colony formation. d: day. iBJ: BJ-hiPSC. Representative scale bars are shown in C, F and I. Scale bars: A-C: 200 µm; D-I: 100 µm.

The transfected cells were observed daily. In addition to the transcription factors (hOKSML) the mRNA cocktail contained mRNA encoding nGFP thereby offering the opportunity to assess the cells transfection efficiency by nGFP expression. The transfected BJ- as well as HTF-2 cells showed nGFP expression uniform throughout the culture dish since day 3 (Fig. 12 D-F). During the transfection series BJ cells underwent morphology changes and formed small, compact clusters distinguishable from the NuFF feeder layer. Finally these changes ended up in the formation of BJ-hiPSC (iBJ) colonies with their typically defined colony edges (Fig. 12 G+H). In contrast to cells within iBJ colonies, surrounding cells further expressed nGFP indicating ongoing application of mRNA cocktail. This morphology development was not observed with HTF-2 cells, whereas transfection efficiency was confirmed by nGFP expression until the end of the transfection series (Fig. 12 I). The striking difference between

BJ- and HTF-2 cells was the proliferation rate during the reprogramming process. In comparison to BJ cells, HTF-2 cells exhibited a very low proliferation rate.

iBJ colonies were picked manually since day 21 on freshly prepared MEF and NuFF feeder layer, respectively (Fig. 13 A+B), and cultured in hiPSC medium 1 (hiPSC-M1; Tab. 6) prepared according to Stemgent's protocol. Because there was no notable difference in the use of MEF and NuFF feeder layer, further culture was performed using the more available MEF feeder layer. iBJs could be propagated in some culture dishes (Fig. 13 C) and cultured until passage No. 4 (Fig. 13 D-E), but could not be successfully cryopreserved before iBJs were lost.

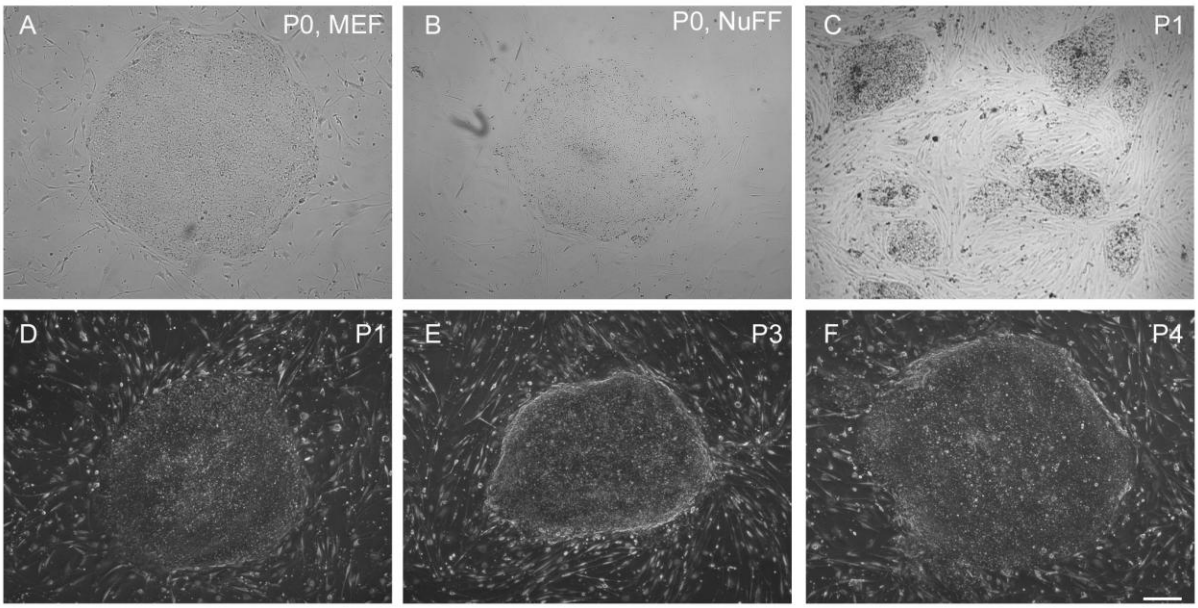


Fig. 13: Manually picking and expansion of iBJ colonies. iBJ colonies (iBJs) of different passage numbers cultured on MEF or NuFF feeder layer are shown. Passage (P) numbers are indicated within the pictures. **(A + B)** Emerged iBJs were picked manually on MEF and NuFF feeder layer, respectively, since day 21 of reprogramming time table, **(C)** could be propagated in some culture wells and **(D-F)** cultured until passage No. 4 using hiPSC-M1 and MEF feeder layer. Representative scale bar in F: 200 µm.

During the attempt of culture establishment, iBJs revealed a positive AP-staining as well as OCT4 (Octamer-binding Transcription Factor 4) staining demonstrating their pluripotent character (Fig. 14).

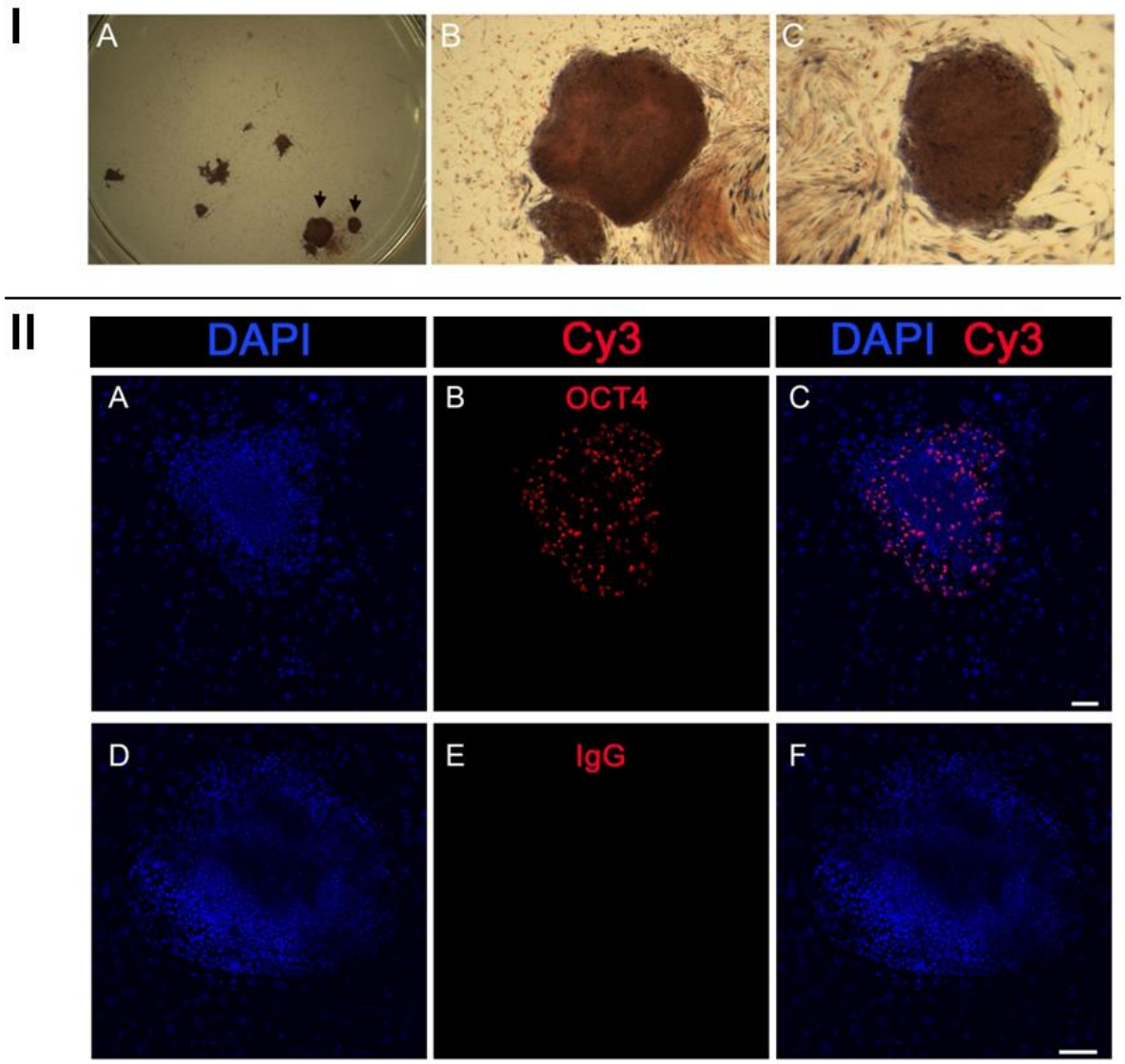


Fig. 14: AP- and OCT4 staining demonstrating the pluripotent character of iBJ colonies. (I) iBJ colonies (iBJ) revealed a positive AP-staining. Arrow marked colonies **(A)** are shown in a higher magnification in **B** and **C**. **(II)** Immunocytochemical staining was performed with iBJs using a specific antibody for the nuclear pluripotency marker OCT4 **(A-C)**. Representative overlay image is shown in C. Incubation with IgG instead of first antibody was used as negative control **(D-F)**. Representative scale bars in C and F: 100 µm.

3.2.2.2 "Feeder Free" mRNA/miRNA Reprogramming

The successfully generation of iBJs proofed the functionality of the used Stemgent® mRNA Reprogramming System, whereas it was not sufficient for the generation of hiPSCs from a cell line derived from an infertile man (3.2.2.1). Upon the advice of Johanna Goldmann (PhD-student of Rudolph Jaenisch's group at the Whitehead Institute for Biomedical Research, Cambridge, USA) some parameters within the mRNA reprogramming protocol were changed, which provided a shortened reprogamming protocol and finally led to successful generation of patient hiPSCs (Fig. 15).

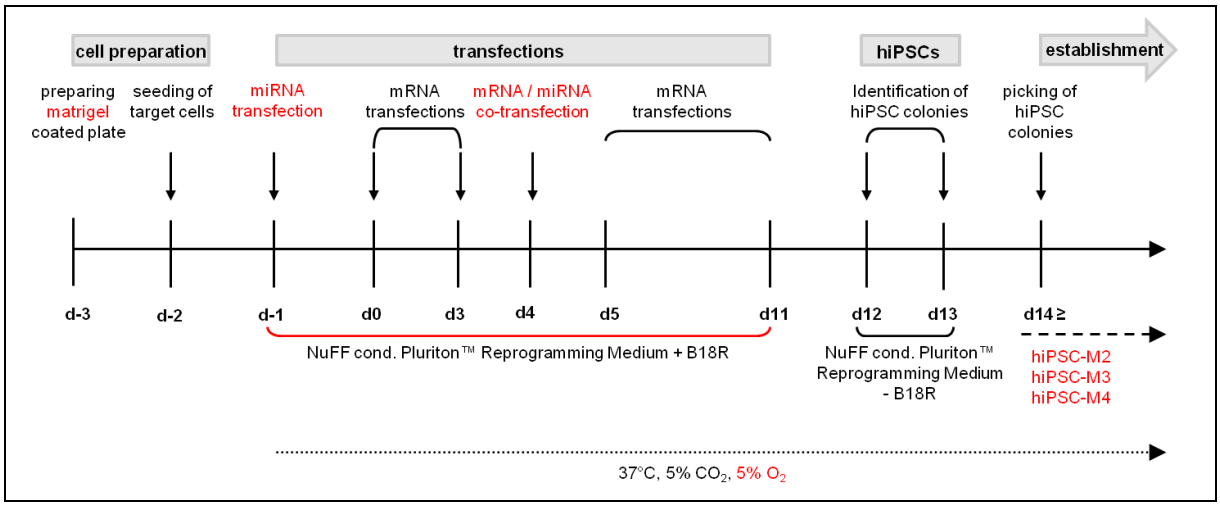


Fig. 15: Schematic overview of "Feeder Free" mRNA/miRNA Reprogramming. The Stemgent® mRNA Reprogramming System for generation of hiPSCs was modified in several parameters, which are highlighted in red. The final "Feeder Free" mRNA/miRNA Reprogramming was performed with target cells seeded on matrigel, additional miRNA transfections with miRNA cluster 302-367 on day -1 (d-1) and day 4 (d4) and culture of cells with conditioned medium during the whole reprogramming process under hypoxic conditions (37°C, 5% CO_2, 5% O_2). These modifications led to a faster emergence of hiPSC colonies. For establishment of stable hiPS cell lines further three hiPSC media were tested. d: day; hiPSC-M1, -2, -3: different hiPSC media.

Improvement of reprogramming was achieved by substitution of NuFF feeder layer with matrigel coated reprogramming plates, reprogramming in hypoxic environment and additional transfections with miRNA cluster 302-367. The modifications in the so called "Feeder Free" mRNA/miRNA Reprogramming are highlighted in Fig. 15. miRNAs are described to modulate several ESC functions such as self renewal (Xu et al., 2009) and differentiation as well as cell cycle progression (Wang et al., 2008). Additionally Ruiz et al. (2011) showed a positive correlation between cell proliferation and reprogramming efficiency. Because the overexpression of the miRNA cluster 302-367 is known to increase the mouse iPSC generation by effecting the mesenchymal-to-epithelial transition as well as the cell cycle (Liao et al., 2011), additional application of this miRNA cluster might increase the proliferation rate of target cells, which was the main difference between BJ- and HTF-2 cells during the reprogramming process explained above (3.2.2.1). One miRNA 302-367 transfection was performed before the start of the transfection series using the hOKSML mRNA cocktail, whereas cells were co-transfected using miRNA 302-367 and mRNA cocktail at day 4. All transfections were performed in NuFF-C-P + in order to provide additionally NuFF feeder layer derived nutrients during reprogramming on matrigel. Using "Feeder Free" mRNA/miRNA Reprogramming well proliferating cells like BJ fibroblasts can be reprogrammed within a mRNA transfection series of 11 days.

First "Feeder Free" mRNA/miRNA Reprogramming (2nd trial; Tab. 5) was carried out with scrotal human fibroblasts (SHF; 7.5 x 10^4 cells; 1 x 10^5 cells; Fig. 16 B+C) derived from a

patient with an intact spermatogenesis. BJ fibroblasts were used as a reprogramming control (2 x 10^4 cells; Fig. 16 A).

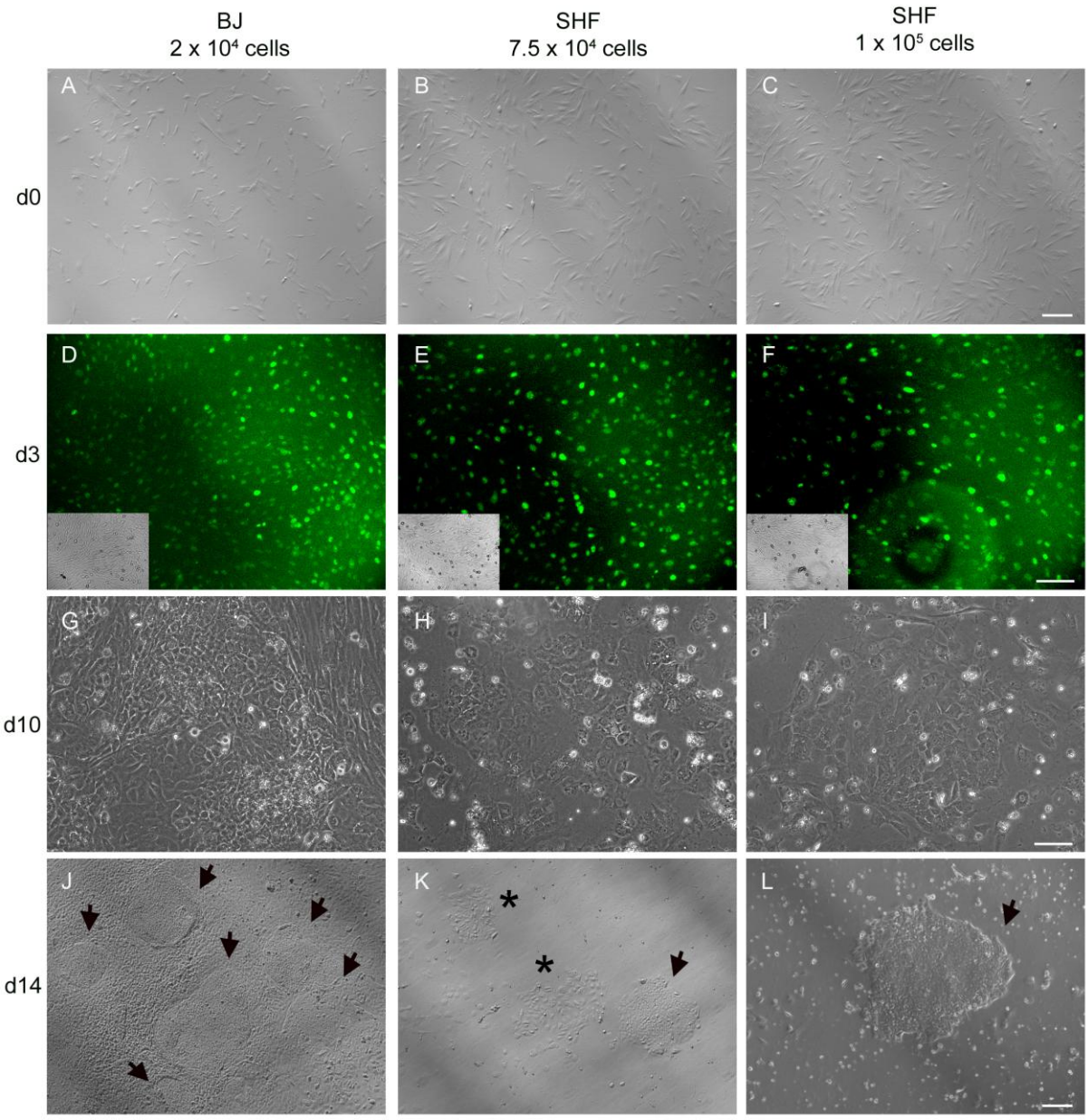


Fig. 16: Morphology changes of BJs and SHF cells during "Feeder Free" mRNA/miRNA Reprogramming (2nd trial). (A-C) The morphology of target cells BJs and SHF, seeded in a density of 2 x 10^4 cells and 7.5 x 10^4 cells / 1 x 10^5 cells, respectively, was checked on day 0 before start of hOKSML-mRNA transfection series. **(D-F)** Uniform nGFP expression confirmed successful transfection of BJs and SHFs on day 3. Brightfield images are shown in inlays. **(G-I)** Both transfected BJs and SHFs revealed formation of small clusters with compact morphology until day 10. SHFs showed a decreased number of attached cells. **(J-L)** Final formation of iBJ- and iSHF colonies was observed (arrows), whereas SHFs also revealed not fully reprogrammed iSHFs (stars). d: day. Scale bars: A-C, J-L: 200 µm; D-I: 100 µm.

The uniform nGFP expression revealed successful transfections in all cell lines tested (Fig. 16 D-F). During the transfection series SHF- and BJ cells formed small clusters with a compact morphology (Fig. 16 G-I), which finally appeared as hiPSCs (Fig. 16 J-L) and proofed the functionality of the "Feeder Free" mRNA/miRNA Reprogramming for generation

of patient-derived hiPSCs. Nevertheless, SHF responded to the transfection series with an increased apoptosis rate from day 10 onwards, which was visible in the decreased number of attached SHF cells in comparison to BJs (Fig. 16 G-L). Moreover SHF cells formed some not completely reprogrammed as well as a less number of hiPSC colonies compared to BJ fibroblasts (Fig. 16 K+L). iBJ colonies could be picked manually from day 14 onwards and cultured in NuFF-C-P - with 20 ng/ml bFGF. During further culture this medium was gradually replaced by hiPSC medium 2 (hiPSC-M2; Tab. 6) prepared according to the protocol provided by Johanna Goldmann. Picked iBJs were lost before a suitable propagation and storage of these cells as well as an evaluation of hiPSC-M2 for culturing the hiPSCs.

A repetition of “Feeder Free“ mRNA/miRNA Reprogramming (3rd trial; Tab. 5) using two different patient cell lines, HTF-2 (1 x 10^5 cells, intact spermatogenesis; Fig. 17 A) and HTF-10 (1 x 10^5 cells, not intact spermatogenesis; Fig. 17 B), yielded in uniform nGFP expression representing successful transfections of both cell lines (Fig. 17 C+D), while only HTF-2 transfected cells revealed hiPSC colony formation starting at day 7 (Fig. 17 E). HTF-10 cells responded to the transfection series with an increased apoptosis rate from day 4 onwards and revealed an insufficient cell density for reprogramming at day 7 (Fig. 17 F), which finally caused the stop of its transfection series at day 8.

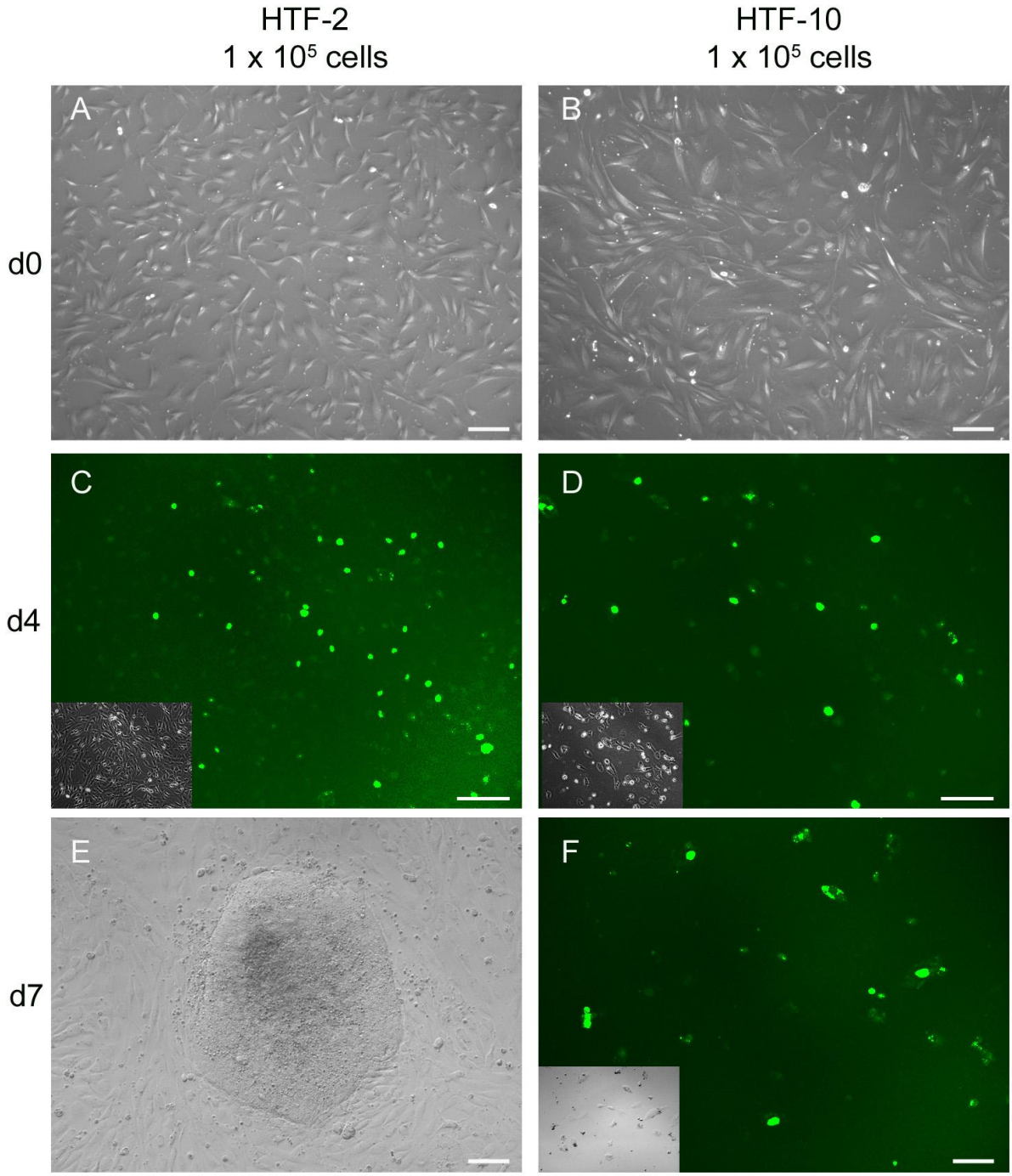


Fig. 17: Morphology changes of HTF-2- and HTF-10 cells during "Feeder Free" mRNA/miRNA Reprogramming (3rd trial). (A+B) The morphology of target cells HTF-2 and HTF-10, each seeded in a density of 1 x 10^5 cells, was checked on day 0 before the start of hOKSML-mRNA transfection series. **(C+D)** Uniform nGFP expression confirmed successful transfection of both cell lines on day 4. Brightfield images are shown in inlays. **(E+F)** Transfected HTF-2 cells revealed colony formation already on day 7, whereas HTF-10 cells showed a highly decreased number of attached cells. d: day. Scale bars: A + B: 200 µm; C-F: 100 µm.

iHTF-2 colonies showed different morphological features. While some iHTF-colonies exhibited their typically defined colony edges (Fig. 18 A-C) or still needed time to grow (Fig. 18 D-F), other colonies did not reveal a compact morphology suggesting an incomplete reprogramming process (Fig. 18 G-I). Firstly, iHTF-2 cell culture was performed in

NuFF-C-P - with 20 ng/ml bFGF, which was gradually replaced by hiPSC-M2. During culture with increased proportion of hiPSC-M2, iHTF-2 colonies revealed morphological changes such as fringed edges and brown-colored cell structures representing their spontaneous differentiation (Fig. 18 J-L). The use of hiPSC-M2 with FCS and a relatively high bFGF concentration (20 ng/ml) probably promotes cell differentiation instead of maintenance of induced pluripotency. Gradually substitution of hiPSC-M2 with hiPSC medium 3 (hiPSC-M3; Tab. 6) containing KO™ serum replacer and a decreased concentration of bFGF (10 ng/ml) was not sufficient to recover iHTF-2 colonies.

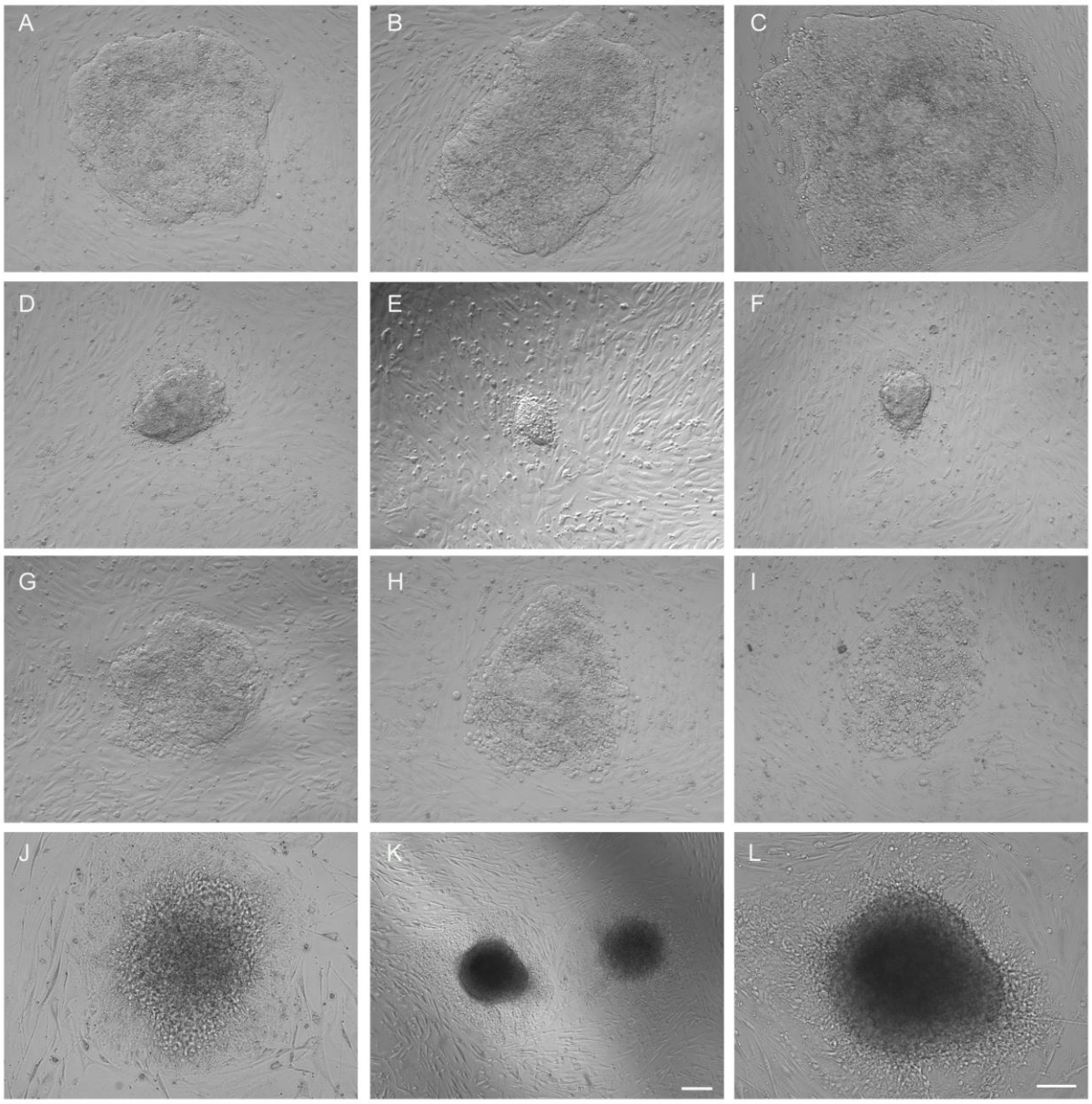


Fig. 18: Morphology changes of iHTF-2 colonies (3rd trial). (A-C) iHTF-2 colonies with defined colony edges representing a characteristic hiPSC morphology. **(D-F)** Small iHTF-2 colonies, which still needed to mature. **(G-I)** HTF-2 cells, which did not complete the fully reprogramming process. **(J-L)** iHTF-2 colonies, which underwent spontaneous differentiation during culture in hiPSC-M2. d: day. Scale bars: K: 200 µm; L, representative for remaining images: 100 µm.

A further performance of “Feeder Free“ mRNA/miRNA Reprogramming (4th trial; Tab. 5) using four SHF cell lines exhibiting different spermatogenesis phenotypes (Fig. 19 I, 1 x 10^5 cells) and BJ fibroblasts as a reprogramming control (2 x 10^4 cells) led to the emergence of hiPSC colonies in all transfected cell lines and finally yielded in the generation of three different iSHF cell lines, namely iSHF-3, iSHF-6 and iSHF-13 (Fig. 19 II).

I

SHF	spermatogenesis phenotype	proliferation
3	spermatogenesis-arrest at the level of primary spermatocytes	+
6	dominant SCO; sporadic detection of spermatogonia	+
9	OA; intact spermatogenesis	+
12	cryptozoospermia; tubuli with hypospermatogenesis	+
13	SCO	~

II

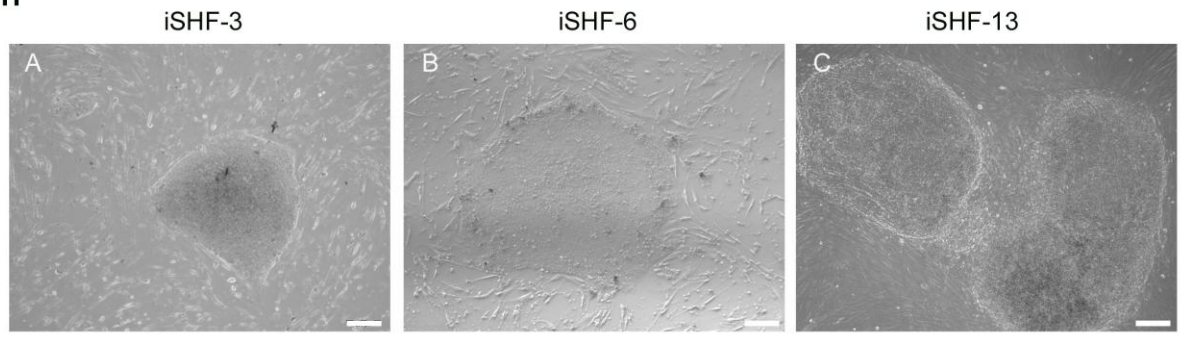


Fig. 19: iSHF colonies derived from “Feeder Free“ mRNA/miRNA Reprogramming (4th trial). (I) SHF cell lines with different spermatogenesis phenotypes were used for “Feeder Free“ mRNA/miRNA Reprogramming. Diagnoses were confirmed by Prof. Schulze, Department of Andrology, University Hospital Hamburg-Eppendorf in Hamburg. OA: Obstructive Azoospermia; SCO: Sertoli Cell Only Syndrome; ~: slow proliferation; +: moderate proliferation. **(II)** Brightfield microscopy images showing generated iSHF-3, iSHF -6 and iSHF -13. Scale bars: 200 µm.

During the transfections series BJ- as well as SHF cells, except SHF-12, revealed a high apoptosis rate, especially in the centre of the reprogramming plate from day 6 onwards. While this cell density was not sufficient to form hiPSCs, these colonies emerged in BJ- as well as SHF-3, -6 and -13 transfected cells predominantly at the border of the reprogramming plate starting at day 9. Although the SHF-9 cell line revealed almost no attached cells from day 6 onwards, one hiPCS colony emerged, which was not sufficient to establish a stable iSHF-9 cell line. SHF-12 became over-confluent during the reprogramming process, which had a negatively impact on transfection efficiency leading to a reduced reprogramming efficiency identified by a decreased nGFP expression. After splitting of SHF-12 cells (1:2 at

day 12), its nGFP expression improved and a few hiPSC colonies emerged, which could not be established in iSHF-12 cell lines.

hiPSC colonies were manually picked from day 12 onwards and cultured in NuFF-C-P - and hiPSC-M3 (50:50). After culturing in pure hiPSC-M3 this medium was replaced with hiPSC medium 4 (hiPSC-M4; Tab. 6) in order to expose hiPSCs to a reduced bFGF concentration of 5 ng/ml and to avoid their spontaneous differentiation observed in the previous reprogramming trial. Using this culture condition iSHF-3, iSHF-6 and iSHF-13 could be established in culture. A detailed protocol of the finally working “Feeder Free“ mRNA/miRNA Reprogramming is described in 2.2.4.11.

3.2.3 Establishment of an efficient cryopreservation method for iSHF cells

Cells, especially hiPSCs, are vulnerable to cell death during freezing and thawing cycles resulting in a poor survival rate of hiPSCs after thawing. Therefore, the establishment of an efficient cryopreservation method of hiPSCs was necessary for facilitation of storage and work with the generated iSHF cells.

The establishment was carried out by testing different ways of enzymatic cell detachment, cell collection, centrifugation, compositions of freezing media and freezing procedures, which is summarized in Tab. 7. The course of establishment as well as the protocols for finally working cryopreservation of iSHF cells using freezing medium containing KO™-SR and DMSO (90:10) supplemented with Y-27632 as well as using CryoStem™ Freezing Medium are described in more detail in 2.2.4.3.11.

Tab. 7: Testing of different cryopreservation methods for iSHF cells.

#	Y-27632 pre-incubation	enzyme	cell collection	centrifugation	freezing medium	freezing procedure	thawing procedure	number of emerged hiPSC colonies
1	-	-	needle	200 x g, 4 min	cell suspension in hiPSC-M : KO™-SR (50:50); addition of hiPSC-M : DMSO (80:20) (Wagner and Welch, 2010)	-80°C or -1°C/min	200 x g, 4 min; + Y-27632 *or* 200 x g,4 min; + Y-27632	-
2	-	Col	needle	400 x g, 4 min	KO™-SR : DMSO (90:10)	-80°C	400 x g, 4 min; + Y 27632	-
3	+	Col	needle	300 x g, 4 min	KO™-SR : DMSO (90:10)	-80°C	300 x g, 4 min; + Y-27632	-
4	-	Col	scratched with pipette and cell scraper	300 x g, 4 min	KO™-SR : DMSO (90:10) + Y-27632	-80°C	300 x g, 4 min; + Y-27632	2 colonies
5	-	Col	scratched with pipette and cell scraper	200 x g, 5 min	CryoStem™ (cold)	-1°C/min	200 x g, 5 min; + Y-27632	4-12 colonies
6	-	Acu	rinsed; cell scraper	500 x g, 3 min	KO™-SR : DMSO (90:10)	-80°C	500 x g, 3 min; + Y-27632	1 colony
7	-	Acu	rinsed; cell scraper	200 x g, 5 min	KO™-SR : DMSO (90:10) + Y-27632	-80°C	200 x g, 5 min; + Y-27632	4 colonies
8	+	Acu	rinsed; cell scraper	300 x g, 4 min	KO™-SR : DMSO (90:10)	-80°C	300 x g, 4 min; + Y-27632	12 colonies
9	-	Acu	rinsed; cell scraper	300 x g, 4 min	KO™-SR : DMSO (90:10) + Y-27632	-80°C	300 x g, 4 min; + Y 27632	20 colonies
10	-	Acu	rinsed; cell scraper	300 x g, 4 min	Biofreeze : hiPSC (90:10) + Y-27632	-80°C	300 x g, 4 min; + Y-27632	-
11	-	Acu	rinsed; cell scraper	200 x g, 5 min	CryoStem™ (cold)	-1°C/min	200 x g, 5 min; + Y-27632	>70 colonies

+: with incubation of indicated reagent; Col: collagenase; Acu: accutase; hiPSC-M: appropriate hiPSC medium; KO™-SR: Knock Out Serum Replacement; Y-27632: ROCK inhibitor.

3.2.4 Characterization of iSHF cell lines

Characterization of iSHF cell lines iSHF-3, -6 and -13 generated by “Feeder Free“ mRNA/miRNA Reprogramming was performed by assessment of AP-staining, the expression of pluripotency markers, karyotype analysis as well as teratoma formation.

iSHF colonies revealed a positive AP-staining and were well distinguishable from the AP negative MEF feeder layer as an internal negative control (Fig. 20).

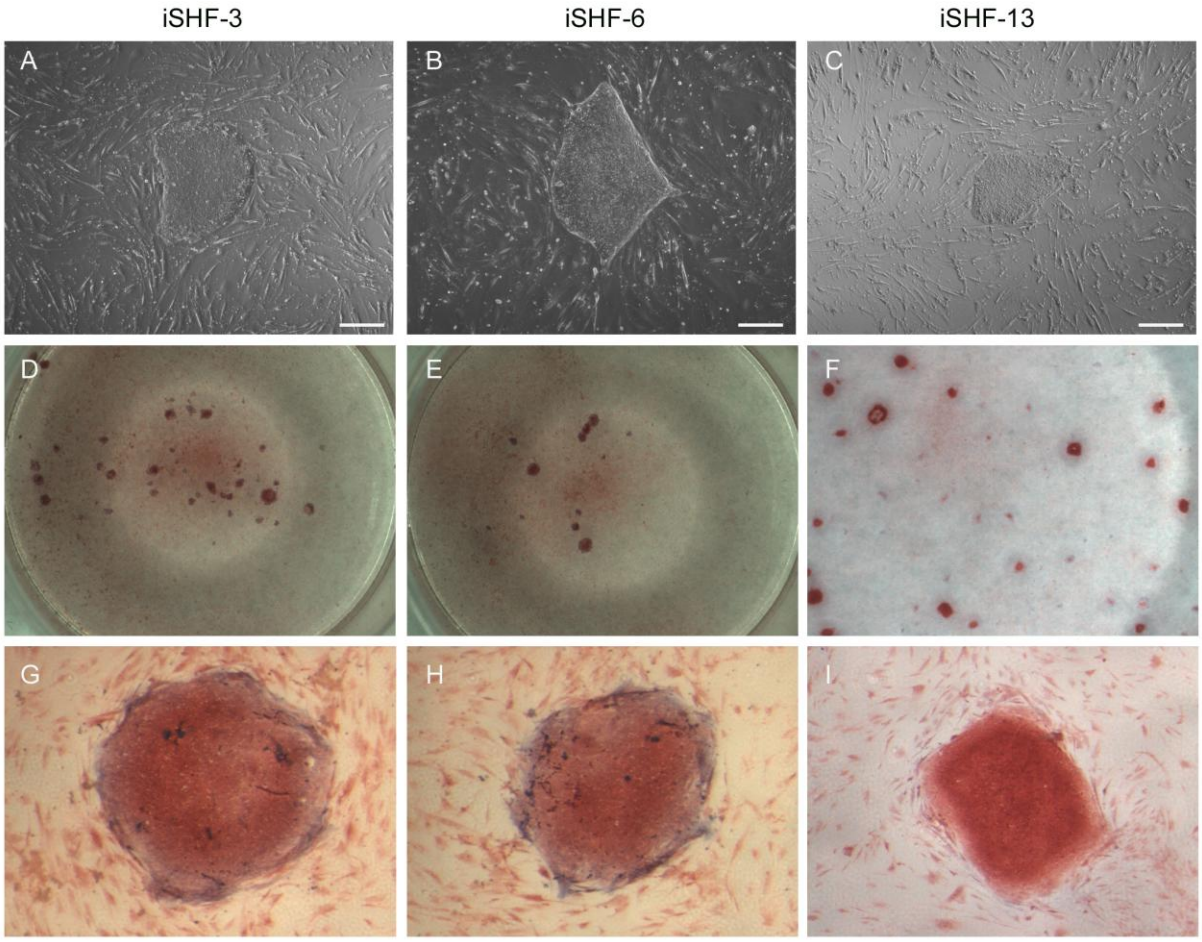


Fig. 20: Alkaline Phosphatase staining of iSHF cell lines. (A-C) Brightfield microscopy images of iSHF-3, -6 and -13. **(D-I)** In contrast to MEF feeder layer, iSHF-3, -6 and -13 are positively stained for AP perceptible from the dark brown staining of these cells. **D-F** provides an overview of culture plate after AP-staining, **G-I** shows AP-positive iSHFs at a higher magnification. AP: Alkaline Phosphatase. Scale bars: 200 µm.

The nuclear marker OCT4 as well as the surface markers SSEA-4, TRA-1-60 and TRA-1-81 were used to further prove the pluripotency character of the generated iSHFs by immunofluorescence staining. The expression of the listed markers was observed in iSHF-3 (Fig. 21) and iSHF-6 (Fig. 22), while iSHF-13 was exclusively characterized by the positive expression of OCT4 and SSEA-4 (Fig. 23). Incubation with IgG antibody was used as negative control (Fig. 21 A-C).

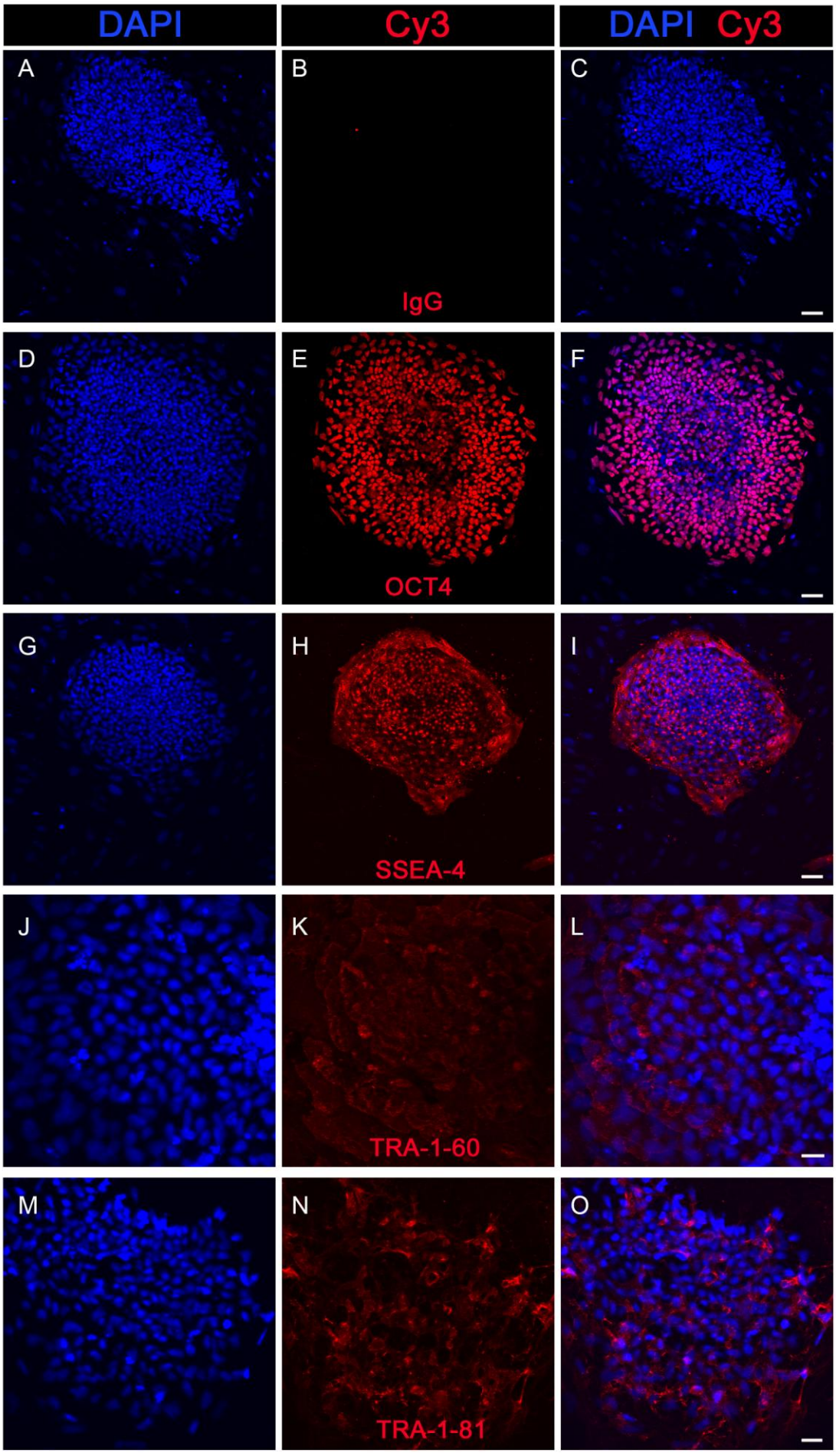

DAPI
Cy3
DAPI Cy3
A
B
C
IgG
D
E
F
OCT4
G
H
I
SSEA-4
J
K
L
TRA-1-60
M
N
O
TRA-1-81

Fig. 21: Immunocytochemical staining of iSHF-3 colonies with different pluripotency markers. iSHF-3 was positively stained for the pluripotency markers OCT4, SSEA-4, TRA-1-60 and TRA-1-81. Representative overlay images are shown in F, I, L and O. Incubations with IgG instead of first antibody were used as negative control (A-C) and is representative for the following immunocytochemical staining of iSHF-6 (Fig. 22) and iSHF-13 (Fig. 23). Representative scale bars are indicated within the overlay images. Scale bars: C, F, I: 50 µm; L, O: 20 µm.

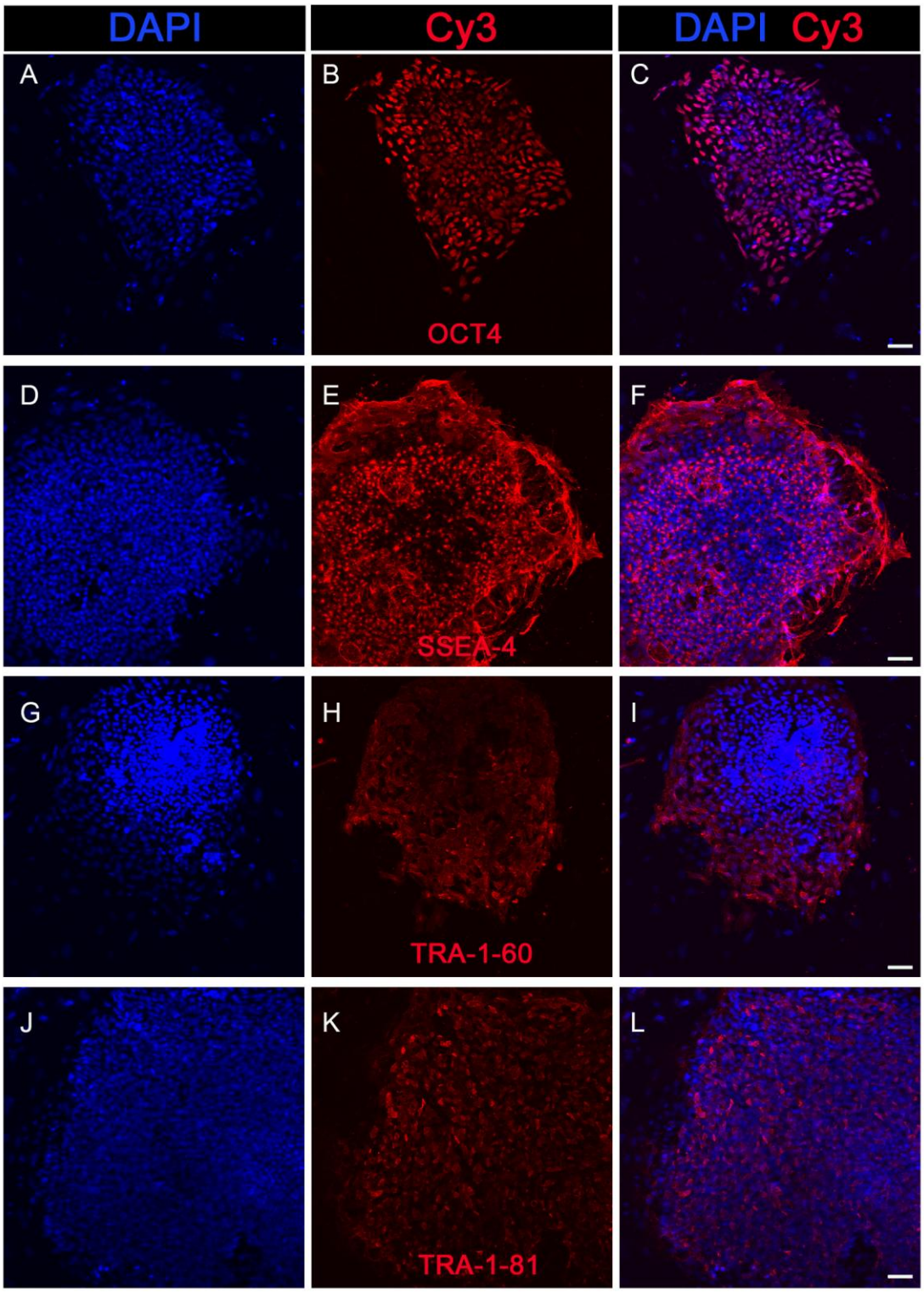


Fig. 22: Immunocytochemical staining of iSHF-6 colonies with different pluripotency markers. iSHF-6 was positively stained for the pluripotency markers OCT4, SSEA-4, TRA-1-60 and TRA-1-81. Representative overlay images are shown in C, F, I and L. Representative incubations with IgG instead of first antibody as negative

controlis shown in Fig. 21 A-C. Representative scale bars are indicated within the overlay images. Scale bars: 50 µm.

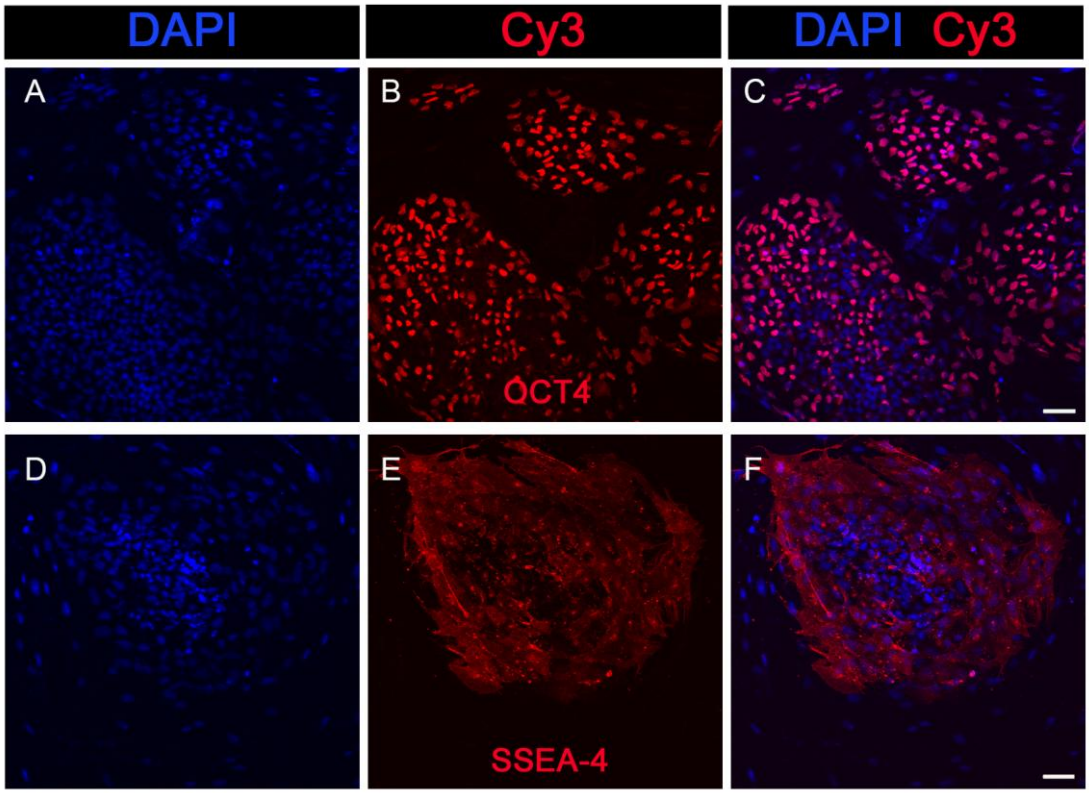


Fig. 23: Immunocytochemical staining of iSHF-13 colonies with different pluritpotency markers. iSHF-13 was positively stained for the pluripotency markers OCT4 and SSEA-4. Representative overlay images are shown in C and F. Representative incubations with IgG instead of first antibody as negative control is shown in Fig. 21 A-C. Representative scale bars are indicated within the overlay images. Scale bars: 50 µm.

Because generation and cultivation of hiPSCs can be associated with karyotypic abnormalities (Martins-Taylor and Xu, 2012), karyotype analyses were performed, which revealed a normal karyotype for all three generated iSHF cell lines (Fig. 24 A-C). Chromosomes from 50 metaphases stained with Giemsa were counted to check for numeric abnormalities. The percentage of metaphases with 45 chromosomes was less than 10% in iSHF cell lines (Fig. 24 D).

Teratoma formation represents robust pluripotency of hiPSCs and is accepted as the gold standard to assess pluripotency. For this assay hiPSCs are injected into immunodeficient NOD/SCID mice and are expected to exhibit a rapid growth of benign tumors consisting of cells derived from all three germ layers (2.2.4.9). As a final proof of their pluripotency iSHF-3 and iSHF-6 were used for this *in vivo* test. In a first trial five mice were injected with each 2×10^6 iSHF-3 cells (passage No. 19) and two mice were treated with 1×10^6 and 2×10^6 iSHF-6 cells (passage No. 23), respectively. No teratoma formation was observed within 75-92 days after injection into immunodeficient mice. A further teratoma assay using iSHF-3 cells is planned to finally validate its pluripotency character.

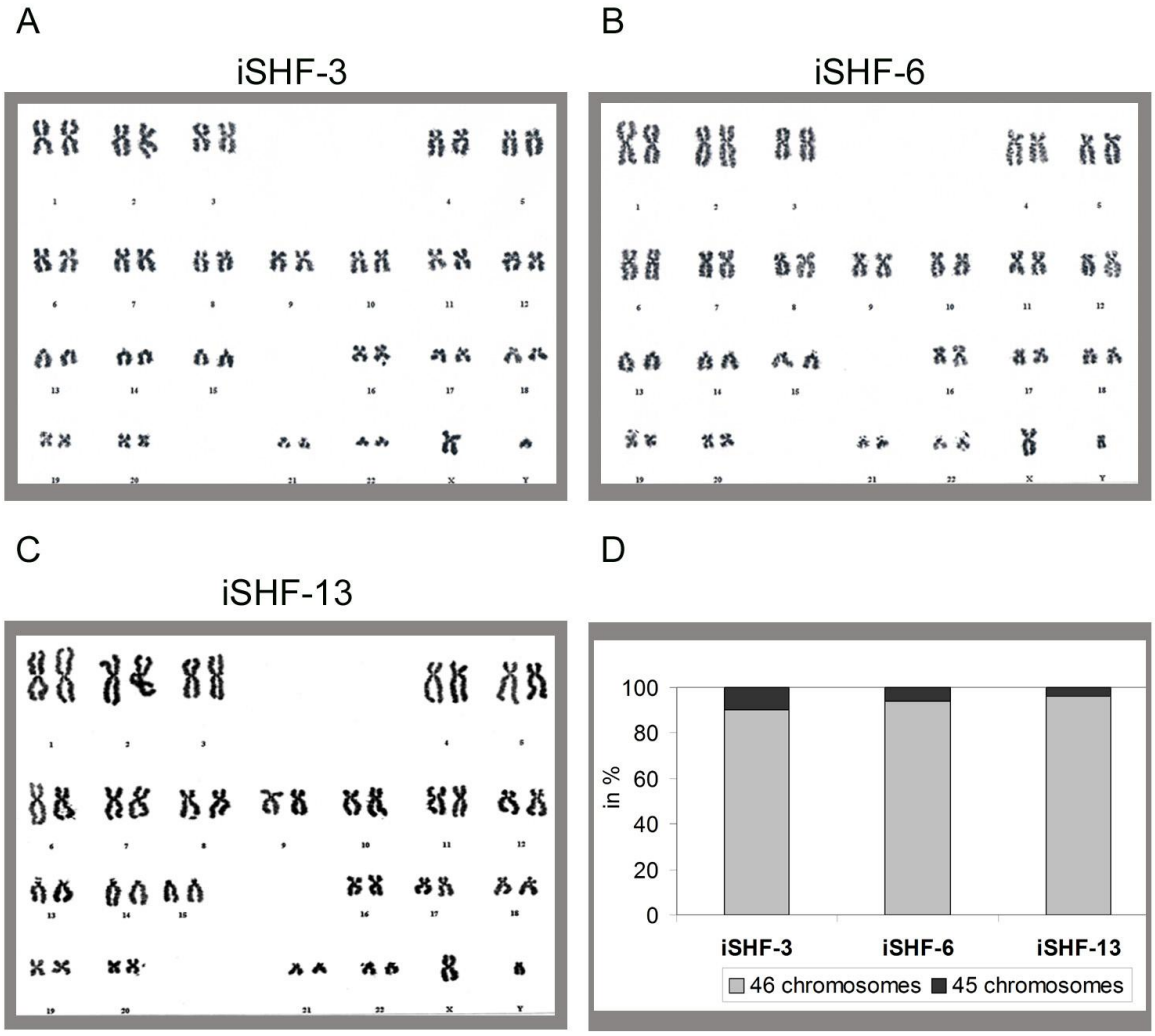


Fig. 24: Karyotyping of iSHF cell lines. (A-C) iSHF-3 (P17), iSHF-6 (P14) and iSHF-13 (P16) revealed a normal karyotype after the reprogramming process. Representative karyograms are shown. **(D)** Counting of chromosomes of 50 metaphases per iSHF cell line showed numeric abnormalities less than 10%. P: passage number.

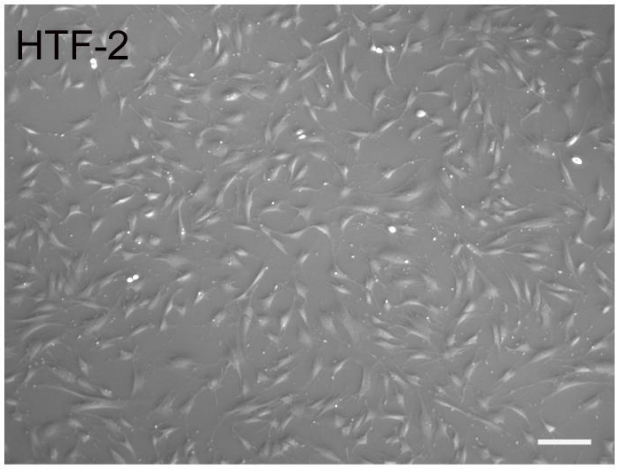
3.3 Co-culture of mouse ESCs with human testicular feeder cells

3.3.1 Preparation of Human Testicular Feeder

The Human Testicular Feeder (HTF) was derived from Magnetic Activated Cell Sortings (MACSortings) with human testicular biopsies from infertile men (3.1; Tab. 2). These biopsies were used to enrich human SSCs via MACSorting using a specific antibody against α6-Integrin, which is described as a surface marker of murine and human SSCs (Shinohara et al., 1999; 2000; Valli et al., 2014). The principle of MACSorting is described in detail in 3.1.2.

Within a two-step procedure the digested testicular cell suspension was incubated with a specific biotinylated α6-Integrin antibody, which was labelled with MicroBeads binding to biotin in a second step. Theoretically, the flow through fraction of this MACSorting should contain the somatic cell populations, which are beside the germ cells part of the testis and have a supportive role during spermatogenesis. These cells were termed as HTF cells, cultured in fibroblast (FB) medium with normal DMEM, 10 % FCS, 2 mM L-glutamine and 1 mM penicillin/ streptomycin on gelatine coated flasks and passaged at 80-90% confluence (2.2.4.3.6; Fig. 25 A). HTF cells at ~80% confluence were used for co-culture with embryonic stem cells and a medium change was performed one day before the start of co-culture. The co-culture experiments and the analyses were performed predominantly with three HTF cell lines derived from MACSortings with human testicular biopsies from three infertile men, whose spermatogenesis were completed and classified at modified Johnsen Score 10 (de Kretser & Holstein, 1976; Fig. 25 B).

A



B

HTF cell lines	testis biopsy mJ-score
HTF-1	10
HTF-2	10
HTF-3	10

Fig. 25: HTF cell lines used in co-culture experiments. (A) Representative brightfield picture of HTF-2 cells derived from MACSorting with testicular biopsy from an infertile man shows the fibroblast morphology of HTF cells. Scale bar: 200 µm. **(B)** Three different HTF cell lines, HTF-1, -2 and -3, were predominatly used for co-

culture experiments derived from MACSorting with testicular biopsies characterized with a spermatogenesis classified at modified Johnsen Score 10. mJ-score: modified Johnsen Score (de Kretser & Holstein, 1976).

3.3.1.1 Characterization of HTF cell lines

Because HTF cell lines were derived from culturing the flow through fractions, it was assumed that these cell lines predominantly consist of human testicular somatic cells. The somatic cell populations within the testis and the seminiferous tubules contribute to the SSC niche, which is a highly specialized microenvironment for the regulation of SSC self renewal and differentiation of SSCs to spermatozoa (1.4). Sertoli cells, leydig cells, peritubular cells as well as the interstitium secrete several factors and thus influence the fate of SSCs residing at the basement membrane of the tubules (de Rooij, 2009; Oatley and Brinster, 2012). For characterization of HTF cell lines, qRT-PCR analyses were performed to evaluate the expression pattern of marker genes of sertoli-, leydig- and peritubular cells. The analysis of several marker genes is necessary, because there are no markers available, which exclusively characterize one of these cell populations.

To validate the origin of HTF cells, we sent RNA derived from all three HTF cell lines, each with two different passages, to two collaborators, who already used different established germ-, sertoli-, leydig- and peritubular cell markers (Kossack et al., 2013; Flenkenthaler et al., 2014). The group of Dr. Neuhaus (née Dr. Kossack; Center of Reproductive Medicine and Andrology, University Hospital Münster) analyzed the expression pattern of the sertoli cell marker genes *FSHR* (Follicle-Stimulating Hormone Receptor), *INHIBIN* and *VIM* (Vimentin) (Fig. 26 A), the leydig cell marker genes *P450*, *LHCGR* (Luteinizing Hormone/Choriogonadotropin Receptor), *3-β-HSD* (3-β-Hydroxysteroid Dehydrogenase) and *STAR (*Steroidogenic Acute Regulatory Protein*)* (Fig. 26 B) as well as the germ cell marker gene *MAGE A4* (Melanoma Antigen Family A4) (Fig. 26 C). Human testis samples, provided by Dr. Neuhaus's group, were used as positive controls and for normalization of qRT data. Sertoli- and leydig cell marker genes were almost not expressed in the analyzed HTF cell lines (Fig. 26). The data for sertoli cell marker gene expression revealed an increased expression of *VIM* in the tested HTF cell lines, which is described to mark not only sertoli cells, but also peritubular cells (Tokuda et al, 2007). Among the tested leydig cell marker genes *STAR* showed an increased to moderate expression in HTF cells. Germ cell marker gene expression of *MAGE A4* was not detectable in any HTF cell lines (Fig. 26 C).

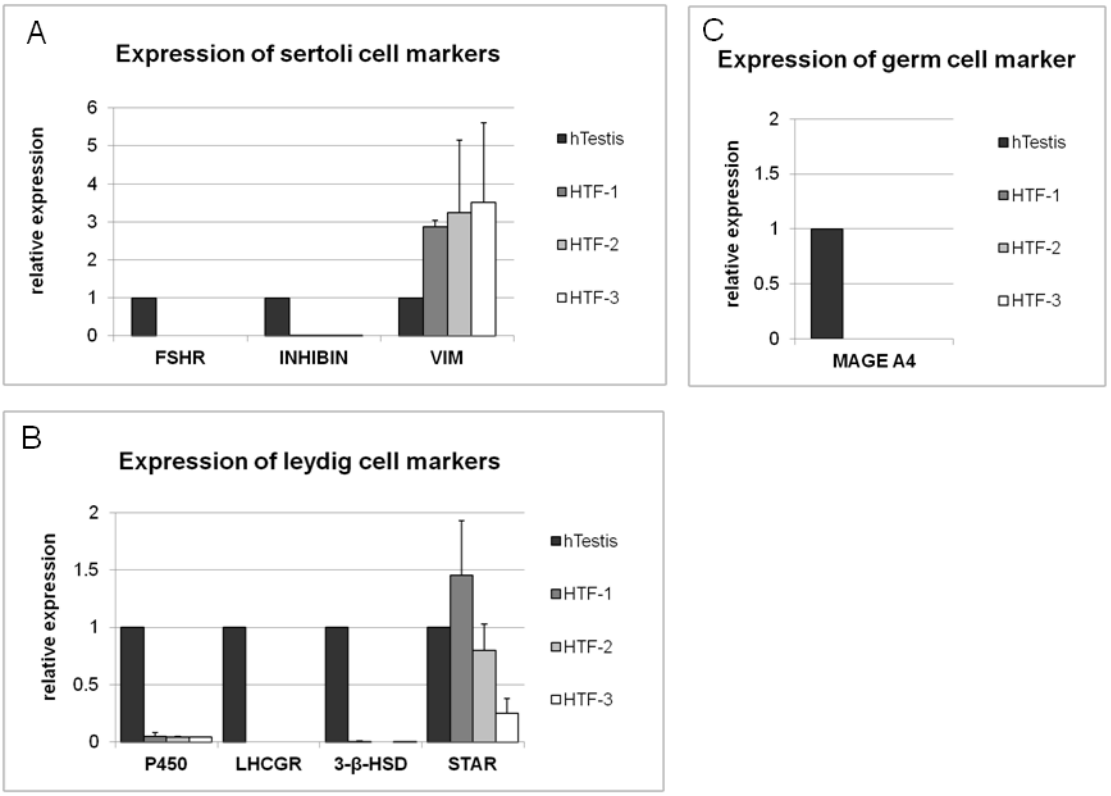


Fig. 26: qRT-expression analyses of sertoli-, leydig- and germ cell marker genes in HTF cell lines. Mean values with standard deviations for **(A)** sertoli cell marker genes *FSHR*, *INHIBIN* and *VIM*, **(B)** leydig cell marker genes *P450*, *LHCGR*, *3-β-HSD* and *STAR* and **(C)** germ cell marker gene *MAGE A4* are shown. Each RNA of two different cell passages (P) were analyzed: HTF-1: P5, P7; HTF-2: P8, P10; HTF-3: P7, P9. Data were collected by the group of Dr. Neuhaus (Münster).

In Prof. Mayerhofer's group (Department for Anatomy and Cell Biology, University of Munich) the expression profile of the peritubular cell marker genes *DCN* (Decorin), *PTX3* (Pentraxin 3), *SMA* (Smooth Muscle Actin), *CAL* (Calponin), *AR* (Androgen Receptor), *GPER* (G-Protein coupled Estrogen Receptor) and *AT1R* (Angiotensin II Type I Receptor) in HTF cells were examined by qRT-PCR analyses. Human testicular peritubular cells (HTPCs; Albrecht et al., 2006), provided by Prof. Mayerhofer's group, were used as positive controls and for normalization of qRT data. All analyzed peritbular cell marker genes were moderate to highly expressed in the HTF cell lines (Fig. 27 I). The peritubular cell marker genes *PTX3*, *CAL*, *GPER* and *AT1R* revealed an expression in HTF cells comparable to HTPCs. Additionally, HTF cells were positively stained for SMA and VIM in immunocytochemical studies, in which these cells showed a heterogeneous expression pattern (Fig. 27 II). Consequently, the results of the qRT-PCR analyses and the immunocytochemical stainings indicated a peritubular cell character of the HTF cells.

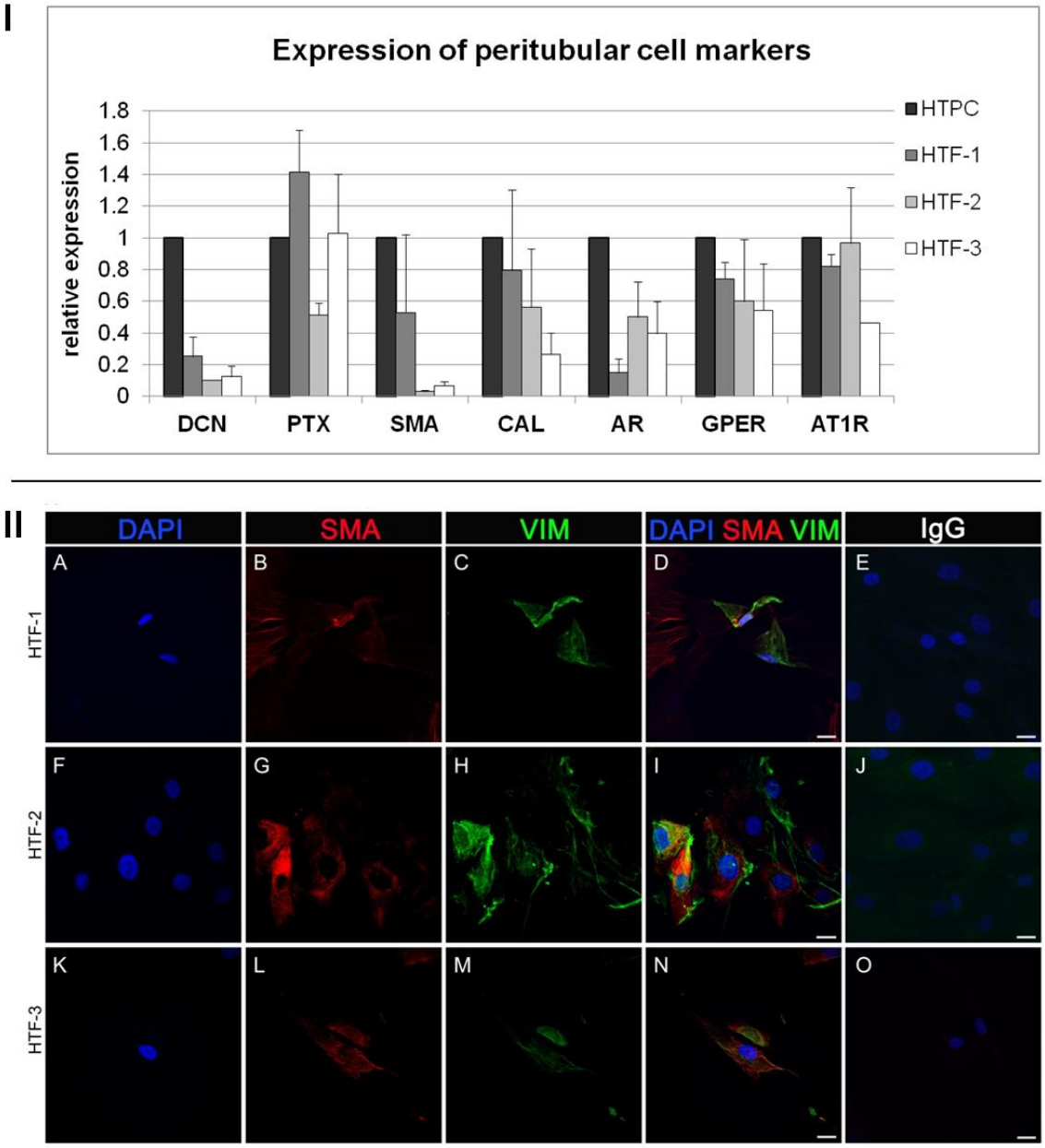


Fig. 27: Peritubular cell marker expression in HTF cell lines analyzed by qRT-PCR experiments and immunocytochemistry. (I) Results of qRT-PCR-expression analyses for peritubular cell marker genes *DCN*, *PTX3*, *SMA*, *CAL*, *AR*, *GPER* and *AT1R*. Mean values with standard deviations are shown. Data were collected by Prof. Mayerhofer's group (Munich). HTPC: Human Testicular Pertibular Cells. **(II)** Immunocytochemical stainings of HTF cells using specific antibodies for SMA and VIM. Representative overlay images are shown in D, I and N. Incubations with IgG instead of first antibody were used as negative controls (E, J and O). Representative scale bars are indicated in the overlay images. Scale bars: 20 µm.

3.3.2 Morphological changes of ESCs co-cultured with HTF cells

In the present work the ESC line “SSC 12”, generated by Nayernia et al. (2006), was used for co-culture experiments. SSC 12 is an ES-RI cell line, established from 129SV-background (Nagy et al., 1993) containing the premeiotic promoter construct *Stra8-EGFP* as well as postmeiotic promoter construct *Prm1-DsRed* for a double selection strategy to establish mSSC lines from mESCs (Nayernia et al., 2006). SSC 12 represents a cell population enriched for *Stra8* expressing cells by twice FACSortings. Because *Stra8* (Stimulated by Retinoic Acid Gene 8) is a premeiotic germ cell marker (Oulad-Abdelghani et al., 1996), but also already expressed in ESCs (Guan et al., 2006), SSC 12 is suggested to be a germ cell directed ESC line.

Co-culture of SSC 12 was performed with HTF cells, which were cultured in FB medium on gelatine coated vessels and used for co-culture experiments at ~80% confluence. A medium change was performed one day before starting the experiment by adding 1×10^5 SSC 12 cells diluted in FB medium on the adherent HTF cells. The whole co-culture experiment was performed with FB medium (Fig. 28 A). The first medium change was performed three days after seeding of SSC 12 cells. Further co-culture comprised medium changes every second to third day and passaging at day ten in a ratio of 1:2-1:3 and further medium changes every second to third day (2.2.4.3.9). At the very first beginning of co-culture SSC 12 cells showed their typical ESC-like morphology. During further culture in FB medium the morphology of the ESCs showed differentiating features, characterized by fringed borders and three dimensional structures, which finally developed into cell clusters with grape-like structures and resembled to putative mouse SSCs (mSSCs) after nearly 20 days of co-culture (Fig. 28 B).

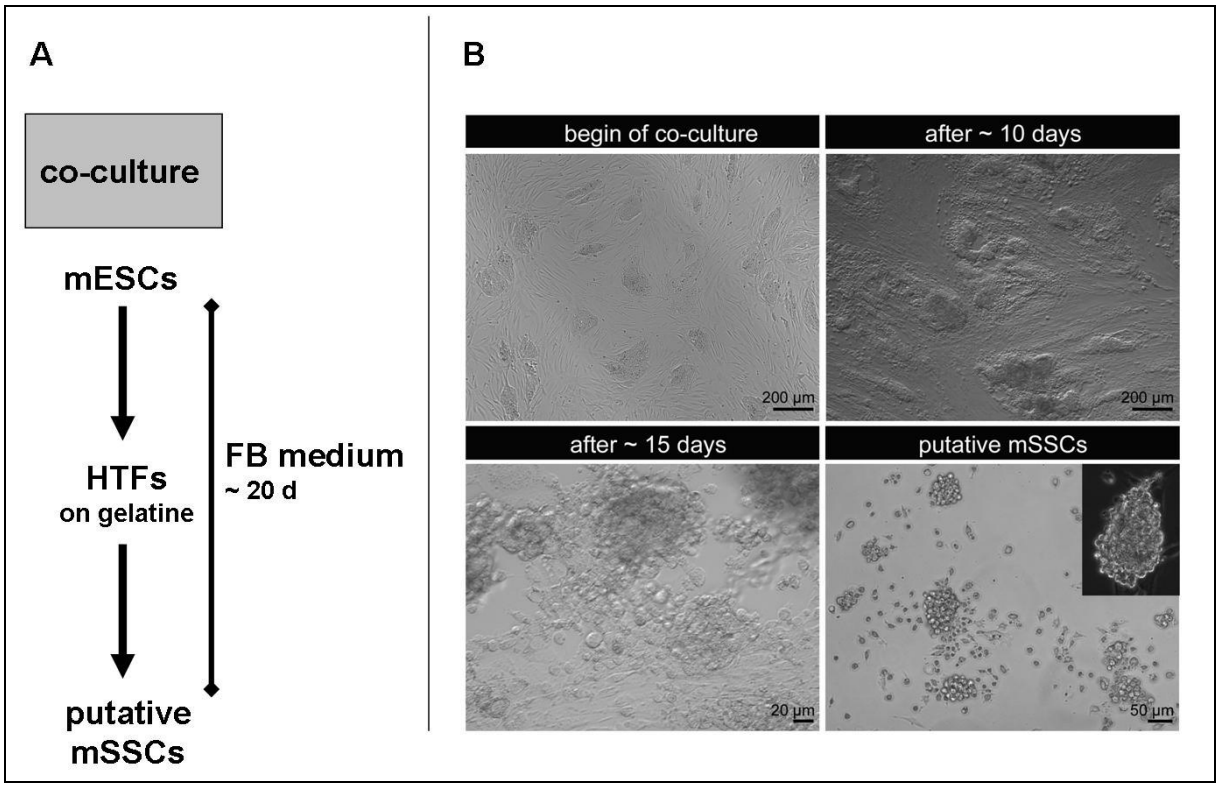


Fig. 28: Experimental outline of co-culture experiments and overview of morphological changes of SSC 12 co-cultured with HTF cells. (A) Schematic outline of a co-culture experiment. **(B)** Brightfield pictures of SSC 12 cells co-cultured with HTF-2 cells at different time points of co-culture. Scale bars are indicated within the pictures.

Below the putative mSSCs are termed according to the HTF cell line used for co-culture with the appendix "-Co". For example the labelling "HTF-1-Co" specifies the putative mSSCs derived from the co-culture of SSC 12 cells with the cell line HTF-1.

3.3.3 Characterization of putative mSSCs derived from co-culture of mESCs with HTF cells

3.3.3.1 Viability of putative mSSCs

For validation of cell viability the putative mSSCs were analyzed by BrdU incubation, which can be incorporated as a thymidine structural analog into replicating DNA in the synthesis phase of cell cycle of dividing cells. Therefore BrdU incorporation into DNA is a useful tool to label DNA in viable and proliferating cells (Givan, 1992; Leif et al., 2004). The putative mSSCs derived from the co-cultures HTF-1-Co, HTF-2-Co and HTF-3-Co underwent a MACSorting using the mSSC surface marker antibody α6-Integrin to enrich the putative mSSCs as α6-Integrin positive cells in culture. These cells were cultured in FB medium with BrdU for 48 h and further analyzed by immunodetection of BrdU positive cells (2.2.4.14; Fig. 29 I). Several cells within the putative mSSC clusters were immunopositive for BrdU (Fig.

29 I C, G, K) indicating the proliferating status of the analyzed cells. No staining was detected in the corresponding stainings with IgG (Fig. 29 I D, H, L). The cell viability was further confirmed by an Annexin V and 7-AAD (7-Amino-Actinomycin) apoptosis assay (2.2.4.13; Khromov et al., 2012). Here freshly α6-Integrin MACSorted putative mSSCs derived from the co-cultures HTF-1-Co, HTF-2-Co and HTF-3-Co were incubated with Annexin V and 7-AAD. While the Ca^{2+} dependent protein Annexin V binds to membrane phospholipid phosphatidylserine, which is exposed at the outer leaflet of the plasma membrane at the latest stage of apoptosis, represents 7-AAD a vital dye, which is excluded from viable cells because of their intact membrane. Flow cytometric analysis allows the determination of viable, early and late apoptotic as well as dead cell fractions. These analyses revealed that approximately 80% of stained co-culture-derived cells were viable (Fig. 29 II).

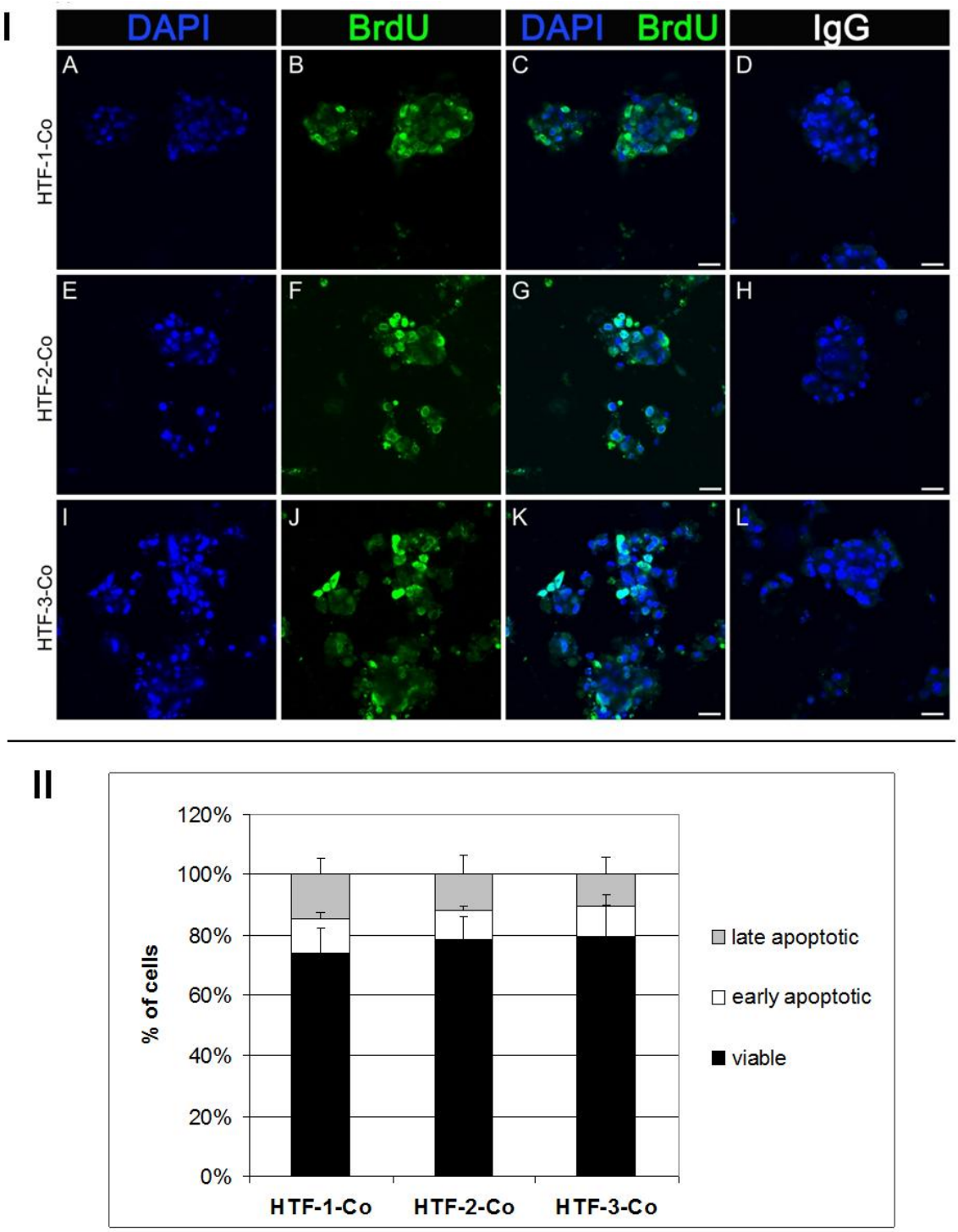


Fig. 29: Validation of co-culture-derived putative mSSCs viability by BrdU staining and apoptosis assay. After MACSorting with α6-Integrin co-culture-derived putative mSSCs (HTF-1-Co, HTF-2-Co and HTF-3-Co) were analyzed **(I)** by BrdU staining for proof of cell viability and proliferation and **(II)** by apoptosis assay. **(I)** Staining of BrdU positive cells after 48 h incubation with the thymidine analog BrdU. Representative overlay images are shown in C, G and K. Incubations with IgG instead of first antibody were used as negative controls (D, H and L). Representative scale bars are indicated in the overlay images. Scale bars: 20 µm. **(II)** Results of flow cytometric measurements show the percentage of viable, early and late apoptotic putative mSSCs. Mean values with standard deviations calculated by averaging three biological replicates are shown.

3.3.3.2 Expression patterns of SSC- and PGC marker genes in putative mSSCs

The characterization of co-culture-derived putative mSSCs was performed by staining with alkaline phosphatase (AP) as well as immunocytochemical and qRT-PCR analyses of phenotypic mSSC markers, which are generally used for the identification of SSCs.

AP-staining as well as immunocytochemical analysis were performed with putative mSSCs co-cultured with HTF cells. The putative mSSCs were positively stained for AP, whereas the HTF cells were negative for AP-staining (Fig. 30 B). Additionally the AP-staining revealed the high number of mSSC-like cells generated in co-culture (Fig. 30 C).

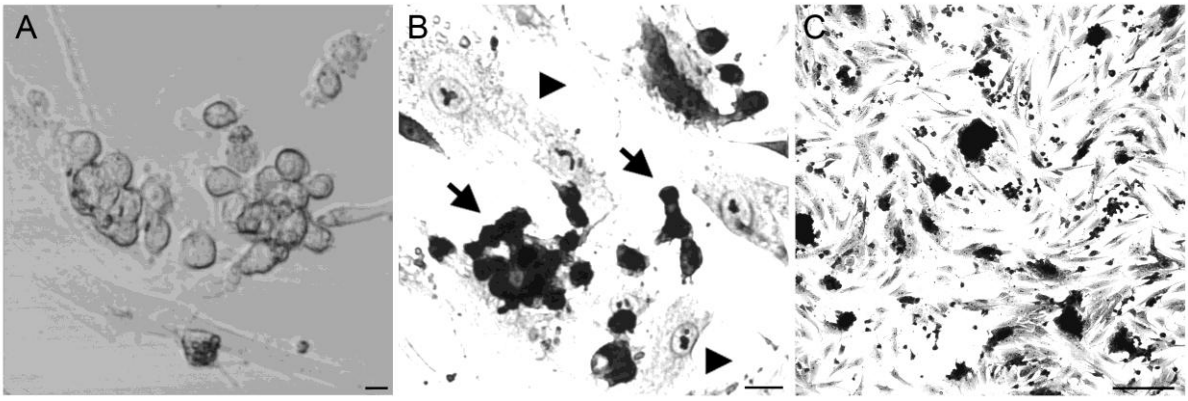
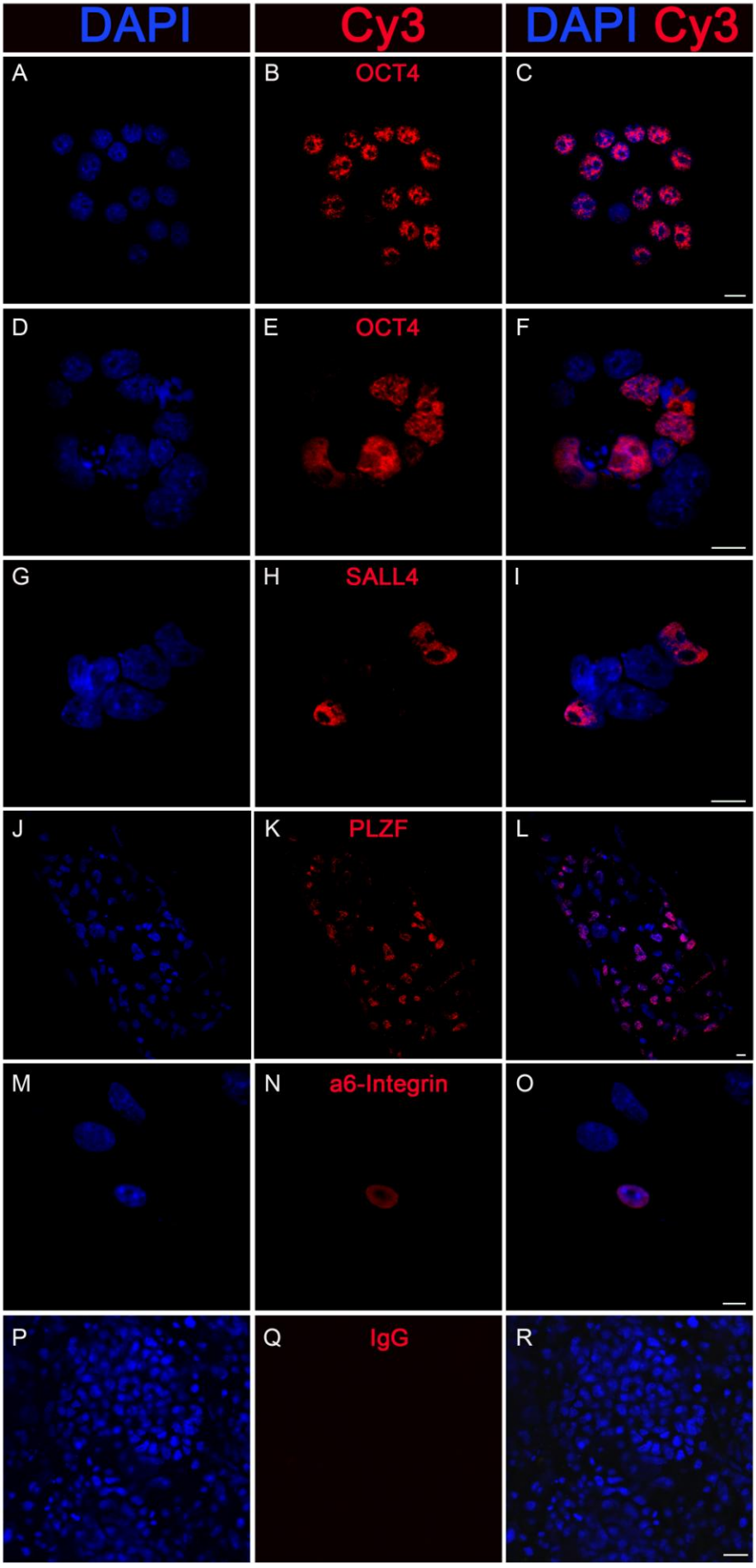


Fig. 30: Alkaline Phosphatase staining of co-culture-derived putative mSSCs. (A) Brightfield microscopy image of putative mSSCs derived from HTF-2-Co before AP-staining showed the typical colonies of round cells with grape-like structures. **(B)** In contrast to HTF-2 cells (arrow heads), the putative mSSCs were positively stained for AP perceptible from the dark brown staining of the cells (arrows). **(C)** Overview of culture plate after AP-staining demonstrated the high number of putative mSSCs generated in co-culture. AP: Alkaline Phosphatase. Scale bars: A: 10 µm; B: 20 µm; C: 100 µm.

Within cell clusters both immunopositive and immunonegative cells could be observed for all tested SSC markers OCT4, SALL4 (Sal-Like Protein 4), PLZF and α6-Integrin (Fig. 31). OCT4 showed a heterogeneous expression pattern within cell clusters, some cells were strongly immunopositive, whereas some remaining cells depicted a weaker staining for OCT4 (Fig. 31 C+F). In comparison to the OCT4 expression the SALL4-, PLZF- and α6-Integrin stainings showed a weaker signal (Fig. 31 I, L, O).


DAPI
Cy3
DAPI Cy3
A
B
OCT4
C
D
E
OCT4
F
G
H
SALL4
I
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K
PLZF
L
M
N
a6-Integrin
O
P
Q
IgG
R

Fig. 31: Immunocytochemical stainings of putative mSSCs derived from co-culture with HTF-2 cells. Immunocytochemical stainings were performed with HTF-2-Co cells using specific antibodies for SSC markers **(A-F)** OCT4, **(G-I)** SALL4, **(J-L)** PLZF and **(M-O)** α6-Integrin. Representative overlay images are shown in the last column (C, F, I, L and O). Incubation with IgG instead of first antibody was used as negative control (P-R). Representative scale bars are indicated in the overlay images. Scale bars: C, F, I, L, O: 10 µm; R: 20 µm.

A similar staining pattern was observed in co-stainings using antibodies against OCT4 and SALL4 as well as OCT4 and DDX4 (). Within cell clusters OCT4 and SALL4 or rather OCT4 and DDX4 (DEAD (Asp-Glu-Ala-Asp) box polypeptide 4; aliases: VASA) were co-localized in some cells (Fig. 32, 4th column), while remaining cells were stained positive for either one or none of the markers (Fig. 32; 2nd and 3rd columns). Mostly the staining intensity of markers differed in cells showing co-localization. No staining was detected in negative controls using IgG instead of first antibody (Fig. 32 M-P).

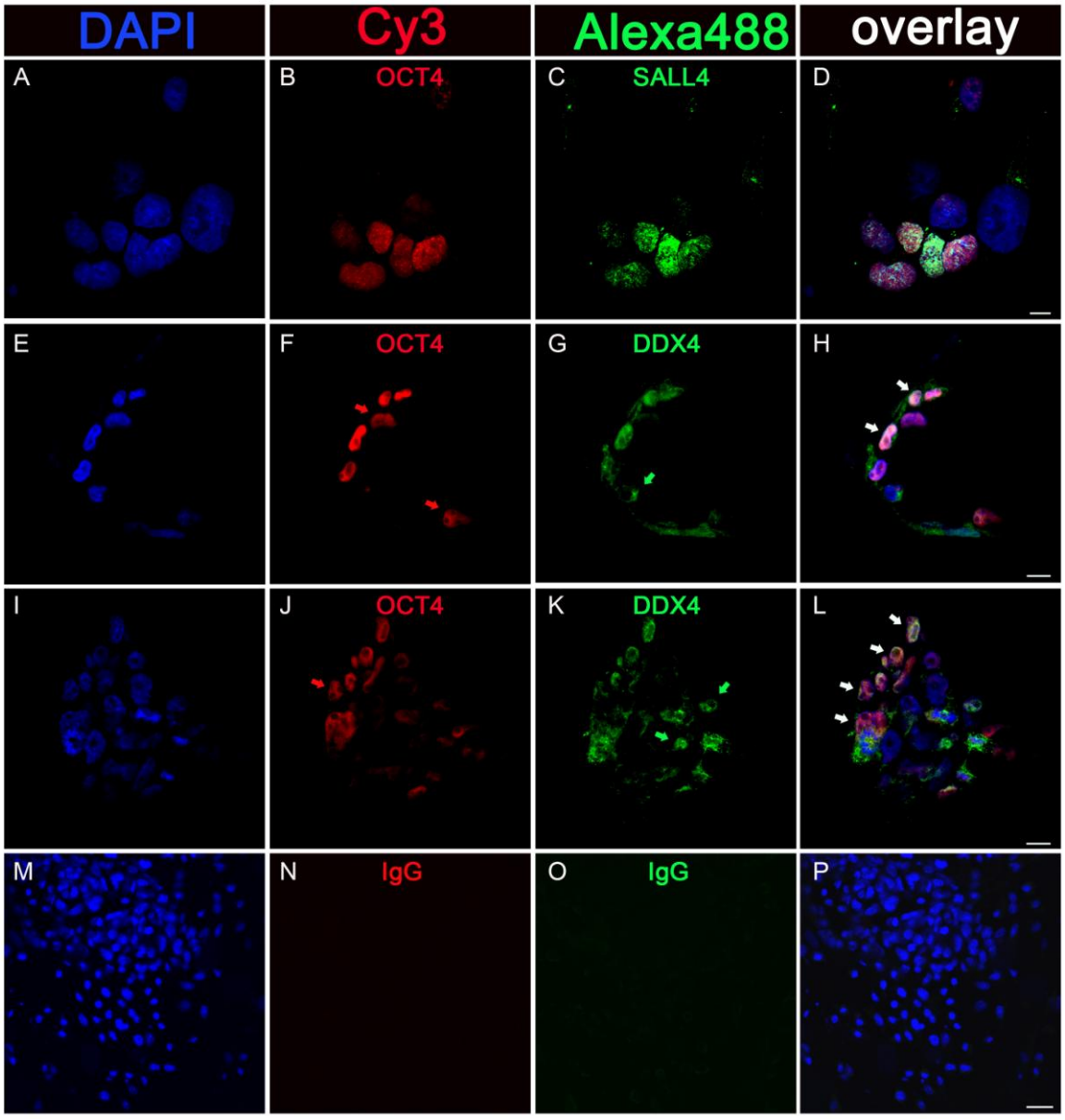
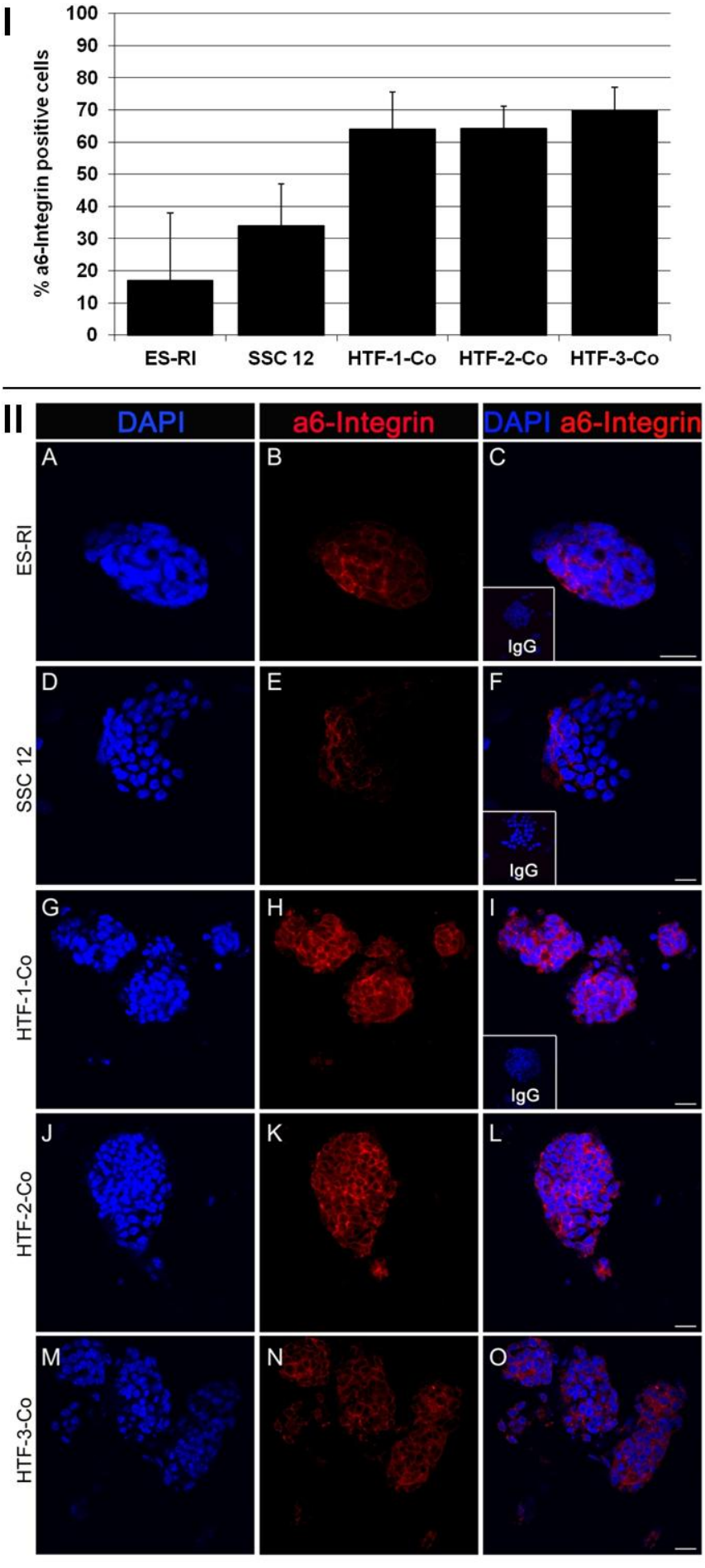


Fig. 32: Co-Immunocytochemical stainings of putative mSSCs derived from co-culture with HTF-2. Co-Immunocytochemical stainings were performed with HTF-2-Co using specific antibodies for SSC markers **(A-H)** OCT4 / SALL4 and **(I-L)** OCT4 and DDX4. Representative overlay images are shown in the last column (D, H and L). Incubation with IgG instead of first antibody was used as negative control **(M-P)**. Red and green arrows indicate cells, which are positive for the respective marker, whereas white arrows indicate those cells that stained positive for both markers. Representative scale bars are indicated in the overlay images. Scale bars: D, H, L: 10 µm; P: 20 µm.

Therefore, the evaluation of the expression pattern of mSSC phenotypic markers by immunocytochemistry indicated that the putative mSSC clusters do not represent a homogeneous mSSC population. An enrichment of those cells in culture could be achieved by MACSorting of the putative mSSCs derived from the co-cultures HTF-1-Co, HTF-2-Co and HTF-3-Co using the mSSC surface marker antibody α6-Integrin, followed by an immunostaining with α6-Integrin in order to quantify the α6-Integrin expression in putative mSSCs after MACSorting. After examination of a total of 500 cells for each cell line regarding their α6-Integrin expression (Fig. 33 II), the putative mSSCs derived from the co-cultures HTF-1-Co, HTF-2-Co and HTF-3-Co revealed 64-70% α6-Integrin positive cells after MACSorting (Fig. 33 I). This comprised a two times higher percentage of α6-Integrin positive compared to the starting cell line SSC 12 of co-culture (33%) and even a five times higher percentage than the original ES cell line ES-RI (13%).


I
100
90
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60
50
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10
0
% a6-Integrin positive cells
ES-RI
SSC 12
HTF-1-Co
HTF-2-Co
HTF-3-Co
II
DAPI
a6-Integrin
DAPI a6-Integrin
A
B
C
IgG
D
E
F
IgG
G
H
I
IgG
J
K
L
M
N
O
ES-RI
SSC 12
HTF-1-Co
HTF-2-Co
HTF-3-Co

Fig. 33: Enrichment of α6-Integrin positive cells by MACSorting of co-culture-derived putative mSSCs. After MACSorting and immunostaining with α6-Integrin a total of 500 cells for HTF-1-Co, HTF-2-Co and HTF-3-Co were examined regarding their α6-Integrin expression, respectively. **(I)** Counting of positive cells revealed a two times- and five times increase of α6-Integrin positive cells in the co-culture-derived mSSCs compared to SSC 12 and ES-RI, respectively. **(II)** Representative images of α6-Integrin immunostaining with SSC 12, ES-RI, HTF-1-Co, HTF-2-Co and HTF-3-Co used for evaluation of α6-Integrin positive cells. Overlay images are shown in C, F, I, L and O. Representative overlay images of incubations with IgG instead of first antibody used as negative controls are shown in inlays in C, F and I. Representative scale bars are indicated in the overlay images. Scale bars: 20 µm.

In addition, qRT-PCR experiments were performed to analyze the expression of a subset of mPGC- and mSSC markers in putative mSSCs derived from co-culture (Fig. 34). These analyses were carried out with putative mSSCs co-cultured with HTF cells as well as with freshly α6-Integrin MACSorted putative mSSCs derived from co-cultures HTF-1-Co, HTF-2-Co and HTF-3-Co in comparison to ES-RI cells as well as the starting cell line SSC 12. Synthesized cDNA derived from mouse testis was used as positive control, while negative controls consisted of the expression analysis in pure HTF cell lines as well as in mouse brain and mouse spleen. Recently, *Fkbp6* (FK506 Binding Protein 6) and *Mov10l1* (Moloney Leukemia Virus 10-like 1) were identified as genes which are specifically expressed in developing fetal PGCs, but not in ESCs and somatic tissues (Sabour et al., 2011). For this reason the expression of these genes indicates the commitment of cells to the germ line lineage and provides the opportunity to discriminate between PGCs and ESCs *in vivo* and *in vitro*. Independent of preparation of co-culture samples, the results of PGC marker gene expression demonstrated a significantly increased expression level of *Fkbp6* and *Mov10l1* in cells derived from co-cultures in comparison to the ESC line ES-RI as well as to SSC 12 cells (Fig. 34 A). The calculated significances normalized to ES-RI are indicated within Fig. 34 A. The expression analysis of *Gpr125* (G-Protein-coupled Receptor 125), which is a well established germ cell marker and expressed in undifferentiated spermatogonia within the testis (Seandel at al., 2007), revealed a moderate expression compared to ES-RI (Fig. 34 B).

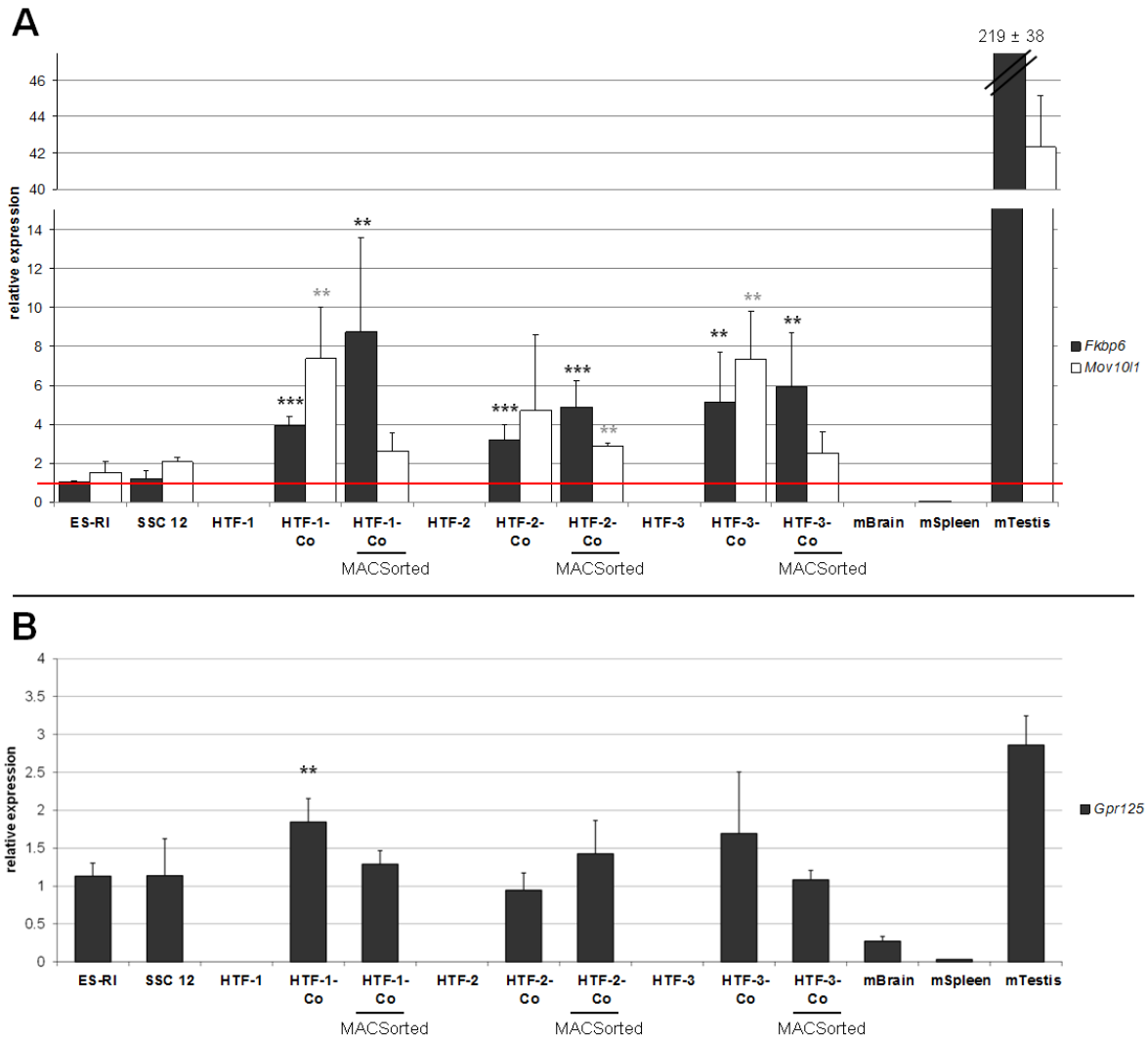


Fig. 34: qRT-PCR expression analyses of PGC marker genes *Fkbp6* and *Mov10l1* and the SSC marker gene *Gpr125* in co-cultured cell lines. Mean values with standard deviations for **(A)** PGC marker genes *Fkbp6* and *Mov10l1* and **(B)** SSC marker gene *Gpr125* are shown. Data comprised at least two biological replicates before and after MACSorting of putative mSSCs derived from HTF-1-Co, HTF-2-Co and HTF-3-Co. Significant increase of marker gene expression in co-culture samples was calculated in comparison to ES-RI and indicated with *: $p \leq 0.05$, **: $p \leq 0.01$; ***: $p \leq 0.001$. SSC 12: starting cell line of co-culture. mTestis: positive control; mBrain, mSpleen: negative controls.

3.3.3.3 Methylation patterns of imprinted marker genes in putative mSSCs

The analysis of the methylation status of imprinted genes in putative mSSCs derived from co-culture was performed in order to check if these genes reveal the SSC characteristic androgenic imprinting pattern. These analyses were carried out in collaboration with Prof. Zechner (Institute of Human Genetics, University of Mainz). For this study genomic DNA samples were isolated from putative mSSCs co-cultured with HTF cells as well as from freshly α6-Integrin MACSorted putative mSSCs derived from co-cultures. Bisulfite pyrosequencing of DNA samples as well as data evaluation in comparison to the methylation patterns of the co-culture starting cell line SSC 12 was performed by the Zechner group. The

methylation status of differentially methylated regions (DMRs) of the paternally imprinted *H19* and *Meg3* genes as well as the maternally imprinted *Igf2r* and *Snrpn* genes was analyzed (Fig. 35).

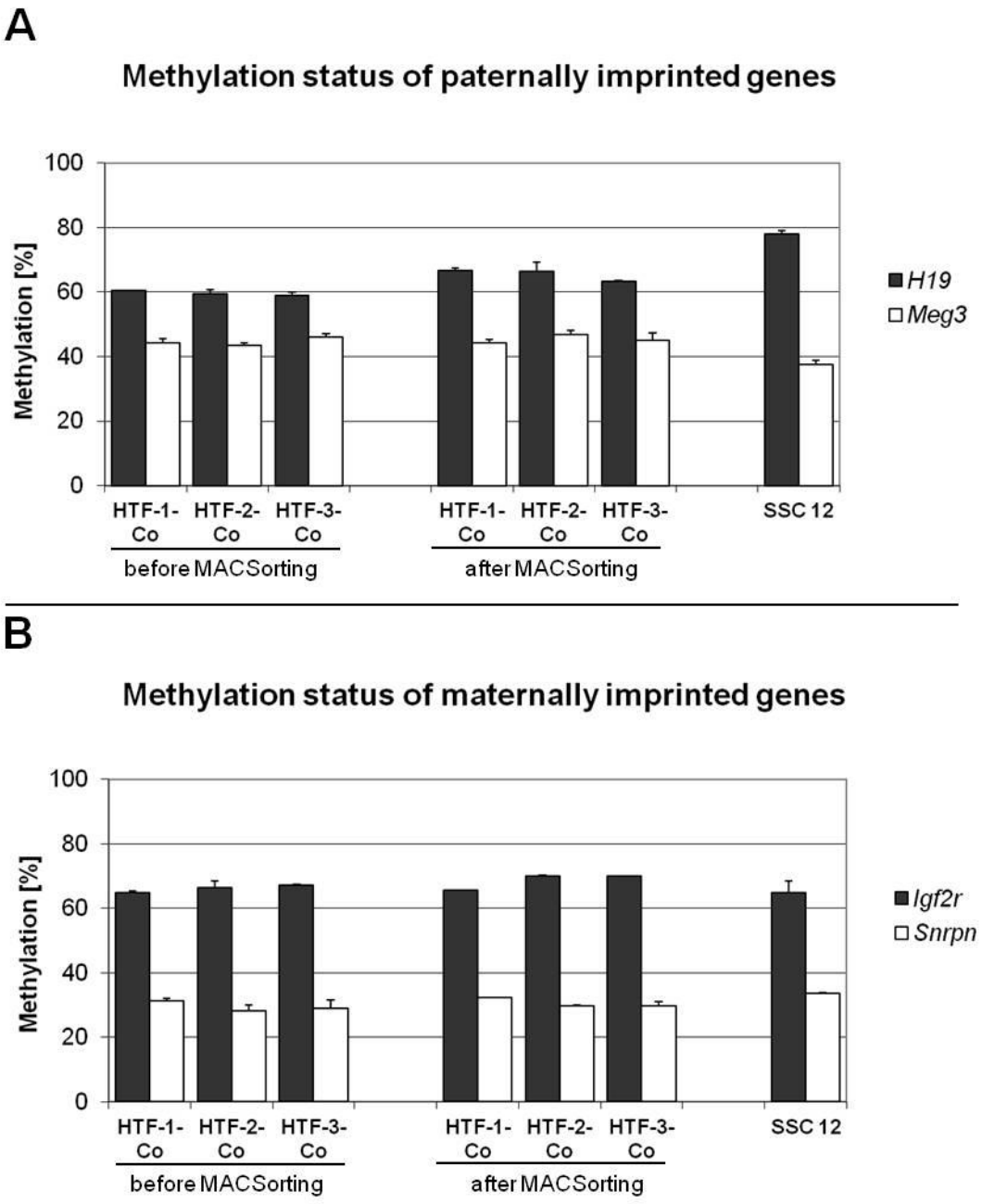


Fig. 35: Methylation analyses of imprinted genes in co-culture-derived putative mSSCs before and after MACSorting with α6-Integrin. The methylation status of **(A)** the paternally imprinted *H19* ICR and *Meg3* IG-DMR and **(B)** the maternally imprinted *Igf2r* DMR2 and *Snrpn* DMR1 in co-culture-derived putative mSSCs before and after MACSorting with α6-Integrin was analyzed by bisulfite pyrosequencing. Mean values with standard deviations are shown. Data comprised three biological replicates before MACSorting and two biological replicates after MACSorting. Data collection and analysis were performed the Zechner group (Mainz).

The analyses revealed minor differences in methylation levels of imprinted genes between putative mSSCs derived from the co-cultures before and after MACSorting with α6-Integrin

and the starting cell line SSC 12. In putative mSSCs derived from HTF-1-Co, HTF-2-Co and HTF-3-Co the paternally imprinted gene *H19* showed a methylation level of ~60% before sorting that very slightly increased to levels ~65% after MACSorting with α6-Integrin (Fig. 35 A). The *Meg3* intergenic (IG) DMR displayed a lower methylation level than *H19* of ~45% that did not change during sorting. Similar observations were made for the methylation patterns of the maternally imprinted *Igf2r* and *Snrpn* genes (Fig. 35 B). *Igf2r* showed a moderate hypermethylation of its DMR2 (~65% before MACSorting, ~68% after MACSorting), while the majority of CpG repeats of the *Snrpn* DMR1 were moderately hypermethylated (~30% before and after MACSorting). The methylation levels of imprinted genes in SSC 12 were nearly consistent with those of ESCs described by Zechner et al., 2009.

3.3.3.4 Derivation of putative mSSCs from HTF-co-cultures with other pluripotent stem cells

Co-culture experiments were also performed with other pluripotent stem cells. The co-culture of HTF cells with the wild type ESC line ES-RI (Nagy et al., 1993) and a wild type iPS cell line (Wernig et al., 2007) also led to the formation of putative mSSC resembling a grape-like structure (Fig. 36 A), which revealed a positive staining for α6-Integrin (Fig. 36 B). This result indicated the suitability of the co-culture system for the generation of SSC-like cells. In the presented thesis a detailed characterization of SSC-like cells was performed with putative mSSC derived from co-cultures of SSC 12 with HTF cells (3.3.3).

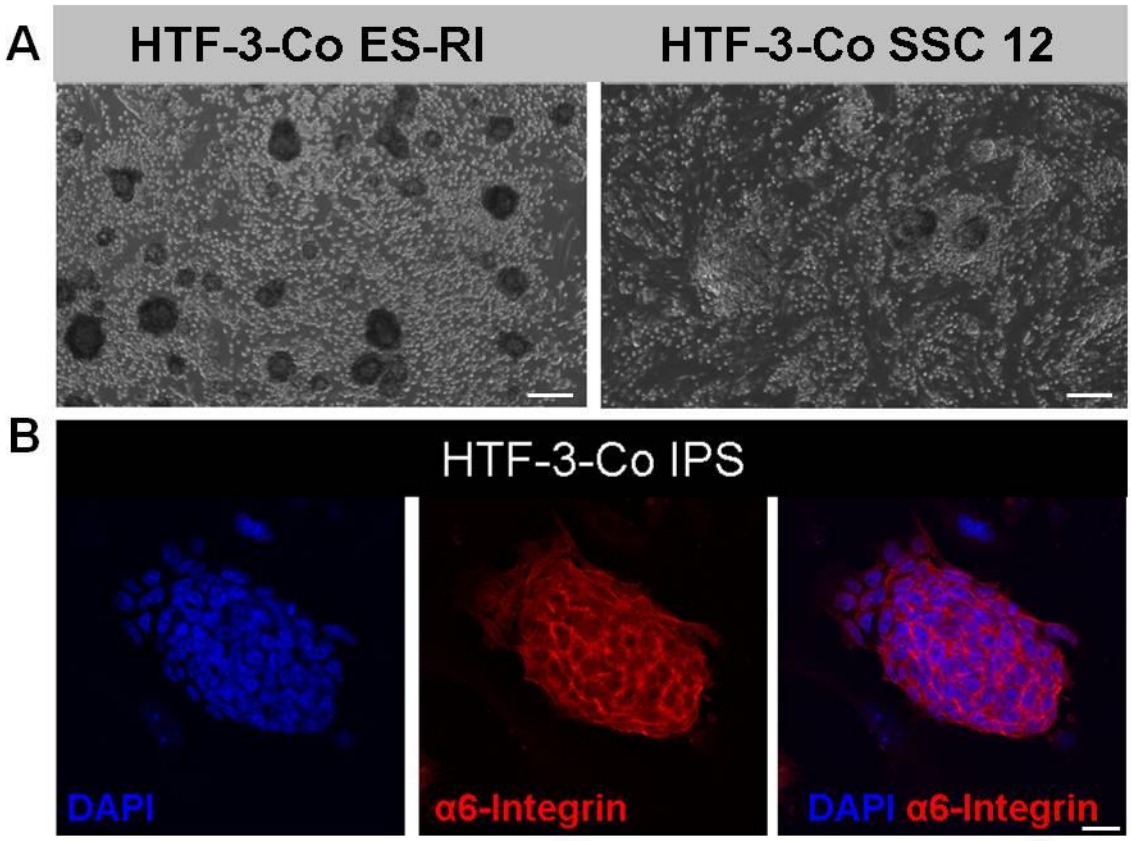


Fig. 36: Morphological features of other pluripotent stem cells for the generation of putative mSSCs in co-culture experiments. (A) SSC-like cells could be also generated by using wild type ES cell lines ES-RI and revealed a similar morphology as putative mSSCs derived from co-culture with SSC 12 (HTF-3-Co SSC 12). **(B)** SSC-like colonies derived from co-culture experiments using a wild type iPS cell line were positively stained for α6-Integrin. Scale bars: A: 200 µm; B: 20 µm.

3.3.4 Transplantation of putative mSSCs in germ cell depleted mice

For functional analysis of cells, co-culture-derived putative mSSCs were transplanted into the seminiferous tubules of germ cell depleted mice (2.2.4.8). While the recipient's endogenous spermatogenesis was destroyed by busulfan treatment, subsequent transplantation of co-culture-derived putative mSSCs may lead to colonization and repopulation of SSCs within the tubules as well as induction and regeneration of spermatogenesis. In general, four to six weeks after busulfan treatment mice were mated with females for at least four weeks to ensure the infertility of males before their use for transplantations. In the transplantation assay, freshly α6-Integrin MACSorted putative mSSCs were either directly transplanted into one testis of the host or cultured for 24 hours in StemPro+3 medium on MEF, which was removed before transplantation procedure. 30-40 µl of the cell suspension (~1 x 10^8 cells/ml) were injected. The remaining testis was not transplanted and therefore used as an internal negative control. Tab. 8 summarizes the performed cell transplantations in germ cell

depleted CD-1 and 129SV mice and indicates the analyses, which were performed in order to check for colonization of transplanted cells as well as for repopulation of the seminiferous tubules.

Tab. 8: Overview of performed cell transplantations in germ cell depleted mice.

	Analysis after transplantation	number of transplanted mice	
		CD-1	129SV
A	Test mating with wild type females for at least 4 weeks following: H&E Staining	6	3
B	Genotyping-PCR for *Stra8-EGFP* construct at certain time points after transplantation	5	10
C	Histochemical analysis at certain time points after transplantation	2	3

The first transplantations of putative mSSCs were carried out with germ cell depleted mice of CD-1 genetic background (Tab. 8 A). Because a complete spermatogenesis and its regeneration after depletion take about 35 days, mice were mated with females approximately four weeks after transplantation. Since after several weeks of mating no offspring was born as well as no teratoma formation was observed, transplanted mice were sacrificed and their testes histologically analyzed by H&E staining. The H&E staining of testis sections revealed no seminiferous tubules with regenerated spermatogenesis (Fig. 37).

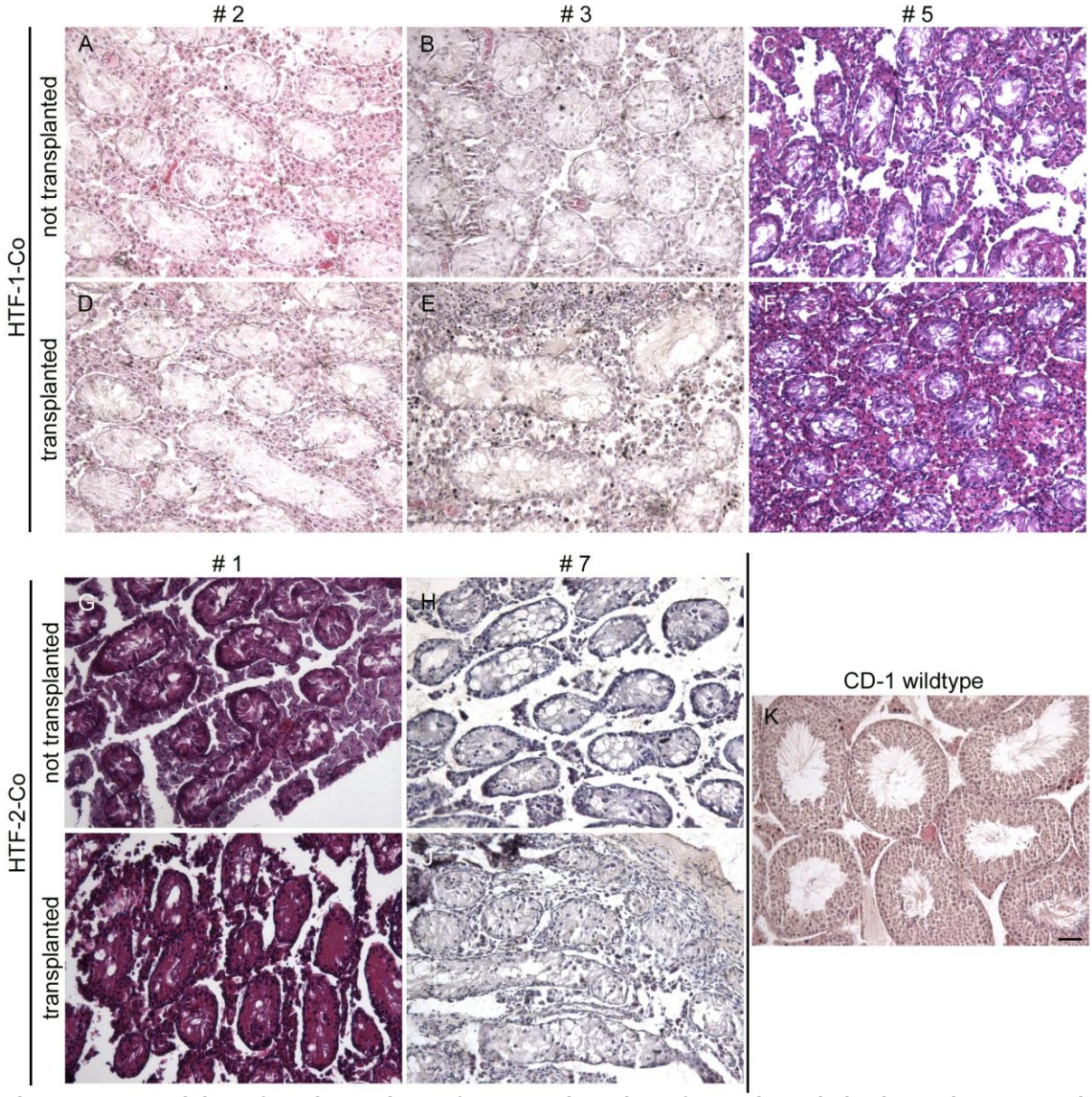


Fig. 37: H&E staining of testis sections after transplantation of co-culture-derived putative mSSCs in germ cell depleted CD-1 mice. After transplantation of putative mSSCs derived from **(A-F)** HTF-1-Co and **(G-J)** HTF-2-Co in germ cell depleted CD-1 mice, their testes were histologically analyzed by H&E staining, which revealed no regeneration of spermatogenesis in seminiferous tubules. Transplanted and non-transplanted testis as an internal negative control were analyzed. H&E staining with CD-1 wild type testis demonstrated normal spermatogenesis. #: mouse number. Representative scale bar in K: 50 µm.

Aiming to check whether putative mSSCs derived from co-culture were able to colonize within the seminiferous tubules after transplantation, germ cell depleted CD-1 mice were transplanted with cell suspension containing the prepared putative mSSCs and sacrificed at certain time points after transplantation. The presence of transplanted cells within the testis was checked by genotyping PCR on genomic DNA derived from transplanted testes (Tab. 8 B) as well as immunohistochemistry on testis cryosections (Tab. 8 C) for the transgene construct *Stra8-EGFP* included in the starting cell line SSC 12 of co-culture. While immunohistochemical analysis did not lead to any results probably attributed to technical

problems to detect EGFP signals on testis cryosections, the presence of *Stra8-EGFP*-specific PCR products was observed 7 days after transplantation by genotyping PCR, whereas no *Stra8-EGFP* PCR bands were detected 14 days after transplantation (Fig. 38 A). The non-transplanted testis was used as an internal negative control and TP2 (Transition Protein 2) -specific PCR was performed as loading control of DNA. Further transplantations were performed to narrow down the time span of settling and loss of transplanted cells within the seminiferous tubules. These transplantations demonstrated the presence of *Stra8-EGFP*-specific PCR products in transplanted testes 6 and 9 days after transplantation by genotyping PCR (Fig. 38 B). No *Stra8-EGFP*-specific PCR bands were detected 12 days after transplantation (Fig. 38 B). Consequently, transplanted mSSCs became lost between 10 and 12 days after transplantations in CD-1 mice.

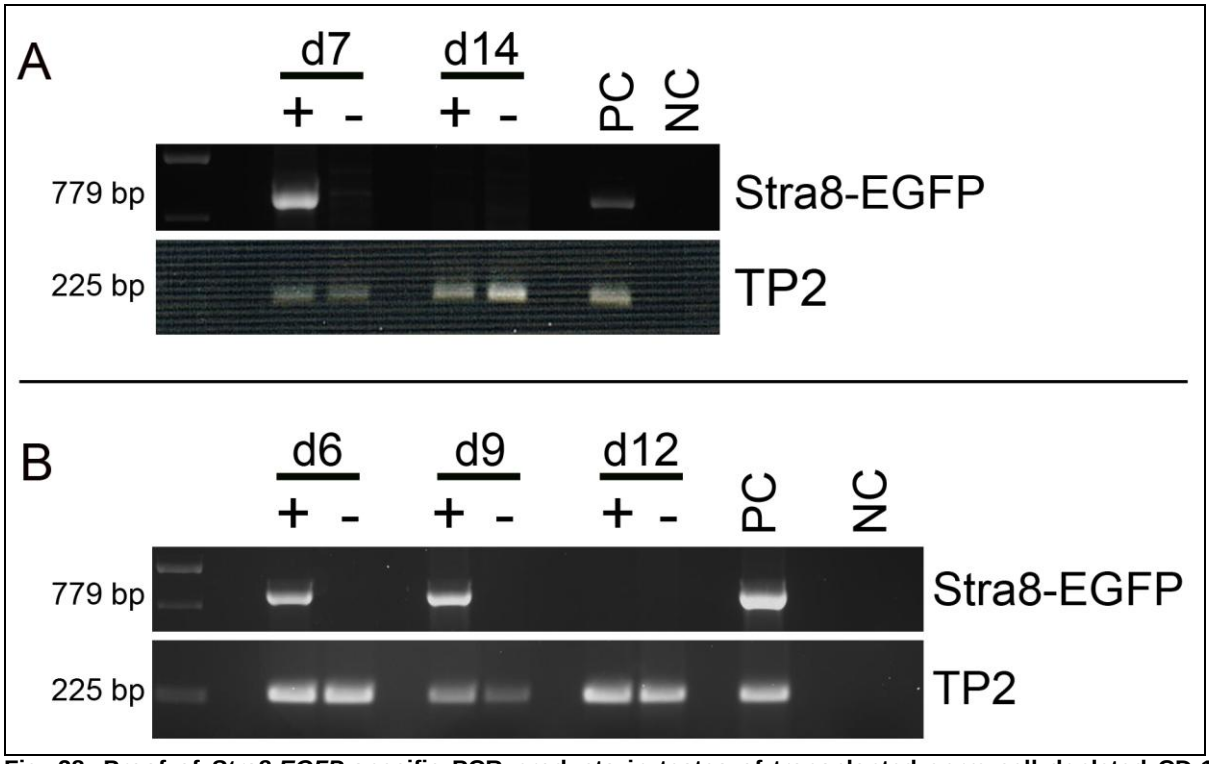


Fig. 38: Proof of *Stra8-EGFP*-specific PCR products in testes of transplanted germ cell depleted CD-1 mice by genotyping PCR. *Stra8-EGFP* genotyping PCR results indicated the presence of *Stra8-EGFP* positive cells **(A)** 7 days as well as **(B)** 6 and 9 days after transplantation of co-culture-derived putative mSSCs in transplanted testes of germ cell depleted CD-1 mice. Putative mSSCs derived from (A) HTF-1-Co and (B) HTF-15-Co were used for transplantation. Fragment sizes of genotyping PCRs are indicated within the figure. TP2: PCR for DNA loading control; d: day after transplantation; +: transplanted testis; -: non-transplanted testis; PC: positive control with SSC 12 genomic DNA; NC: negative control with H_2O.

Because an ES-RI cell line, established from a murine 129SV-background (Nagy et al., 1993), is the origin of the starting cell line SSC 12 of co-culture, further transplantations of putative mSSCs derived from co-culture were performed using germ cell depleted 129SV mice in order to exclude any immune response against the transplanted cells within the

testis. Genotyping PCR was performed to check the presence of *Stra8-EGFP*-specific PCR products in transplanted testes (Tab. 8 B). After transplantation into germ cell depleted 129SV mice, *Stra8-EGFP* PCR bands were detected 6, 9 and 12 days after transplantation by genotyping PCR (Fig. 39 A), which was confirmed in three biological replicates. Furthermore the presence of *Stra8-EGFP*-specific PCR products could be confirmed 15 days after transplantation in 129SV mice (Fig. 39 B). The non-transplanted testis and TP2 PCR were used as an internal negative control and loading control of DNA, respectively.

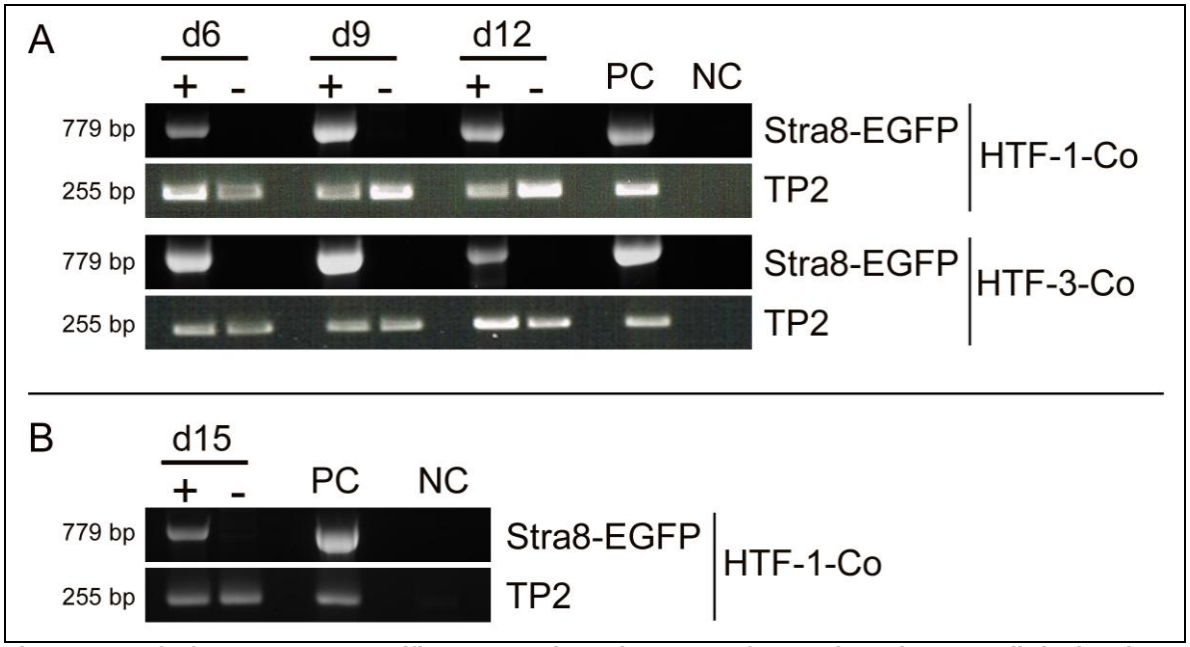


Fig. 39: Proof of *Stra8-EGFP*-specific PCR products in testes of transplanted germ cell depleted 129SV mice by genotyping PCR. *Stra8-EGFP* genotyping PCR results indicated the presence of *Stra8-EGFP* positive cells **(A)** 6, 9 and 12 days as well as **(B)** 15 days after transplantation of HTF-1-Co-derived putative mSSCs in transplanted testes of germ cell depleted 129SV mice. Fragment sizes of genotyping PCRs are indicated within the figure. TP2: PCR for DNA loading control; d: day after transplantation; +: transplanted testis; -: non-transplanted testis; PC: positive control with SSC 12 genomic DNA; NC: negative control with H_2O.

In order to check whether the presence of *Stra8-EGFP*-specific PCR products in transplanted testes revealed by genotyping PCR corresponded to a possible colonization of transplanted cells within the seminiferous tubules, putative mSSCs derived from co-culture were transplanted into germ cell depleted 129SV mice and analyzed at the given time points by immunohistochemical staining (Tab. 8 C). The staining pattern of SALL4, a pluripotency factor, which is expressed in undifferentiated spermatogonia in adult testes (Gassei and Orwig, 2013), should offer a valuable clue to a potential colonization of transplanted cells within the seminiferous tubules. The analyses of SALL4 staining in histological sections of testes prepared 9, 12 and 15 days after transplantation are still in progress.

In addition, germ cell depleted 129SV mice were mated with females approximately four weeks after further transplantations to test for regeneration of spermatogenesis (Tab. 8 A).

No offspring was born as well as no teratoma formation was observed after several weeks of breeding. Two mice are still in test matings, while one transplanted mouse was already sacrificed and its testes histological analyzed by H&E staining, which revealed no seminiferous tubules with regenerated spermatogenesis.

3.3.5 *In vitro* spermatogenesis experiments with putative mSSCs derived from co-culture

3.3.5.1 Molecular marker expression before induction of *in vitro* spermatogenesis

For further functional analysis, co-culture derived putative mSSCs were used for *in vitro* spermatogenesis experiments in order to monitor if these cells were able to undergo differentiation towards haploid male germ cells. Before start of an intended induction of male germ cell differentiation, the expression pattern of molecular markers for germ cell development were examined in order to check if there was any proof of meiotic or even postmeiotic marker gene expression in co-culture samples without induction of differentiation. To monitor the initial situation seven independent HTF-2-Co samples were tested for the expression of different spermatogenesis marker genes by RT-PCR. Fig. 40 A provides an overview of generally used molecular markers for different steps of male germ cell differentiation. In each case two marker genes were chosen for the analysis of premeiotic, meiotic and postmeiotic state of differentiation (Fig. 40 A). The pure HTF-2 cell line was used as an internal negative control. The RT-PCR analysis revealed an expression of premeiotic marker genes *Stra8* and *Vasa* (aliases: *Ddx4*) as well as of meiotic marker genes *Ovol1* (OVO Homolog-like 1) and *Sycp3* (Synaptonemal Complex Protein 3) (Fig. 40 B). Additionally, the expression of the early postmeiotic gene *Acr* (Acrosin) was detected, whereas *Prm1* (Protamine 1) as one of the latest postmeiotic genes was not expressed (Fig. 40 B). No expression of these marker genes was detected in the pure HTF-2 cell line. These RT-PCR results indicated a turning-on of early postmeiotic gene expression in the co-culture system without any induction of germ cell differentiation.

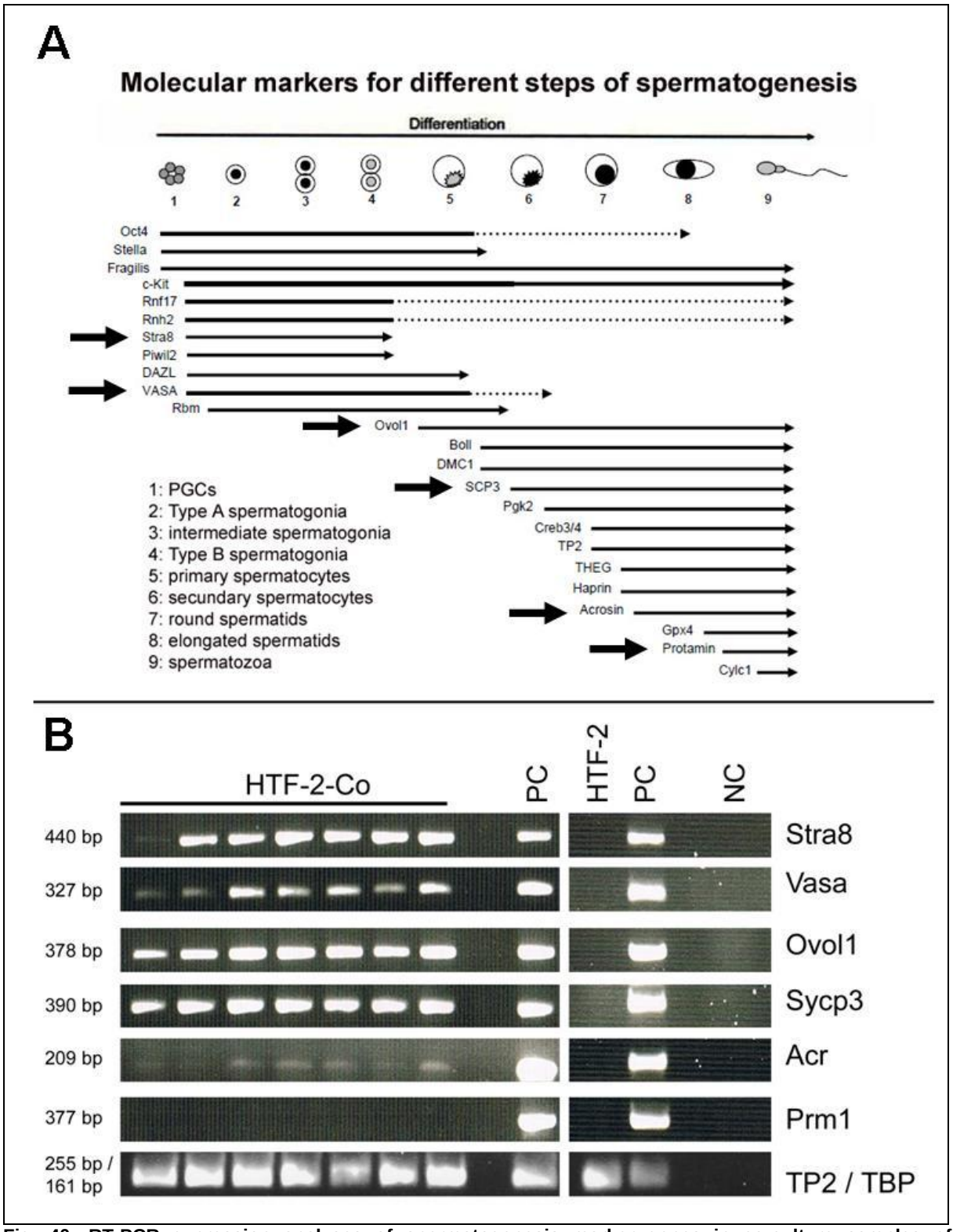


Fig. 40: RT-PCR expression analyses of spermatogenesis marker genes in co-culture samples of HTF-2-Co. **(A)** Overview of molecular markers for characterization of different steps of male germ cell differentiation. Arrows indicate the marker genes used in the presented RT-PCR expression analyses. Modified, Nolte et al., 2007. **(B)** RT-PCR results showed the expression of premeiotic marker genes *Stra8* and *Vasa*, meiotic marker genes *Ovol1* and *Sycp3* and the early postmeiotic gene *Acr*. *Prm1* expression as one of the latest postmeiotic genes was not detected. Fragment sizes of PCR products are indicated within the figure. TP2: PCR for murine DNA loading control; TBP: PCR for human DNA loading control; PC: positive control with SSC 12 genomic DNA; NC: negative control with H_2O.

3.3.5.2 Molecular marker expression and imprinted gene methylation analysis during *in vitro* spermatogenesis

Induction of *in vitro* differentiation is generally performed by application of retinoic acid (RA), which is known to be an important regulator of embryonic development (Ross et al., 2000). Local levels of RA, which is produced by oxidative reactions from vitamin A (retinol) (Ross et al., 2000), are regulated by retinaldehyde dehydrogenases catalyzing the last step of RA synthesis and by CYP26 (cytochrome p450, 26 family of enzymes), which degrades RA (Abu-Abed et al., 2001; Niederreither et al., 2002; Duester et al., 2003). The RA-dependent transcription of premeiotic *Stra8* is essential for inducing meiosis (Baltus et al., 2006; Anderson et al., 2008). Koubova et al. (2006) proposed that RA signalling and metabolism play an important role in initiation of meiosis during embryogenesis. CYP26B1, which is exclusively expressed in embryonic testis, is supposed to degrade RA and prevent expression of *Stra8* resulting in a prevention of meiosis in embryonic testes (Fig. 41). Therefore an application of Talarozole (TA; R115866), a specific CYP26-inhibitor *in vivo* (Stoppie et al., 2000), during induction of germ cell differentiation might increase the number of cells entering meiosis by inhibiting the CYP26B1-dependent RA degradation. Based on this assumption the *in vitro* induction of differentiation of putative mSSCs derived from co-culture was induced by application of RA (10^{-6} M) or TA (1 µM) as well as a combination of both.

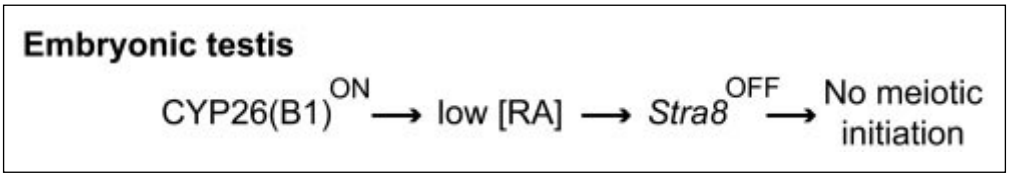


Fig. 41: Proposed model for CYP26 in RA signalling and metabolism to prevent initiation of meiosis during embryogenesis. CYP26B1 is exclusively expressed in embryonic testis and is supposed to degrade RA and prevent expression of *Stra8*, which finally leads to a block of meiosis initiation in embryonic testes. modified; Koubova et al., 2006.

For *in vitro* differentiation HTF-1-Co, HTF-2-Co and HTF-3-Co were cultured in the presence of RA or TA as well as RA and TA and harvested after 5 and 10 days (d5 and d10, respectively) of incubation with these reagents. RNA isolation followed by cDNA synthesis was performed for expression analysis of spermatogenesis marker genes *Stra8*, *Gpx4* (Glutathione Peroxidase 4) and *Acr* by qRT-PCR experiments. Synthesised cDNAs derived from mouse testis and HTF cell lines were used as positive and negative control, respectively. Additionally, isolated genomic DNA from induced co-culture samples was used for analysis of methylation status of imprinted genes. Bisulfite pyrosequencing of samples as well as data evaluation in comparison to the methylation pattern of the co-culture starting cell

line SSC 12 was performed by the Zechner group (Mainz). All data were compared to co-culture samples before induction of differentiation.

The analysis demonstrated that *Stra8* expression can be highly increased by RA application as well as by inhibiting the CYP26B1-dependent RA degradation (Fig. 42 A; Fig. 43 A; Fig. 44 A). Compared to the start of induction, incubation with RA, or TA as well as with both reagents induced a significant increase of premeiotic *Stra8* expression in HTF-1-Co and HTF-2-Co at day 5 and day 10. During induction of differentiation of HTF-3-Co, *Stra8* was also upregulated, however this upregulation was not statistically significant because of the deviations within the tested biological replicates. Moreover, the expression analyses of the postmeiotic marker genes *Gpx4* and *Acr* indicated the presence of postmeiotic cells derived from HTF-1-Co, HTF-2-Co and HTF-3-Co (Fig. 42 A; Fig. 43 A; Fig. 44 A). While inhibiting the CYP26B1-dependent RA degradation caused an upregulation of *Gpx4* expression in HTF-1-Co, HTF-2-Co and HTF-3-Co at day 5 and in HTF-1-Co and HTF-2-Co also at day 10, an increased *Acr* expression was observed at least at one of the tested time points during induction of differentiation. The calculated significances for the comparisons to mRNA levels at the start of induction are indicated within the figures (Fig. 42 A; Fig. 43 A; Fig. 44 A).

During male germ cell differentiation the methylation levels of the paternally imprinted *H19* and *Meg3* genes are expected to increase, while the methylation of maternally imprinted *Igf2r* and *Snrpn* genes should be erased with subsequent maintenance of a largely unmethylated state. Compared to the start of induction with RA and TA as well as to the co-culture starting cell line SSC 12, the methylation patterns of induced HTF-1-Co, HTF-2-Co and HTF-3-Co did not display any trend towards a hypermethylated pattern of *H19* and *Meg3* and a hypomethylated pattern of *Igf2r* and *Snrpn* that would be indicative for *in vitro* differentiation (Fig. 42 B; Fig. 43 B; Fig. 44 B). Considering all analyzed differentiation induced cell lines and corresponding controls, no gross methylation changes were detected for all four genes at day 5 and day 10 of differentiation with RA, or TA as well as with both reagents. Before and after differentiation, *H19* displayed a methylation level ~50-70%, *Meg3* a methylation level ~40-50%, *Igf2r* a methylation level ~60-70% and *Snrpn* a methylation level of ~30-40%. A slightly increased methylation was only seen for the *H19* and *Meg3* genes at day 10 of differentitation of HTF-1-Co, HTF-2-Co and HTF-3-Co with both RA and TA. However, this was not accompanied by a parallel decrease of *Igfr2* and *Snrpn* methylation.

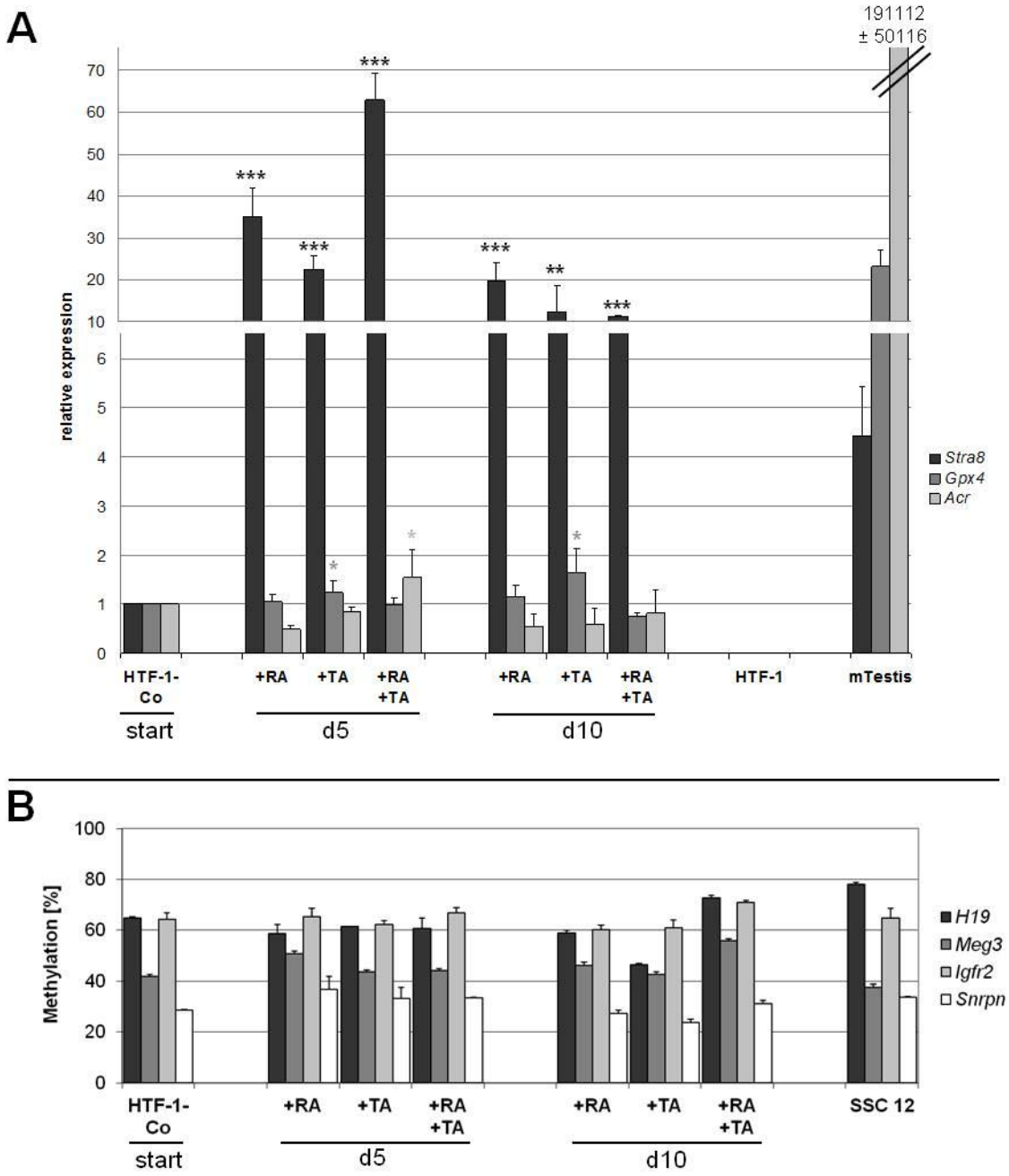


Fig. 42: Analyses of spermatogenesis marker gene expression by qRT-PCR experiments and methylation pattern during *in vitro* differentiation of HTF-1-Co. (A) Results of qRT-PCR-expression analyses for premeiotic *Stra8* and postmeiotic *Gpx4* and *Acr*. Mean values with standard deviations are shown. Data consists of at least two biological replicates. Significant increase of marker gene expression in co-culture samples during differentiation was calculated in comparison to HTF-1-Co before start of induction and is indicated with *: $p \leq 0.05$, **: $p \leq 0.01$; ***: $p \leq 0.001$. **(B)** The methylation status of the paternally imprinted *H19* ICR and *Meg3* IG-DMR and the maternally imprinted *Igf2r* DMR2 and *Snrpn* DMR1 were analyzed by bisulfite pyrosequencing. Mean values with standard deviations are shown. Data collection and analysis were performed by the Zechner group (Mainz). SSC 12: starting cell line of co-culture. HTF-1: pure HTF cell line; start: HTF-1-Co before start of induction. d: day of induction; +: incubated with certain reagent; RA: Retinoic Acid; TA: Talarozole (CYP26-inhibitor).

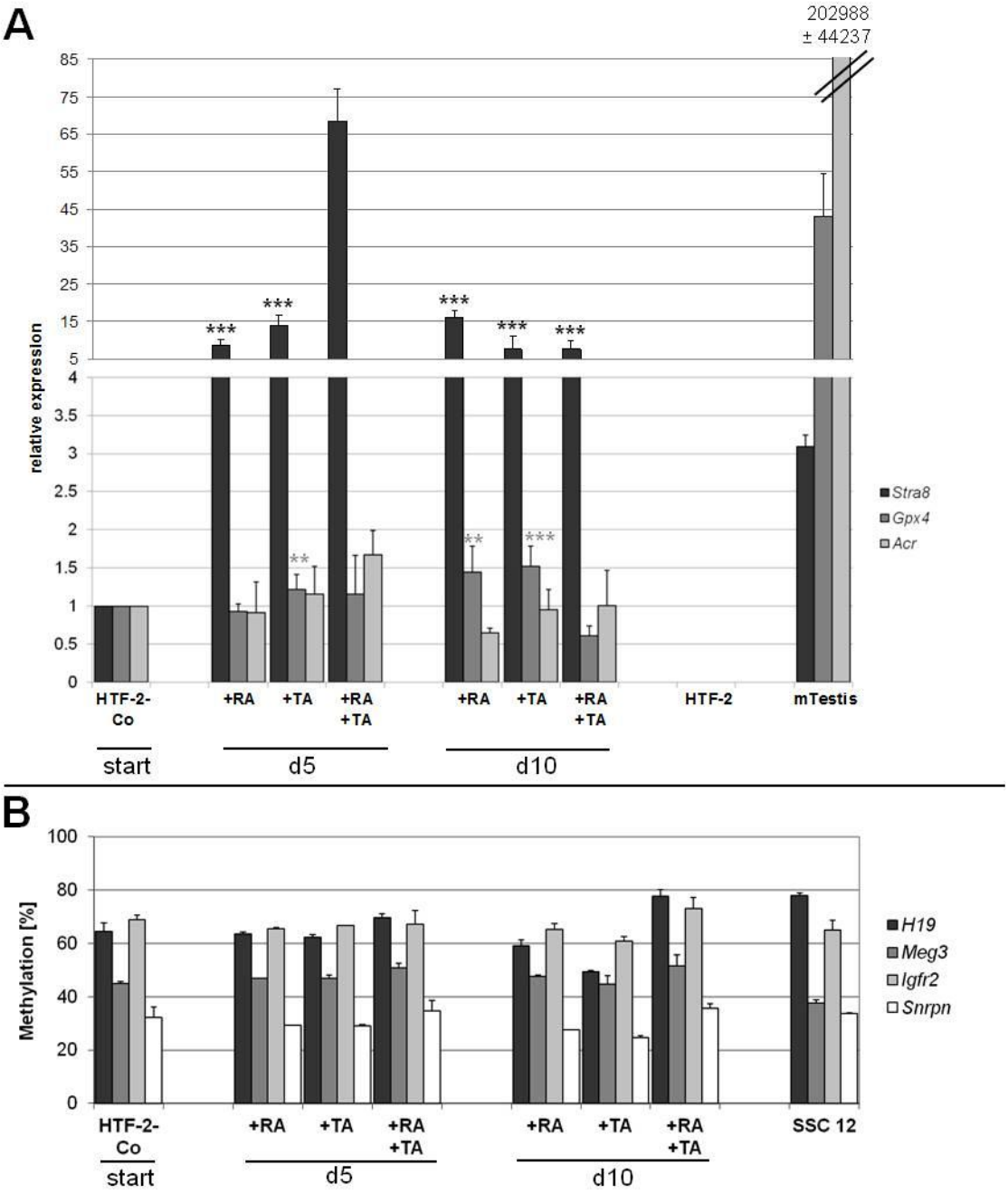


Fig. 43: Analyses of spermatogenesis marker gene expression by qRT-PCR experiments and methylation pattern during *in vitro* differentiation of HTF-2-Co. (A) Results of qRT-PCR-expression analyses for premeiotic *Stra8* and postmeiotic *Gpx4* and *Acr*. Mean values with standard deviations are shown. Data consists of at least two biological replicates. Significant increase of marker gene expression in co-culture samples during differentiation was calculated in comparison to HTF-2-Co before start of induction and is indicated with *: $p \leq 0.05$, **: $p \leq 0.01$; ***: $p \leq 0.001$. **(B)** The methylation status of the paternally imprinted *H19* ICR and *Meg3* IG-DMR and the maternally imprinted *Igf2r* DMR2 and *Snrpn* DMR1 were analyzed by bisulfite pyrosequencing. Mean values with standard deviations are shown. Data collection and analysis were performed by the Zechner group (Mainz). SSC 12: starting cell line of co-culture. HTF-2: pure HTF cell line; start: HTF-2-Co before start of induction. d: day of induction; +: incubated with certain reagent; RA: Retinoic Acid; TA: Talarozole (CYP26-inhibitor).

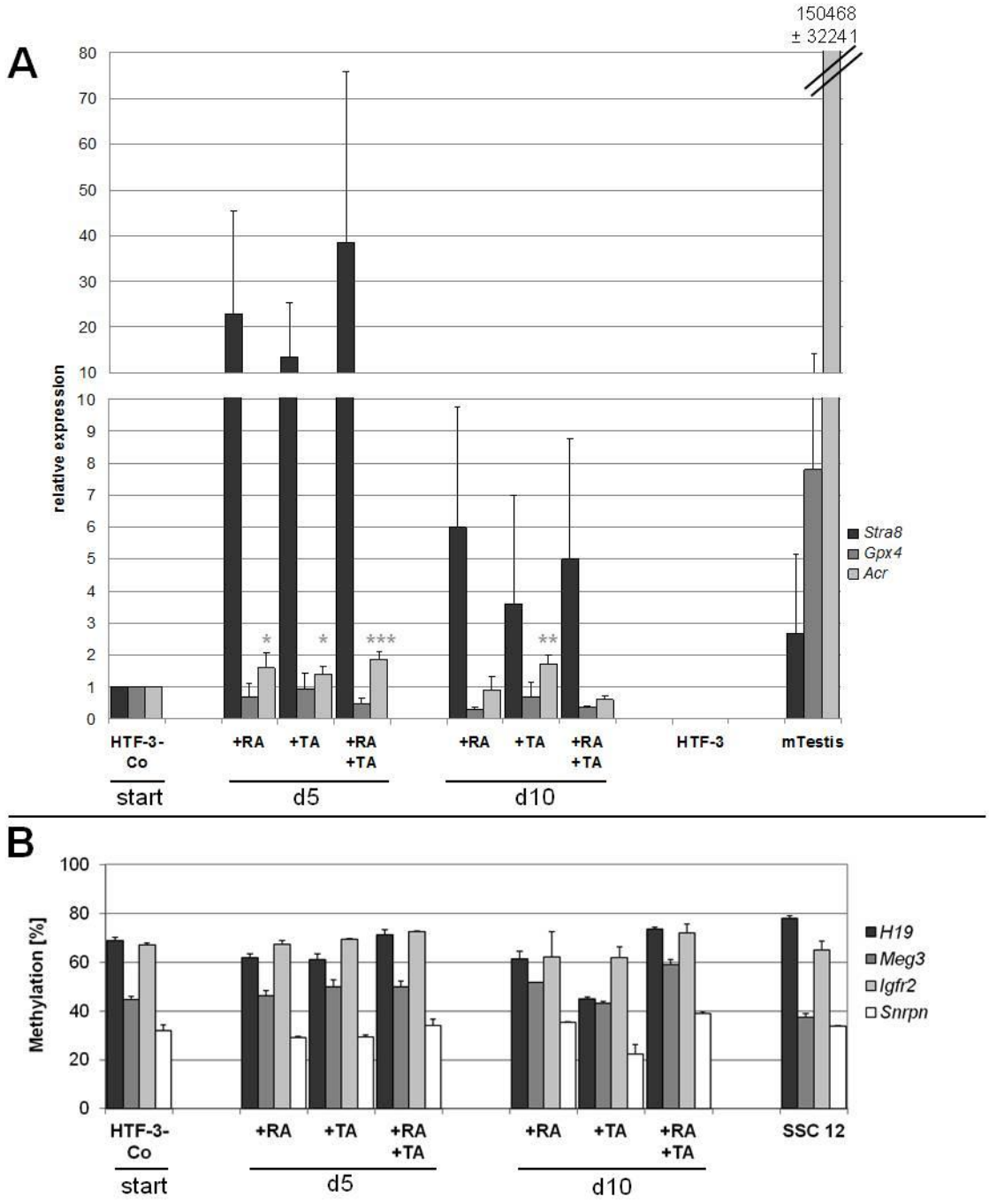


Fig. 44: Analyses of spermatogenesis marker gene expression by qRT-PCR experiments and methylation pattern during *in vitro* differentiation of HTF-3-Co. (A) Results of qRT-PCR-expression analyses for premeiotic *Stra8* and postmeiotic *Gpx4* and *Acr*. Mean values with standard deviations are shown. Data consists of at least two biological replicates. Significant increase of marker gene expression in co-culture samples during differentiation was calculated in comparison to HTF-3-Co before start of induction and is indicated with *: $p \leq 0.05$, **: $p \leq 0.01$; ***: $p \leq 0.001$. **(B)** The methylation status of the paternally imprinted *H19* ICR and *Meg3* IG-DMR and the maternally imprinted *Igf2r* DMR2 and *Snrpn* DMR1 were analyzed by bisulfite pyrosequencing. Mean values with standard deviations are shown. Data collection and analysis were performed by the Zechner group (Mainz). SSC 12: starting cell line of co-culture. HTF-3: pure HTF cell line; start: HTF-3-Co before start of induction. d: day of induction; +: incubated with certain reagent; RA: Retinoic Acid; TA: Talarozole (CYP26-inhibitor).

3.3.6 Role of HTF cells in generation of co-culture-derived putative mSSCs

During co-culture ESCs are seeded on mitotically active HTF cells, which are supposed to have an impact on the generation of putative mSSCs within this co-culture system. HTF cells might secrete important factors, which support the generation of putative mSSC. It is also possible that the cell-cell contact between HTF cells and ESCs promote the process of mSSC differentiation in co-culture. In order to determine a possible impact of secreted factors or the cell-cell contact on mSSC generation, some parameters in co-culture experiments were changed, the morphology changes of ESCs were monitored and compared with the progress in common co-culture system (Fig. 45). Usually the co-culture was performed with SSC 12 seeded on HTF cells, which was cultured in FB medium on gelatine coated vessels until ~80% confluence. Cells were cultured further in FB medium and SSC 12 revealed morphology changes, which resembled to putative mSSCs after nearly 20 days of co-culture (Fig. 45 A). Fig. 45 B and C illustrates the experimental design in order to examine the role of HTF cells in generation of co-culture-derived putative mSSCs. On the one hand SSC 12 was seeded on mitomycin treated MEF (Fig. 45 B) or directly on gelatine coated vessels (Fig. 45 C) in order to test if SSC 12 preferred HTF cells, needed any cell line or even no cells to attach and differentiate into putative mSSCs. On the other hand the use of conditioned (cond) FB medium derived from the culture of pure HTF cell lines should shed light on a possible impact of HTF cells' secreted factors on mSSC generation. For this purpose pure HTF was cultured in FB medium, which was filtered once before its further use for co-culture (cond FB; Fig. 45 II). Additionally SSC 12 was seeded on vessels and cultured in normal ESC culture medium without LIF, which is the usually used medium in differentiating experiments, in order to offer comfortable conditions for SSC 12 to attach. After one day of culture the medium was changed to FB (Fig. 45 III) or cond FB medium (Fig. 45 IV).

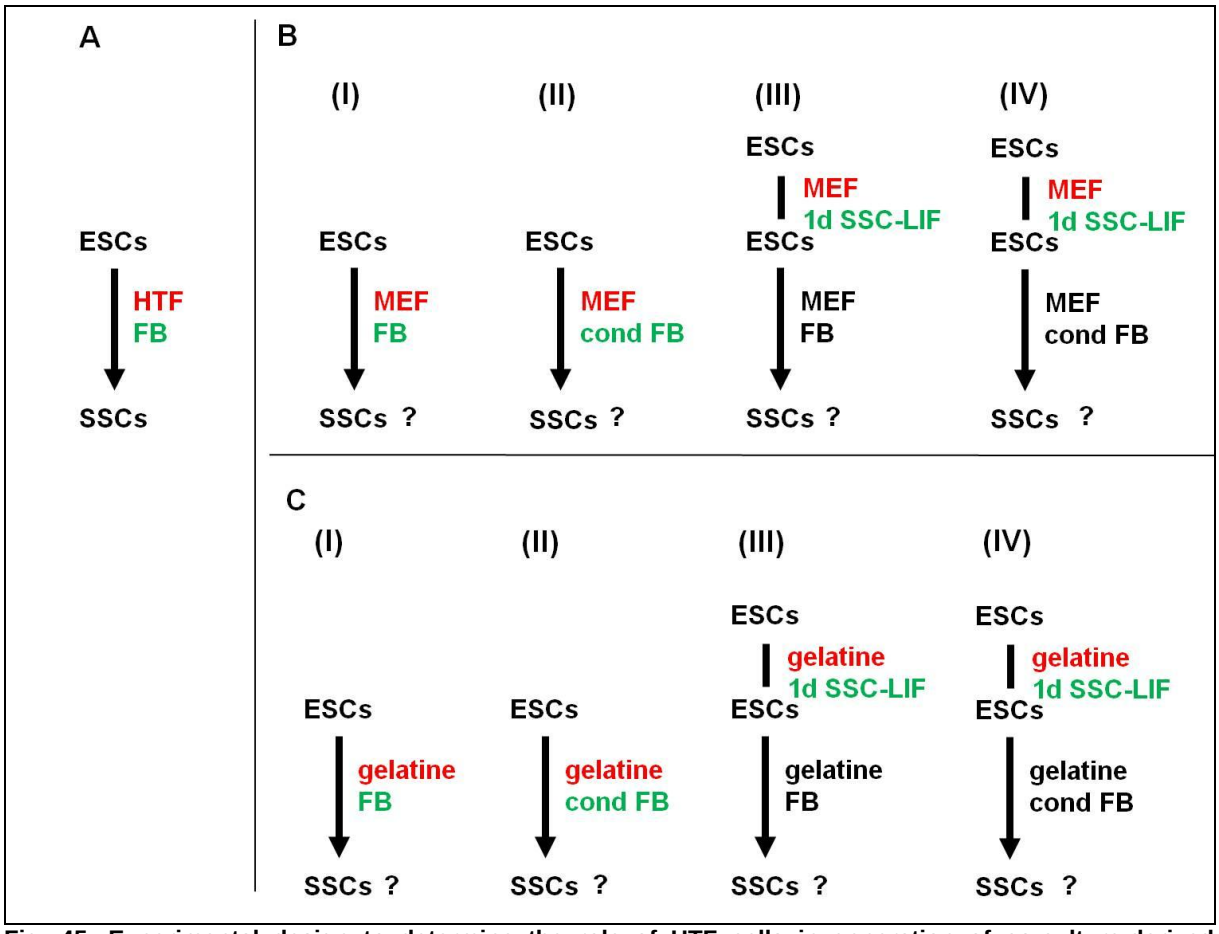
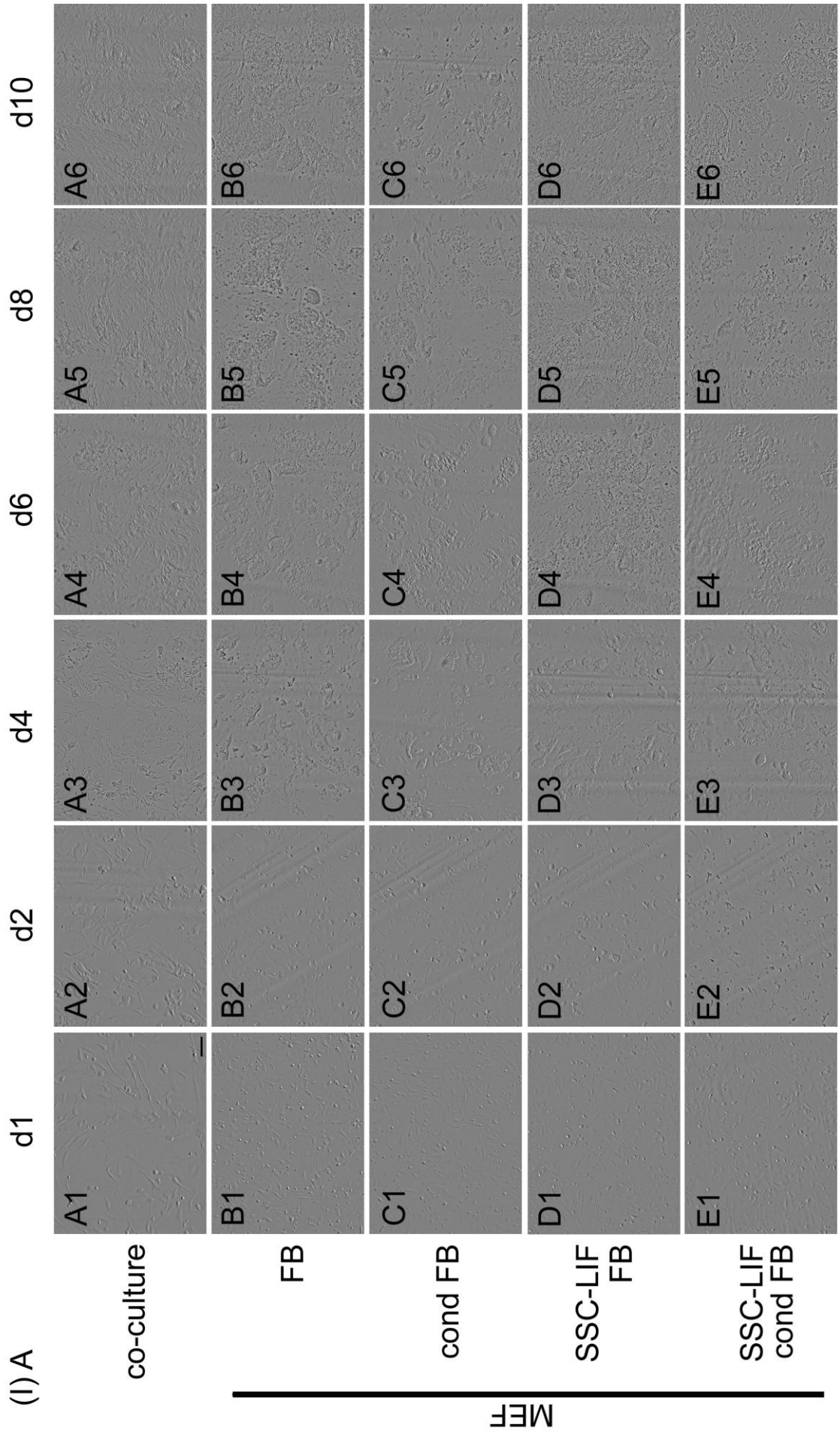


Fig. 45: Experimental design to determine the role of HTF cells in generation of co-culture-derived putative mSSCs. (A) Standard procedure of co-culture of ESCs with HTF cells in FB medium. **(B)** Co-culture experiments performed on mitomycin C treated MEF. **(C)** Co-culture experiments performed on gelatine coated vessels. Co-culture experiments on MEF and gelatine, respectively, were performed using **(I)** FB medium, **(II)** cond FB medium, **(III)** one day SSC-LIF medium followed by further culture in FB medium or **(IV)** cond FB medium. FB: Fibroblast medium; cond FB: conditioned FB medium; SSC-LIF: ESC culture medium without LIF; MEF: mytomycin treated MEF; d: day.

These different co-culture experiments were performed with HTF-1-Co. The procedure was exactly as already described (3.3.2). The morphology changes of SSC 12 were documented by taking brightfield images for all tested co-culture conditions one day after seeding of SSC 12 as well as every second day of co-culture (Fig. 46). Overall none of the tested conditions led to the generation of putative mSSCs derived from initially performed co-culture experiments using SSC 12 and HTF cells. Latest at day 14 cells cultured in different co-culture conditions were detached from the culture vessel (Fig. 46 IB and IIB). Rarely cell attachment of SSC 12 was observed using gelatine coating vessels with all tested medium conditions (Fig. 46 II A), while SSC 12 did attach very well to mitomycin C treated MEF independent from the used culture medium and enabled culture of SSC 12 till day 12 (Fig. 46 I B). However, none of these co-cultures using MEF for cell attachment led to a similar morphology change and putative mSSC development as observed in the initially performed co-culture (Fig. 46 I B, A6). Nevertheless, SSC 12 cultured in cond FB medium revealed a

healthier appearance than in FB medium. Altogether, these experiments demonstrated that cell attachment was facilitated by offering mitomycin C treated MEF for SSC 12, but it did not result in the generation of putative mSSCs. Based on these results HTF cells seem to be needed for cell attachment of ESCs as well as for providing important factors for differentiation of putative mSSCs in the co-culture system.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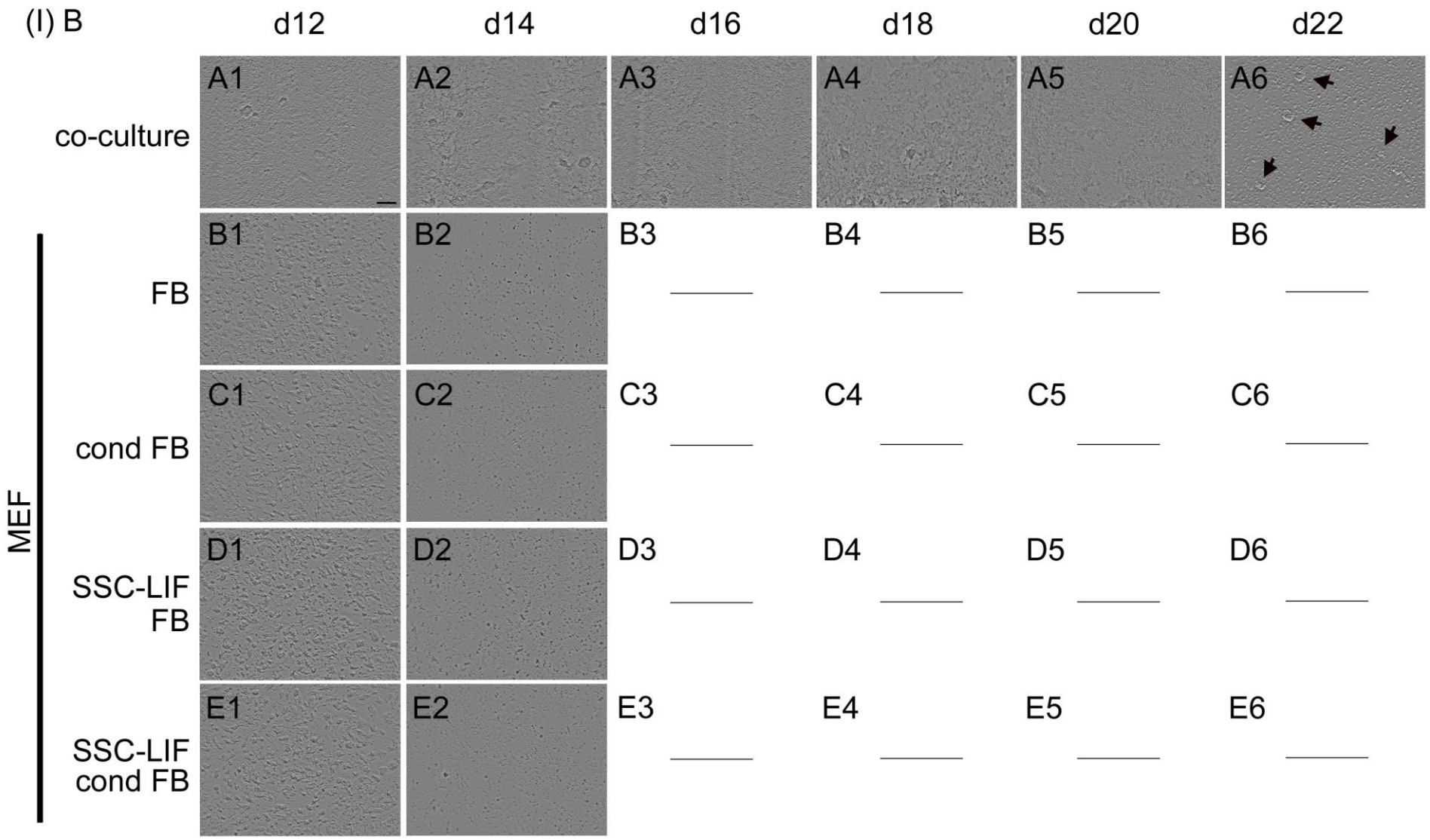
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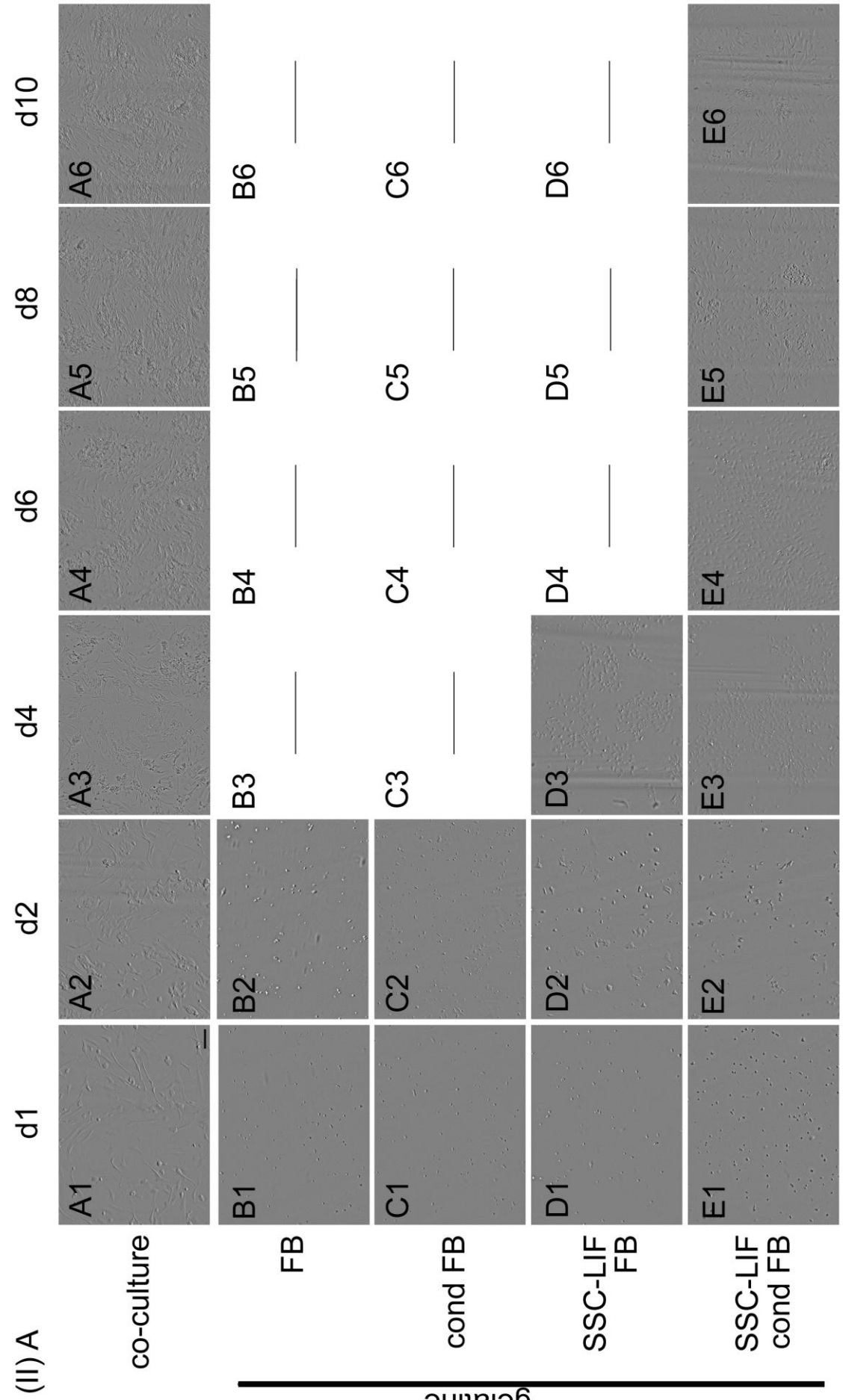
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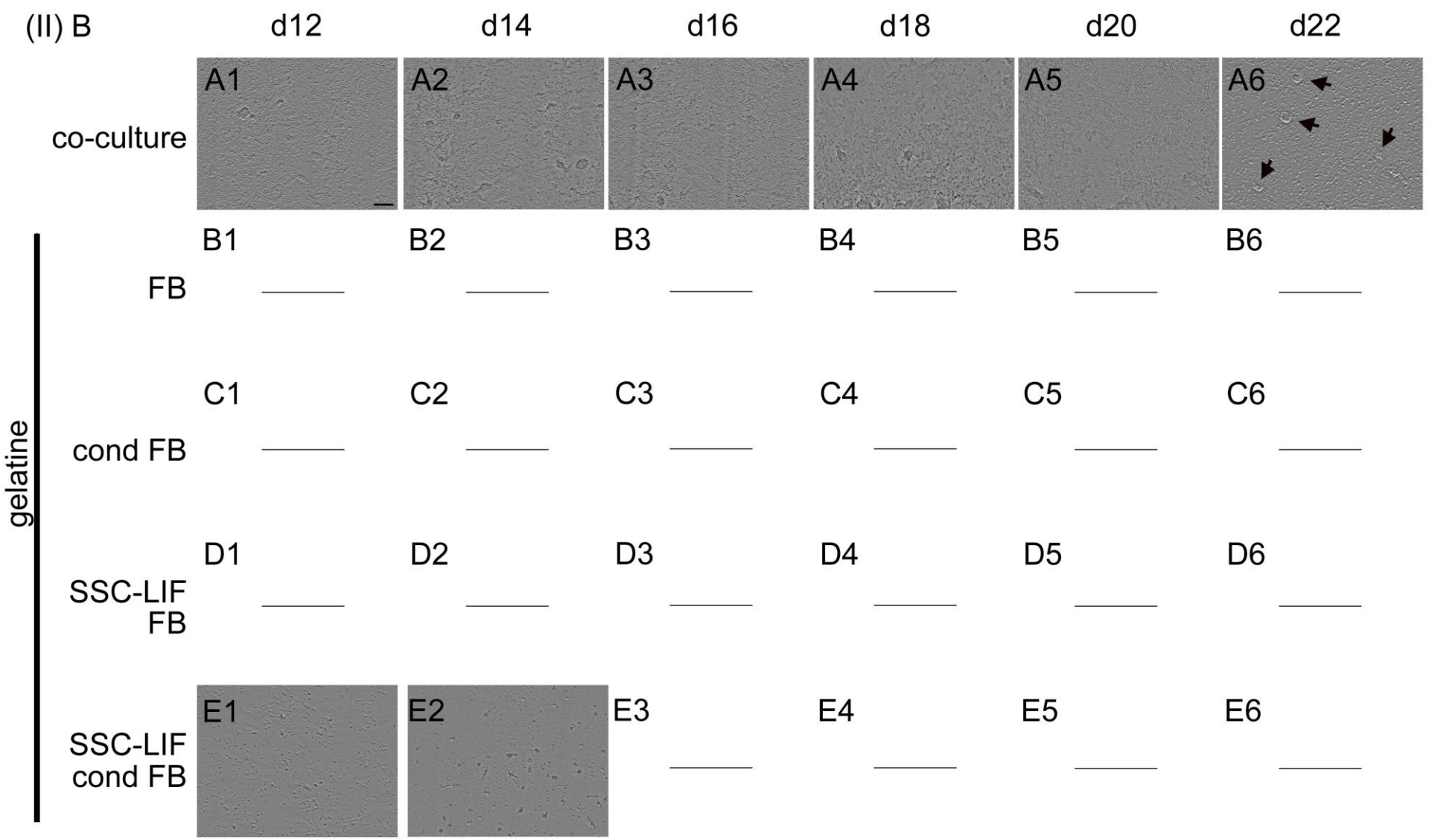
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B5
B6
FB
C1
C2
C3
C4
C5
C6
cond FB
D1
D2
D3
D4
D5
D6
SSC-LIF FB
E1
E2
E3
E4
E5
E6
SSC-LIF cond FB

Fig. 46: Morphology changes of HTF-1-Co during day 1 and day 22 cultured under different conditions to determine the role of HTF cells in generation of co-culture-derived putative mSSCs. HTF-1-Co was cultured on **(I)** MEF and **(II)** gelatine coated culture vessels. **A and B** shows morphology changes during day 1- day 10 and day 12- day 22, respectively. HTF-1-Co was performed as a positive control **(A1-A6)** revealing the SSC-like cells at day 22 (A6, arrows). Furthermore HTF-1-Co was cultured in FB **(B1-B6)**, in cond FB **(C1-C6)**, in SSC without LIF with medium change on FB **(D1-D6)** and in SSC without LIF with medium change on cond FB **(E1-E6)**. d: day of co-culture; FB: Fibroblast medium; cond FB: conditioned FB medium; SSC-LIF: ESC culture medium without LIF; -: no cells attached anymore. Representative scale bar in A1: 200 µm.

3.4 *In vitro* spermatogenesis experiments

3.4.1 Strategy for *Stra8* overexpression during *in vitro* spermatogenesis experiments

In general the generation of male germ cells derived from stem cells was performed according to the strategy, which was established in this institute (Nayernia et al., 2006; Nolte et al., 2010). In brief, the strategy bases on the transfection of stem cells with two promoter constructs, *Stra8-EGFP* and *Prm1-DsRed*, which enable the enrichment of SSC-like cells and the identification of haploid germ cells. The promoter construct *Stra8-EGFP* provides *EGFP* under the promoter control of the premeiotic gene *Stra8*. EGFP positive cells are enriched by two FACSortings and resemble a spermatogonial cell population. Cultivation of these cells under differentiating culture conditions yielded in formation of some haploid cells. These cells can be detected by *DsRed* expression, which is driven by the promoter of the postmeiotic gene *Prm1* (Nayernia et al., 2006; Nolte et al., 2010). Here a potential impact of *Stra8* on the efficiency and the quality on differentiation of male germ cells should be examined by the overexpression of this premeiotic gene during *in vitro* spermatogenesis. These experiments were planned to be carried out with two different cell lines, whose generation is specified in Fig. 47. The ESC line SSC 12 already contained the promoter constructs *Stra8-EGFP* and *Prm1-DsRed* (Nayernia et al., 2006) and could be directly used for *Stra8* overexpression studies (Fig. 47 A). Moreover ESC lines derived from a double transgenic mouse containing premeiotic *Stra8-EGFP* and meiotic *Sycp3-DsRed* constructs (Smorag et al., 2012) were generated to be used for these experiments (Fig. 47 B). Their use provides the advantage that all cells, which might undergo meiosis, can be detected by their red fluorescence due to the activation of the *Sycp3* promoter. An influence of *Stra8* on the *in vitro* spermatogenesis was planned to be examined by its permanent overexpression (Fig. 48 B) during differentiation (Fig. 48 C).

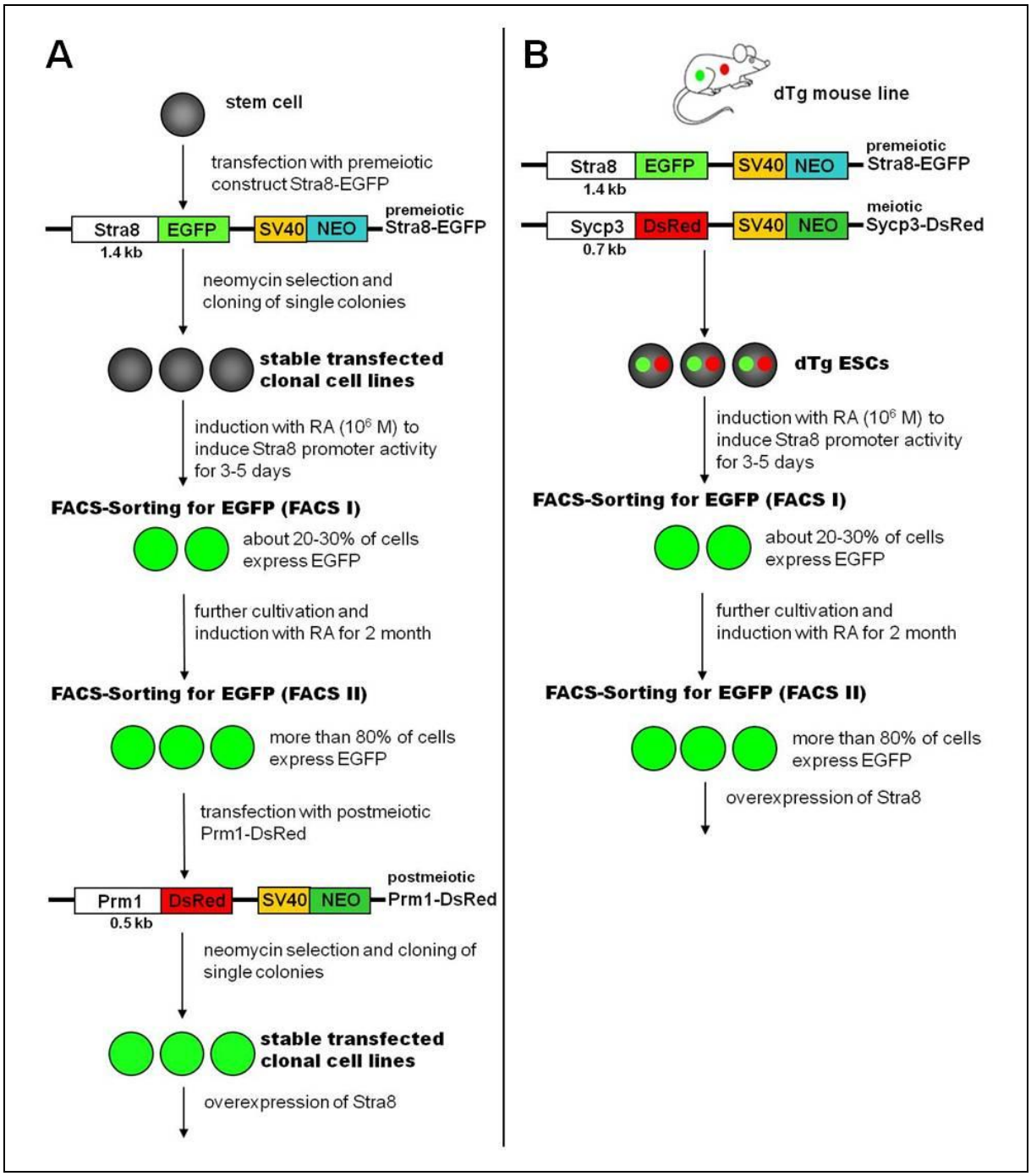


Fig. 47: Scheme of the experimental procedure for the generation of male germ cells derived from different ESCs. (A) Wild type ESCs were transfected with the promoter construct *Stra8-EGFP* and two times FACSorted for the enrichment of EGFP positive cells, which were further transfected with *Prm1-DsRed*. This stable transfected clonal cell line SSC 12 was already available (Nayernia et al., 2006). **(B)** ESCs containing *Stra8-EGFP* and *Sycp3-DsRed* were generated from double transgenic mice and EGFP positive cells were collected within two FACSortings. ESCs described in A and B should be used for *Stra8* overexpression studies during *in vitro* spermatogenesis. RA: Retinoic Acid; FACS: Fluorescence Activated Cell Sorting; dTg: double transgene; ESCs: Embryonic Stem Cells. Modified, Nolte et al., 2010.

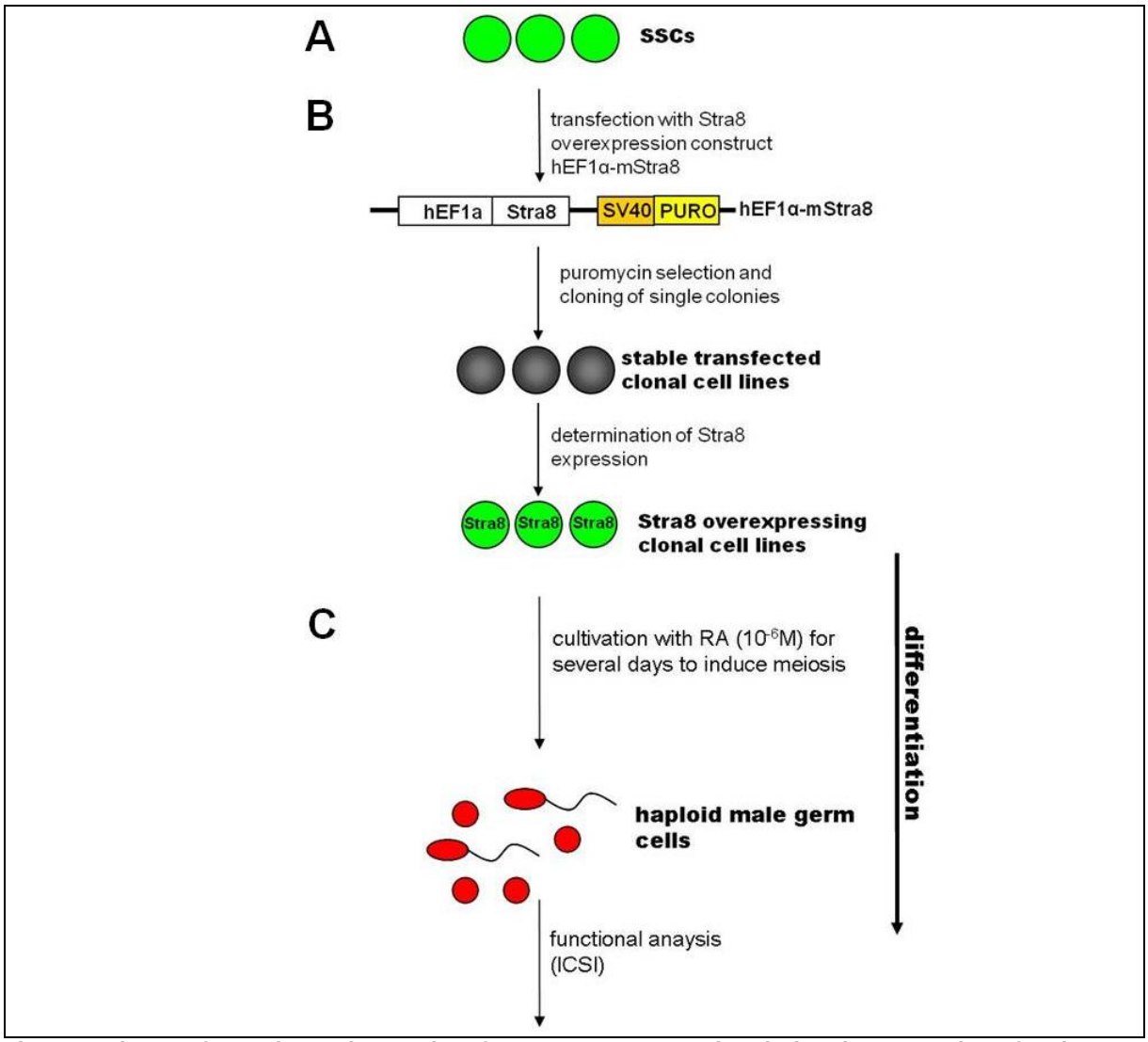


Fig. 48: Scheme of experimental procedure for *Stra8* overexpression during the generation of male germ cells. (A)Two times FACSorted ESCs (Fig. 47) resemble a spermatogonial cell population (SSCs). **(B)** SSC-like cells were transfected with hEF1α-mStra8 construct for stable Stra8 overexpression. **(C)** Transfected cells should be used for *Stra8* overexpression studies during *in vitro* spermatogenesis. SSCs: Spermatogonial Stem Cells; RA: Retinoic Acid; ICSI: Intracytoplasmic Sperm Injection. Modified, Nolte et al., 2010.

3.4.2 ES cell lines used for *in vitro* spermatogenesis experiments

3.4.2.1 ES cell line SSC 12 (Nayernia et al., 2006)

The ESC line SSC 12, generated by Nayernia et al. (2006), was used for *in vitro* spermatogenesis experiments. SSC 12 is an ES-RI cell line, established from a murine 129SV-background (Nagy et al., 1993) and transfected with the premeiotic promoter construct *Stra8-EGFP* and postmeiotic promoter construct *Prm1-DsRed* (Fig. 49 A). Before its use the presence of *Stra8-EGFP* and *Prm1-DsRed* in SSC 12 was validated by genotyping PCR using primers located in the promoter region of the *Stra8* or *Prm1* and in the EGFP or DsRed coding region, respectively (Fig. 49 B).

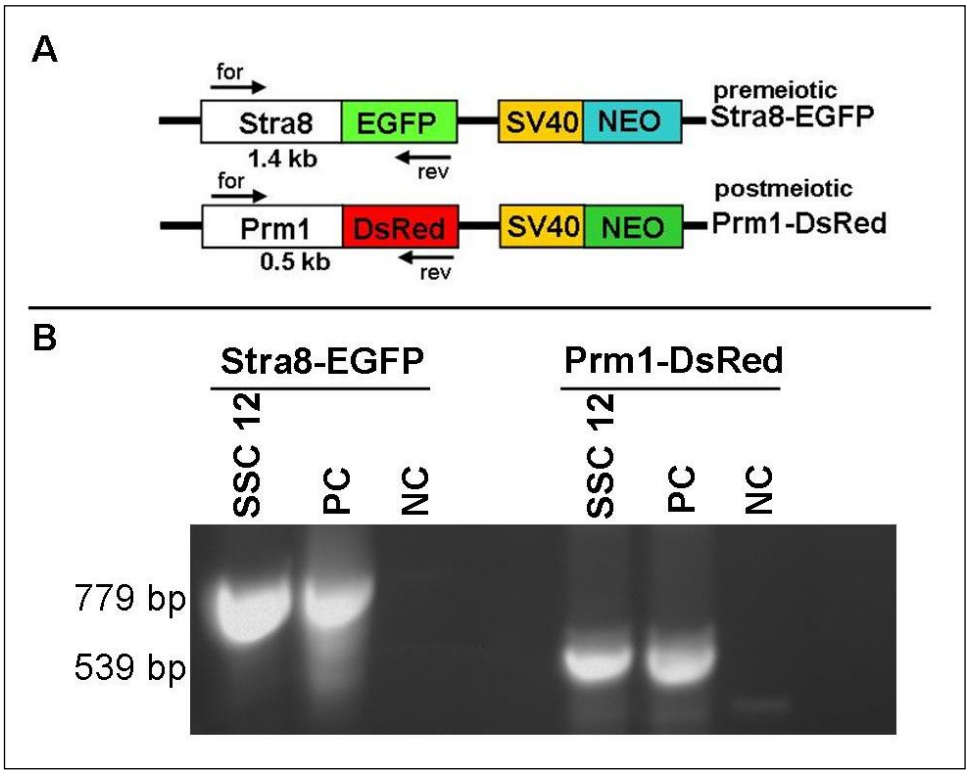


Fig. 49: Examination of SSC 12 containing the promoter constructs *Stra8-EGFP* and *Prm1-DsRed*. (A) Schematic representation of the promoter constructs *Stra8-EGFP* and *Prm1-DsRed*. Primers used for genotyping PCR are indicated with arrows. kb: kilo base of promoter region. for/rev: forward/reverse primers used for genotyping PCR. Modified, Nolte et al., 2010. **(B)** Genotyping PCR of SSC 12 proved the presence of *Stra8-EGFP* and *Prm1-DsRed*. Fragment sizes are indicated within the figures. PC: positive control; NC: negative control with H_2O; bp: base pairs.

3.4.2.2 ES cell lines derived from Stra8-EGFP/Sycp3-DsRed mice

ES cell lines derived from double transgenic (dTg) *Stra8-EGFP* and *Sycp3-DsRed* mouse line (Smorag et al., 2012) were generated to be used in *in vitro* spermatogenesis experiments. These transgenic mice provide the premeiotic *Stra8-EGFP* and meiotic *Sycp3-DsRed* constructs (Fig. 50).

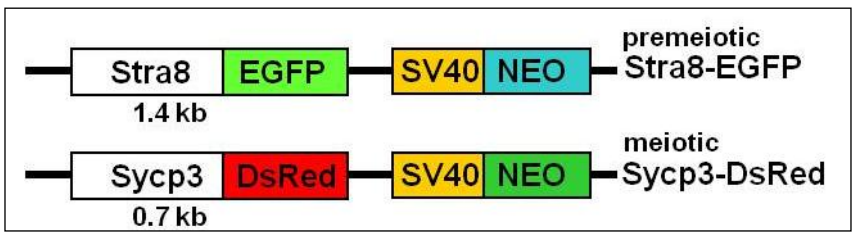


Fig. 50: Schematic representation of promoter constructs of double transgenic *Stra8-EGFP* and *Sycp3-DsRed* mice. kb: kilo base of promoter region. Modified, Nolte et al., 2010.

3.4.2.2.1 Generation of Stra8-EGFP/Sycp3-DsRed ES cell lines

The dTg *Stra8-EGFP* and *Sycp3-DsRed* mouse line (Smorag et al., 2012) was used for the generation of Stra8-EGFP/Sycp3-DsRed ES cell lines. In brief, blastocysts were isolated from gestating mice at stage 3.5 days post coitum (dpc) and cultured on MEF feeder layer in KSOM medium at 37°C and 5% CO_2. After outgrowth of the inner cell mass of the blastocysts, it was disaggregated and seeded on fresh MEF feeder layer. During further culture in ESC medium cells grew in colonies and revealed a mouse ES cell like morphology (2.2.4.10).

3.4.2.2.2 Characterization of Stra8-EGFP/Sycp3-DsRed-2 ES cells

While several ES cell lines could be generated from dTg mice, Stra8-EGFP/Sycp3-DsRed-2 cells were chosen for further characterization and experimental procedure (Fig. 51 A). Its pluripotent character was proven by the detection of OCT4 protein expression using western blot analysis. ES-RI was used as a positive control, whereas the negative control CRL-2691, a male human fibroblast cell line, did not reveal any expression of the pluripotency marker (Fig. 51 B). Furthermore, a male genotype of Stra8-EGFP/Sycp3-DsRed-2 was required for its intended use in *in vitro* spermatogenesis experiments. Aiming to check for its male genotype, Stra8-EGFP/Sycp3-DsRed-2 cells were examined for the presence of the androgen receptor (AR) and sex determining region of chromosome Y (SRY). Genomic DNA of a male wild type mouse was used as a positive control and revealed detection of both genes analyzed, whereas *Sry* could not be detected in female wild type mouse used as a negative control. Compared to the controls Stra8-EGFP/Sycp3-DsRed-2 demonstrated a male genotype (Fig. 51 C).

Finally, genotyping PCR confirmed that Stra8-EGFP/Sycp3-DsRed-2 cells indeed derived from dTg animals and contained the transgenes *Stra8-EGFP* and *Sycp3-DsRed* (Fig. 51 D). Analysis was performed according to Smorag et al. (2012) and genomic DNA of a wild type ES cell line was used for control.

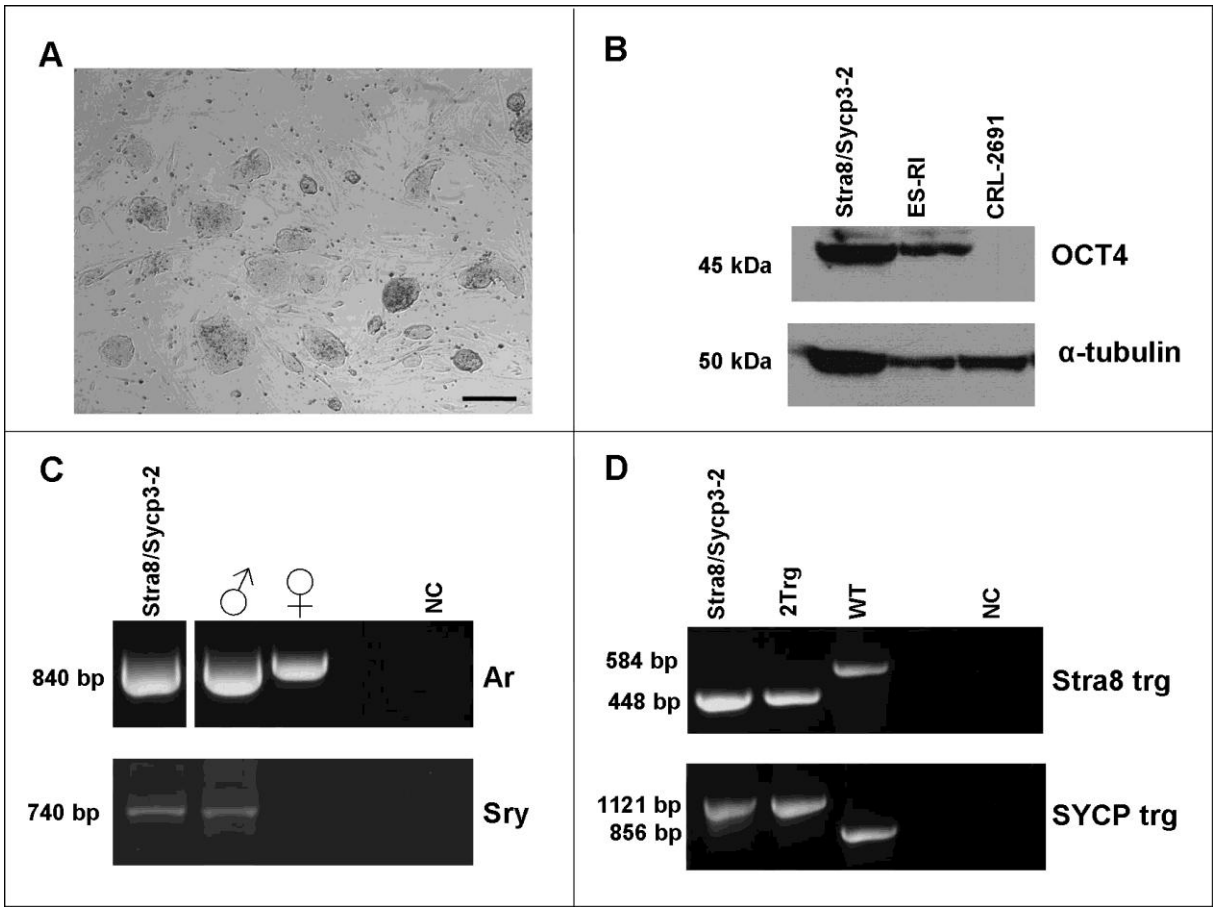


Fig. 51: Characterization of the generated ES cell line Stra8-EGFP/Sycp3-DsRed-2. (A) Representative brightfield image of the generated ES cell line Stra8-EGFP/Sycp3-DsRed-2. Scale bar: 200 µm. **(B)** Western blot analysis revealed the expression of the pluripotency factor OCT4 in Stra8-EGFP/Sycp3-DsRed-2 cells. Wild type ES cell line ES-RI was used as positive control, human fibroblast cell line CRL-2691 as negative control. Detection of α-tubulin served as loading control. Molecular weights of proteins are indicated. kDa: kilo Dalton. **(C)** Stra8-EGFP/Sycp3-DsRed-2 cells possessed a male genotype by expressing *Ar* and *Sry*. Genomic DNA of male and female mice served as controls. Ar: Androgen receptor; Sry: sex determining region of chromosome Y; NC: negative control with H_2O. **(D)** Genotyping PCR proved the derivation of Stra8-EGFP/Sycp3-DsRed-2 cells from homozygous double transgenic mice. 2Trg: genomic DNA of double transgenic mice; WT: wild type genomic DNA; NC: negative control with H_2O; trg: transgene. Stra8 trg: genotyping PCR for Stra8 transgene; SYCP trg: genotyping PCR for Sycp3 transgene. PCR fragment sizes are indicated within the figures C and D.

3.4.2.2.3 Enrichment of EGFP positive cells by FACSorting

According to the strategy for the derivation of male germ cells from stem cells, an enrichment of EGFP positive ($EGFP^+$) cells resembles a spermatogonial population (Nayernia et al., 2006; Nolte et al., 2010), which requires a retinoic acid (RA) inducible EGFP expression in the used cell line. For validation of RA inducible EGFP expression, Stra8-EGFP/Sycp3-DsRed-2 cells were cultured in the presence of RA (10^{-6} M) for three days and checked for EGFP expression by fluorescence microscopy (Fig. 52 I). In comparison to non-RA-induced cells, application of RA activated the *Stra8* promoter and induced the EGFP expression visible in the strong EGFP fluorescence of

Stra8-EGFP/Sycp3-DsRed-2 cells. For enrichment of $EGFP^+$ cells Stra8-EGFP/Sycp3-DsRed-2 cells were induced with RA for three days and $EGFP^+$ cells were collected by FACSorting (Fig. 52 II). The fraction of $EGFP^+$ cells could be raised by ~32% resulting in a population of ~82% $EGFP^+$ cells (Fig. 52 IIA). These cells were cultured on MEF feeder layer in ESC medium containing RA in order to further increase the $EGFP^+$ cell population, which should be obtained by a second FACSorting. Here the initial $EGFP^+$ cell population revealed ~56% $EGFP^+$ cells, which could be enriched in a fraction consisting of ~87% $EGFP^+$ cells by repeated FACSorting (Fig. 52 IIB). These cells were further cultured on MEF feeder layer in ESC medium containing RA. ES cells derived from a murine C57BL/W background were used as control in FACSorting analyses and Stra8-EGFP/Sycp3-DsRed-2 cells before and after FACSortings were cyropreserved in a sufficient number of stocks for subsequent analyses.

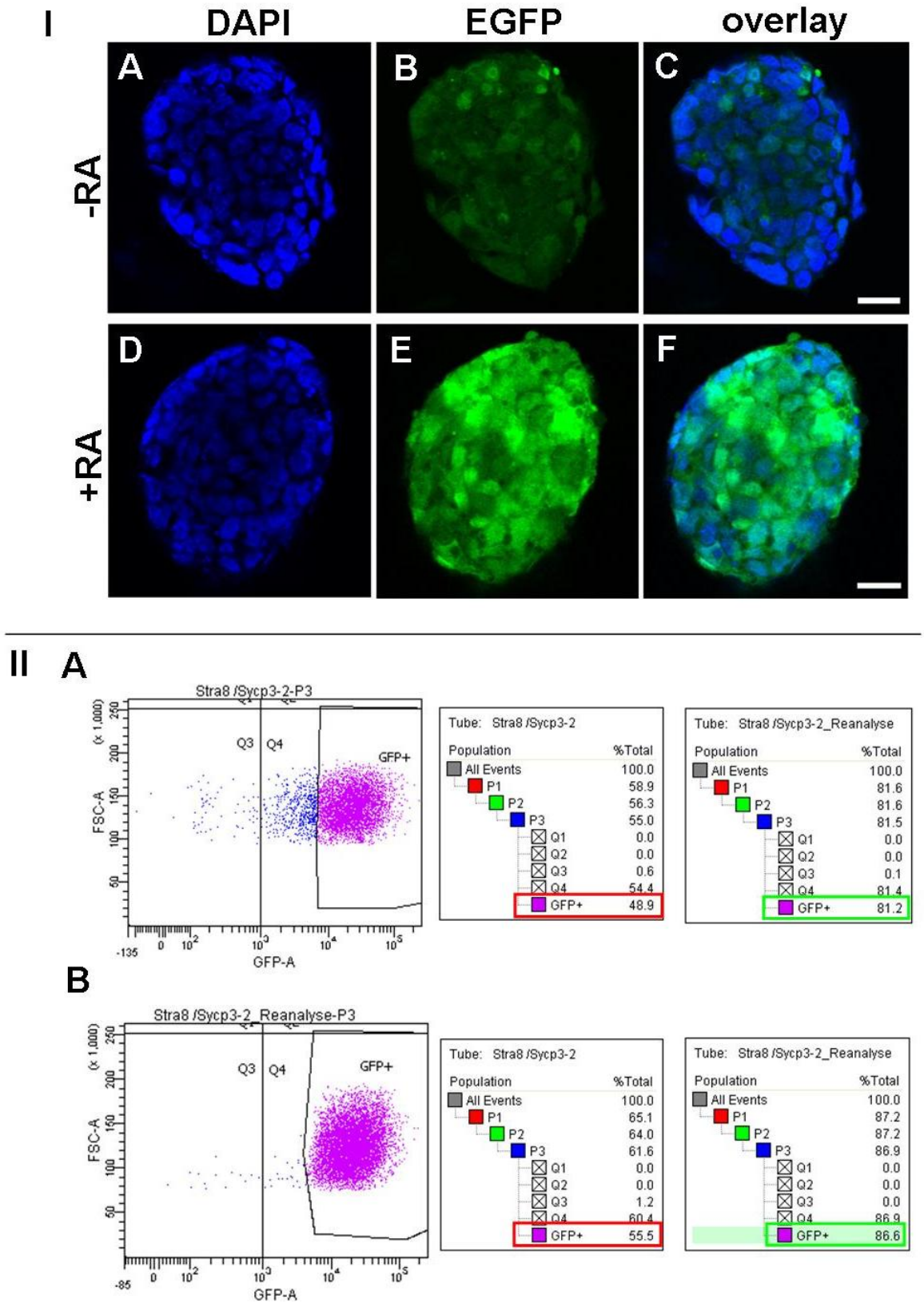


Fig. 52: RA inducible EGFP expression used for FACSortings of Stra8-EGFP/Sycp3-DsRed-2 ES cells. (I) Stra8-EGFP/Sycp3-DsRed-2 colonies revealed strong EGFP expression after three days of RA induction. -: without RA induction; +: with 10^{-6} M RA induction for three days; RA: Retinoic Acid. Representative scale bars are indicated within overlay images. Scale bars: 20 µm. **(II)** EGFP positive cells were enriched by two FACSortings **(A + B)** of RA induced Stra8-EGFP/Sycp3-DsRed-2 cells. Percentages of EGFP positive cells before and after FACSortings are highlighted in red and green, respectively.

3.4.3 Generation of *Stra8* overexpressing cell lines

It was shown that male gametes derived *in vitro* from ES cells were able to activate unfertilized oocytes and gave rise to offspring (Nayernia et al., 2006; Nolte et al., 2010). Nevertheless, these offspring died prematurely and revealed incorrect reprogramming of imprinted genes, which is necessary for normal development. It is suggested that germ cell related genes influence meiosis as well as imprinting during spermatogenesis (Yu et al., 2009; Medrano et al., 2012). The premeiotic gene *Stra8* is known to be essential for induction of meiosis (Baltus et al., 2006; Anderson et al., 2008) and is suggested to interact with chromatin remodelling proteins, which are regulators of imprinting control regions (Pantakani, unpublished data). In the presented thesis it should be tested, if a *Stra8* overexpression during *in vitro* spermatogenesis can enhance the progression of meiosis and support correct imprinting.

3.4.3.1 Stable *Stra8* overexpression

3.4.3.1.1 Generation of phEF1α-puro-mStra8

For stable overexpression of *Stra8* cells had to be transfected with a plasmid providing the mouse *Stra8* (m*Stra8*) ORF (Open Reading Frame) for expression. Due to the already included promoter constructs *Stra8-EGFP* and *Prm1-DsRed*, which each have a neomycin resistance cassette, SSC 12 already possessed a strong resistance towards neomycin.

Therefore, the expression plasmid phEF1α-puro-mStra8, which contains m*Stra8* under the control of the h*EF1α* promoter and a puromycin resistance cassette for selection, was generated (Fig. 53 A). The plasmid Integrin β1-tev-BHCys (addgene) with the vector backbone pEF-puro (phEF1α-puro, ~7400 bp) provided the h*EF1α* promoter for target gene expression as well as the selectable marker puromycin. Correct nucleotide sequences of h*EF1α* promoter and *Puromycin* gene were validated by sequencing analysis. phEF1α-puro was prepared by cutting out the insert integrin (2500 bp) using *Bam*HI/*Not*I, followed by gel extraction and purification of the vector (Fig. 53 B). The ORF of m*Stra8* (1182 bp) was cut out of phEF1α-mStra8 using *Bam*HI/*Not*I (Fig. 53 C), followed by ligation with phEF1α-puro and transformation in DH5α cells. Identification of positive clones was performed by test digestions using *Bam*HI/*Not*I (Fig. 53 D). After linearization with *Bst*I and sterile purification (Fig. 53 E), phEF1α-puro-mStra8 was used for electroporation of SSC 12 (3.4.3.1.2).

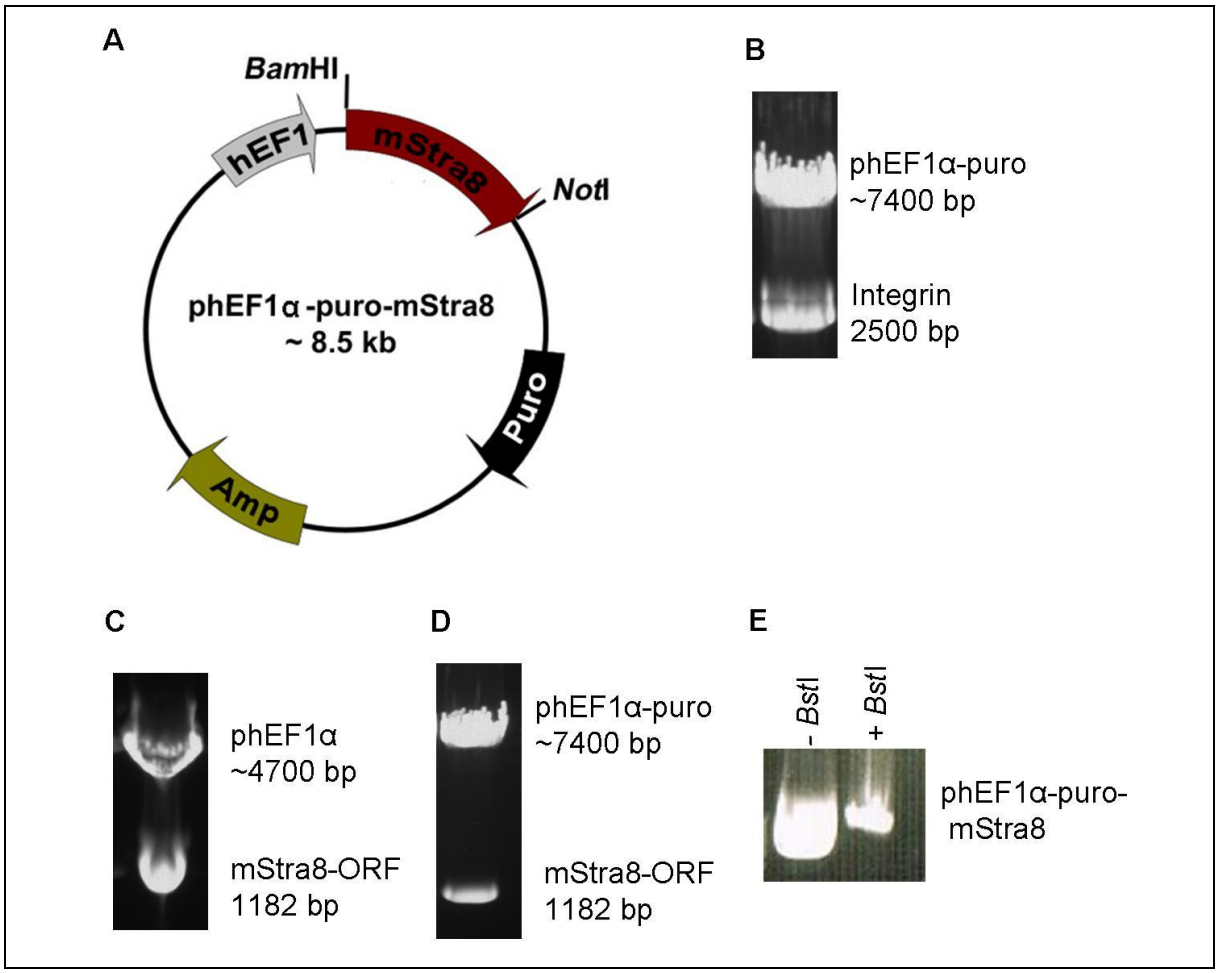


Fig. 53: Generation of phEF1α-puro-mStra8 construct. (A) Plasmid map of generated phEF1α-puro-mStra8 construct, which provides m*Stra8* driven by the hEF1α promoter as well as a puromycin (Puro) and ampicillin (Amp) resistance cassette for cloning and bacteria selection, respectively. m*Stra8* ORF was cloned into *Bam*HI/*Not*I restriction sites. **(B**) Extraction of phEF1α-puro. **(C)** Extraction of mStra8 ORF (1182 bp) from phEF1α-mStra8. **(D)** Validation of correct phEF1α-puro-mStra8 by test digestion using *Bam*HI/*Not*I. **(E)** Linearization of phEF1α-puro-mStra8 using *Bst*I for stable cell transfection by electroporation. Fragment sizes are indicated within the figures. ORF: Open Reading Frame; bp: base pairs.

3.4.3.1.2 Generation of stable *Stra8* overexpressing SSC 12 cell lines

A puromycin tolerance test using SSC 12 was performed to determine the puromycin concentrations, which should be used for the selection of successful transfected cells after electroporation and further culturing. The test demonstrated that puromycin selection of transfected SSC 12 could be performed using 1.25 µg/ml puromycin (selection medium), while later established cell lines should be cultured in medium containing 0.75 µg/ml puromycin (culture medium) in order to ensure plasmid uptake. After electroporation of SSC 12 with phEF1α-puro-mStra8 positive selection of transfected cells was performed with ESC culture medium containing puromycin (1.25 µg/ml) for two days. Because of increased apoptosis, selection medium was replaced with ESC culture medium without puromycin for cell recovery for four days. Growing clones were further cultured in ESC culture medium

containing culture concentration of puromycin and processed according standard procedure including picking and propagation of single clones (2.2.4.5) and their DNA as well as RNA isolation for genotyping and qRT-PCR analyses.

Genotyping PCR to check for phEF1α-puro-mStra8 by using primers located in the h*EF1α* promoter and m*Stra8* ORF revealed several clones, which might be successful transfected with phEF1α-puro-mStra8 (Fig. 54 I). The clones #11 and #13, which were selected for *Stra8* expression analysis by qRT-PCR, revealed stable *Stra8* overexpression and were termed as SSC 12/11 and SSC 12/13, respectively. Both cell lines showed an about six to seven fold increased *Stra8* expression in comparison to ES-RI, which represented the original wild type ES cell line used for generation of SSC 12 (Fig. 54 IIA). SSC 12 itself revealed already a three times increased Stra8 expression compared to ES-RI (Fig. 54 IIA). Additionally, *Stra8* was about two times higher expressed in SSC 12/11 and SSC 12/13 (Fig. 54 IIB), thus the *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 were further used for *in vitro* spermatogenesis experiments.

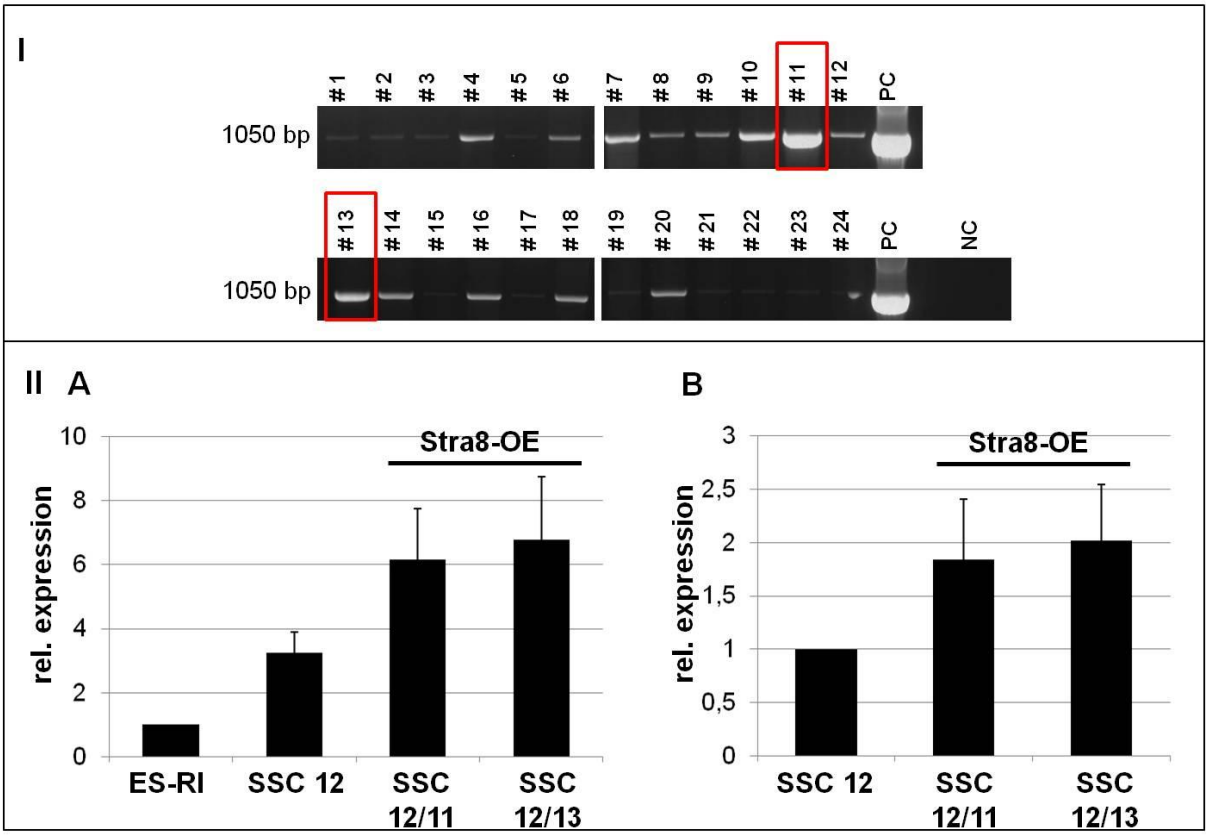


Fig. 54: Determination of *Stra8* expression in phEF1α-puro-mStra8 transfected SSC 12 cells. (I) Genotyping PCR for the construct phEF1α-puro-mStra8 revealed several potential clones after transfection of SSC 12 with phEF1α-puro-mStra8. # 11 and # 13, highlighted in red, were selected for *Stra8* expression analysis by qRT-PCR. Fragment size is indicated within the figure. bp: base pairs; #: clone number; PC: positive control with phEF1α-puro-mStra8 DNA; NC: negative control with H_2O. **(II)** SSC 12/11 and SSC 12/13 showed an **(A)** about six to seven times increased *Stra8* expression compared to wild type ES cell line ES-RI and **(B)** about two times increased expression as compared to SSC 12. Stra8-OE: *Stra8* overexpression.

3.4.4 *Stra8* overexpression during *in vitro* spermatogenesis

3.4.4.1 Morphological changes of *Stra8* overexpressing cells during induction of differentiation

The analysis of an effect of permanent *Stra8* overexpression during differentiation of male germ cells was performed with the *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 in comparison to the originally cell line SSC 12. SSC 12/11 and SSC 12/13 revealed a two times increased *Stra8* expression compared to SSC 12 (3.4.3.1.2).

To maintain their undifferentiated pluripotent state, ESCs are cultured on MEF feeder layer in the presence of the cytokine LIF, whose activation of the JAK/STAT signalling pathway regulates the transcriptional expression of pluripotency- and tissue-specific genes (Arabadjiev et al., 2012). For induction of *in vitro* differentiation, cells were seeded on gelatine coated vessels und cultured in ESC medium without LIF. Additionally, the differentiation medium was supplemented with RA as a widely used reagent for ESC differentiation as well as for induction of *Stra8* expression. During differentiation morphology changes of the cells were documented by taking brightfield images before induction as well as every third day after induction (Fig. 55).

During the first six days of induction SSC 12 and *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 did not reveal differences in morphological changes. Cells lost cell their ESC-like structure and showed differentiating features characterized by fringed borders at day 3 (Fig. 55 D-F) and formation of embryoid body (EB)- like structures at day 6 (Fig. 55 G-I). After nine days of induction *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 revealed a clearly less number of attached cells including EB-like structures. The remaining EB-like structures exhibited a decreased size (Fig. 55 J-L). While at day 12 *Stra8* overexpressing cells SSC 12/11 and SSC 12/13 were rarely attached, SSC 12 revealed cell detachment to a minor extend and the remaining EB-like structures increased in their size (Fig. 55 M-O). While SSC 12/11 and SSC 12/13 could not be cultured further, SSC 12 could be induced until day 15. At this time point some cells including smaller EB-like structures were still attached (Fig. 55 P).

Cells as well as the supernatant, which could contain meiotic cells released into the medium, were harvested at the indicated time points. RNA and gDNA was isolated for further analyses by RT- and qRT-PCR analyses for the expression of spermatogenesis markers and bisulfite pyrosequencing for methylation studies, respectively.

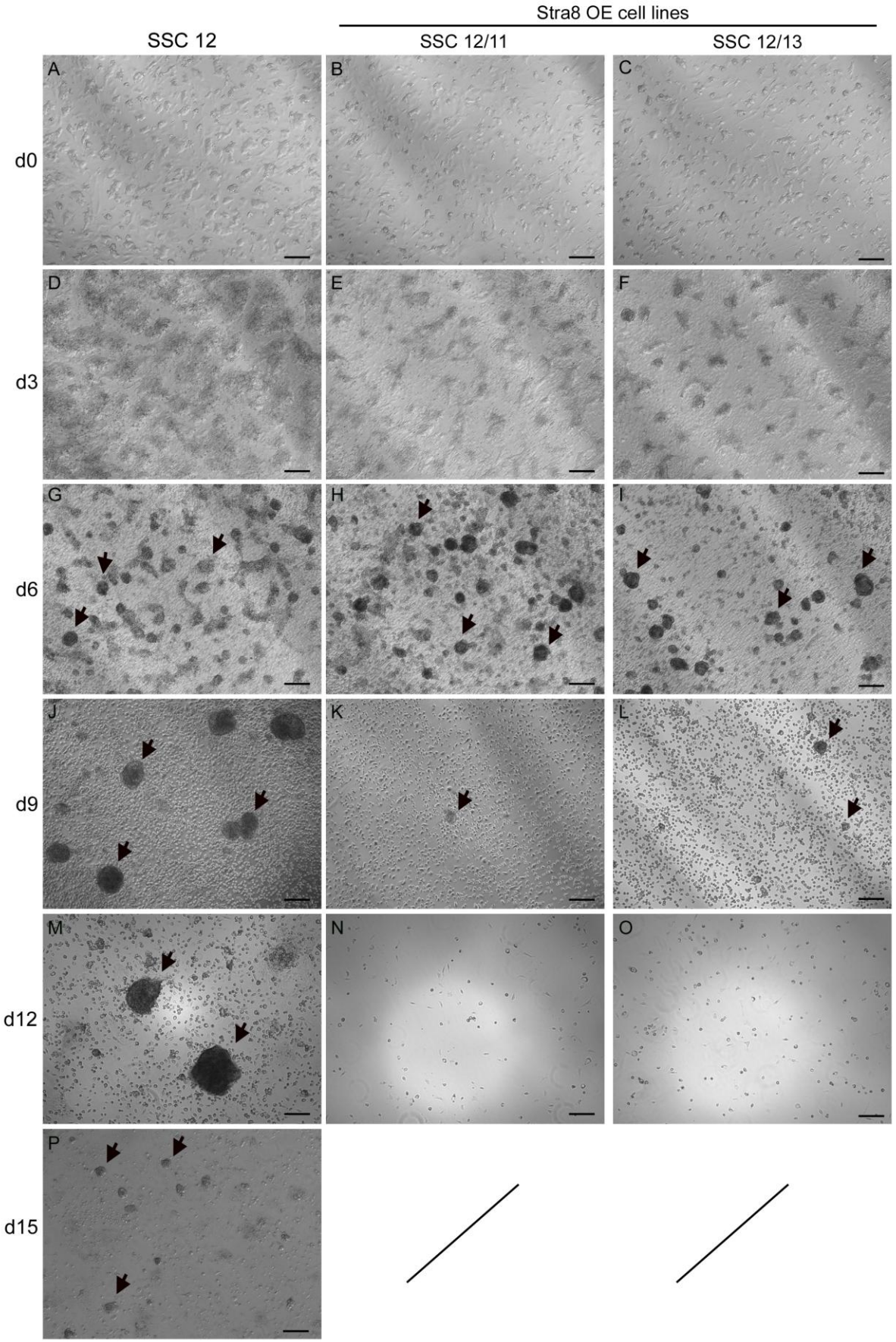


Fig. 55: Microscopic documentation of morphological changes of SSC 12 and *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 during *in vitro* differentiation. SSC 12 and *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 were cultured in differentiation medium for 12 to 15 days. Their morphology was

documented with brightfield images each third day. **(A-C)** Cells before start of *in vitro* differentiation at day 0. **(D-F)** Cells revealed fringed borders at day 3. **(G-I)** Cells formed EB-like structures at day 6. **(J-L)** *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 showed a decreased number and smaller EB-like structures than SSC 12 at day 9. **(M-O)** SSC 12/11 and SSC 12/13 showed an increased cell detachment, while EB-like structures in SSC 12 further increased in size at day 12. **(P)** SSC 12 revealed still adhered cells and small EB-like structure at day 15. Arrows within images indicate exemplarily EB-like structures. Stra8 OE cell lines: *Stra8* overexpressing cell lines; d: day. EB: Embryoid Body. Scale bars: 200 µm.

3.4.4.2 Analyses of spermatogenesis marker expression during *in vitro* differentiation

3.4.4.2.1 Immunocytochemical analysis of SSC 12 and *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13

At the indicated days of *in vitro* differentiation SSC 12 and *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 were checked for the expression of DsRed by microscopy, which would indicate an activation of the postmeiotic *Prm1* promoter and thus a postmeiotic germ cell development. No DsRed-positive cells could be detected at any of the time points suggesting that the *Prm1* promoter was not activated and cells obviously did not develop into haploid male germ cells. Because *Prm1* is expressed at the final stage of germ cell development, cells undergoing the differentiation process were additionally analyzed by immunostaining using DAZL (Deleted In Azoospermia-Like) specific antibody. The RNA binding protein DAZL plays an important role during germ cell differentiation and is localized in the nucleus of spermatogonia, but translocates into the cytoplasm at the onset of meiosis (Reijo et al., 2000). SSC 12 was already DAZL positive at day 3 showing a weak nuclear and partly intensive cytoplasmic expression in single cells. During the differentiation process the DAZL signal increased clearly, but was mostly restricted to the cytoplasm of cells within the EB-like structures until day 12 (Fig. 56). In contrast, DAZL expression was just visible in the *Stra8* overexpressing cell line SSC 12/11, while SSC 12/13 did not reveal any DAZL expression. In SSC 12/11 cells DAZL expression was mostly restricted to the cytoplasm of cells within EB-like structures at day 6 (Fig. 57). At this time point of differentiation *Stra8* overexpressing cell lines exhibited the most and largest EB-like structures.

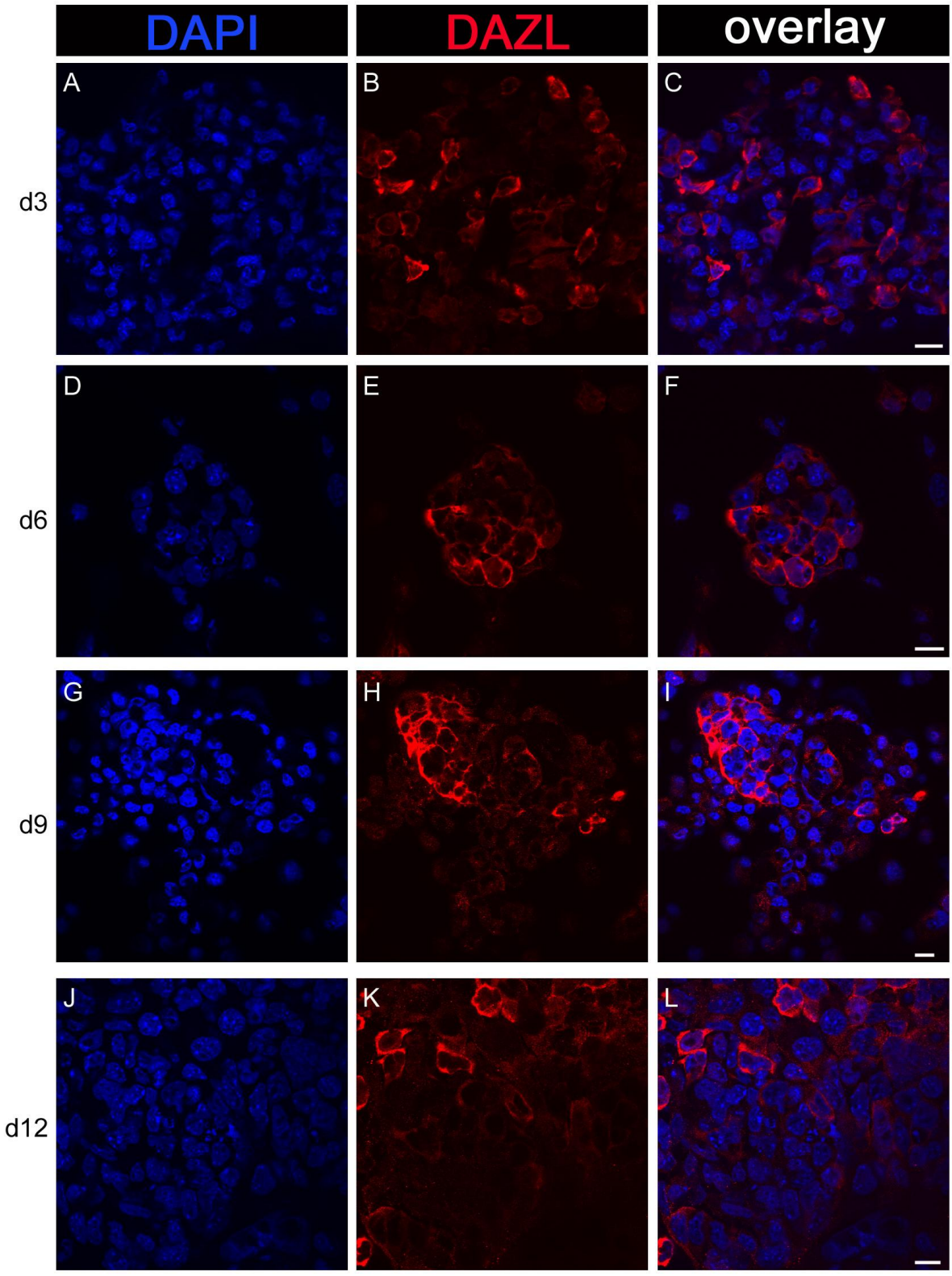


Fig. 56: DAZL immunostaining of SSC 12 during *in vitro* differentiation. DAZL immunostaining of SSC 12 at day (d) 3, d6, d9 and d12 of *in vitro* differentiation. During the differentiation process DAZL expression was detected weakly in the nuclei and predominantly in the cytoplasm of SSC 12. Immunonegative cells served as an internal negative control. Representative scale bars are shown in overlay images. d: day. Scale bars: 10 µm.

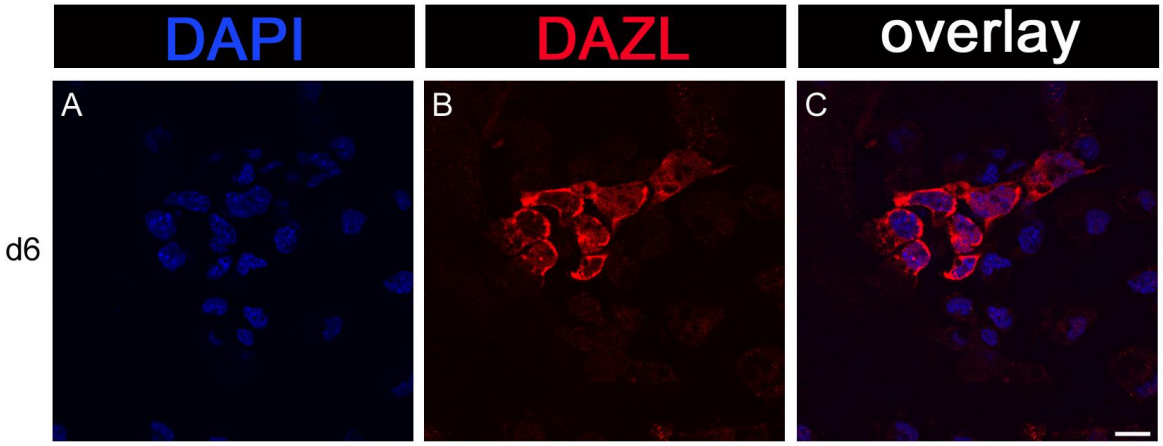


Fig. 57: DAZL immunostaining of SSC 12/11 at day 6 of *in vitro* differentiation. In the *Stra8* overexpressing cell line SSC 12/11 DAZL expression was just detectable at day 6 of *in vitro* differentiation revealing mostly a cytoplasmic staining. Immunonegative cells served as an internal negative control. Representative scale bar is shown in the overlay image. d: day. Scale bar: 10 µm.

Because cytoplasmic DAZL expression indicated that cells entered meiosis, cells should be also checked for the formation of the meiosis specific synaptonemal complex using an specific antibody against the synaptonemal complex protein 3 (SYCP3). Appearance of these structures would be an additional indication of meiotic differentiation. In spite of several attempts these analyses did not reveal any results due to unsufficient SYCP3 antibody staining. Therefore, the DAZL immunostaining results suggested that SSC 12 and the *Stra8* overexpressing cell line Stra8 12/11 entered meiosis indicated by cytoplasmic DAZL expression, but a further progress of meiosis could not be proven by immunostaining analyses.

3.4.4.2.2 Expression analysis of postmeiotic genes by qRT-PCR in SSC 12 and *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13

qRT-PCR experiments were performed to analyze the expression of the haploid markers *Acr*, *Gpx4* (Glutathione Peroxidase 4) and *Prm1* in cells with and without *Stra8* overexpression during *in vitro* differentiation. These analyses revealed no expression of *Acr* and *Prm1* at any time points of differentiation. In contrast, an upregulation of *Gpx4* expression was detected, which is known to be expressed in the mitochondria of sperm and indicates a possible tail formation within the cells (Nayernia et al., 2004; Nolte et al., 2010). Fig. 58 shows the qRT-PCR results for *Gpx4* expression in SSC 12 as well as in *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 before and at day 3, day 6 and day 9 of differentiation induction. Additionally, cells collected from the supernatant from all cell lines at day 6 and day 9 were included in the expression analysis. Because of the increased cell detachment of SSC 12/11 and SSC 12/13 (Fig. 55 N+O), just the initial cell line SSC 12 could be analyzed at day 12.

The calculated significances normalized to each cell line at day 0 are indicated within the figures (Fig. 58) as well as the *Gpx4* expression in mouse testis and the wild type ES cell line ES-RI, which served as a positive and negative control, respectively.

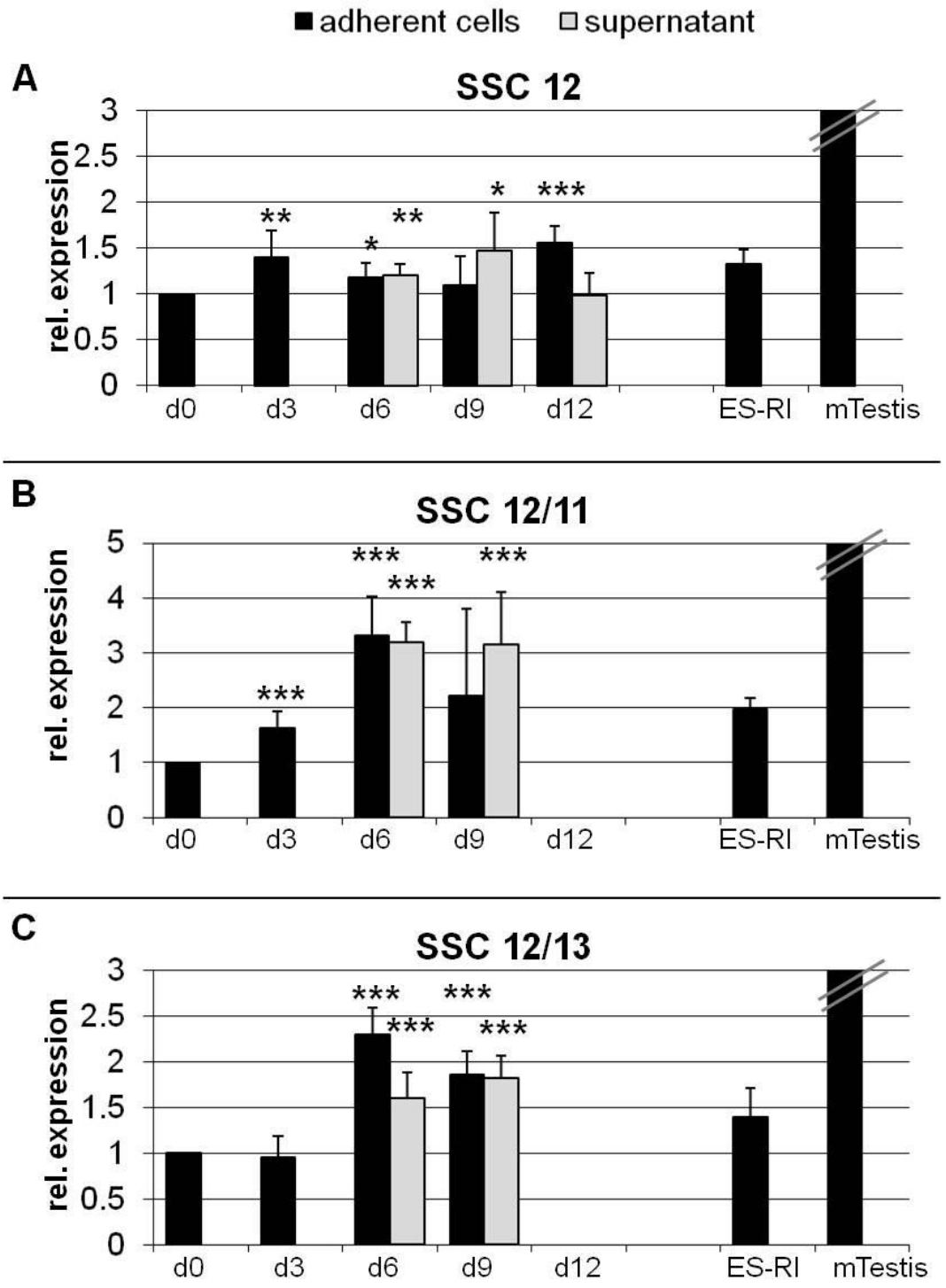


Fig. 58: qRT-PCR expression analysis of the spermatogenesis marker gene *Gpx4* during *in vitro* differentiation of SSC 12 and *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13. Mean values with standard deviations for *Gpx4* expression in adherent and supernatant-derived cells of **(A)** SSC 12 and *Stra8* overexpressing cell lines **(B)** SSC 12/11 and **(C)** SSC 12/13 are shown. Data comprised two biological replicates. Significant increase of marker gene expression was calculated in comparison to day 0 of the analyzed cell line

and indicated with *: $p \leq 0.05$, **: $p \leq 0.01$; ***: $p \leq 0.001$. ES-RI: wild type ES cell lines served as negative control; mTestis: positive control. d: day.

While SSC 12 revealed an increase of *Gpx4* expression, which scored with a high significance at day 12 (Fig. 58 A), the *Stra8* overexpressing cell lines SSC 12/11 (Fig. 58 B) and SSC 12/13 (Fig. 58 C) showed a significantly increased *Gpx4* expression already at day 6 in adherent and supernatant-derived cells. This expression level sustained until day 9 in supernatant-derived cells of SSC 12/11 and in both analyzed cell types of SSC 12/13. Therefore, these qRT-PCR data suggested an earlier onset of *Gpx4* expression in *Stra8* overexpressing cells during *in vitro* differentiation. Additionally, the increased expression of cells derived from the supernatant indicated that detached cells entered meiosis.

3.4.4.2.3 *In vitro* differentiation of SSC 12 and *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 without RA induction

Based on the qRT-PCR results it was assumed that the process of *in vitro* differentiation was forced in *Stra8* overexpressing cell lines by the early induction with RA from day 0. Fig. 59 shows the expression profile of different spermatogenesis marker genes in SSC 12 and the *Stra8* overexpressing cell line SSC 12/13 at different passages of *in vitro* differentiation without RA application. Here, different cell passages were analyzed, because the cell lines exhibited disparate proliferation rates and had to be passaged at different time points. RT-PCR experiments with cDNA derived from mouse testis and the wild type ES cell line ES-RI served as controls. *Hprt*-PCR was performed as a loading control of cDNA. Weak *Acr* positive bands could be detected in passage No. 8 and 11 in adherent SSC 12 cells (Fig. 59; yellow stars), while *Acr* was already detectable in passage No. 1 of SSC 12/13 adherent cells (Fig. 59; orange stars) and in supernatant derived cells of passage No. 3 (Fig. 59; red star). These results also indicated a forced *in vitro* differentiation in the *Stra8* overexpressing cell line SSC 12/13. Further qRT-PCR analyses could give some detailed information about the development of spermatogenesis marker expression, which have to be still performed.

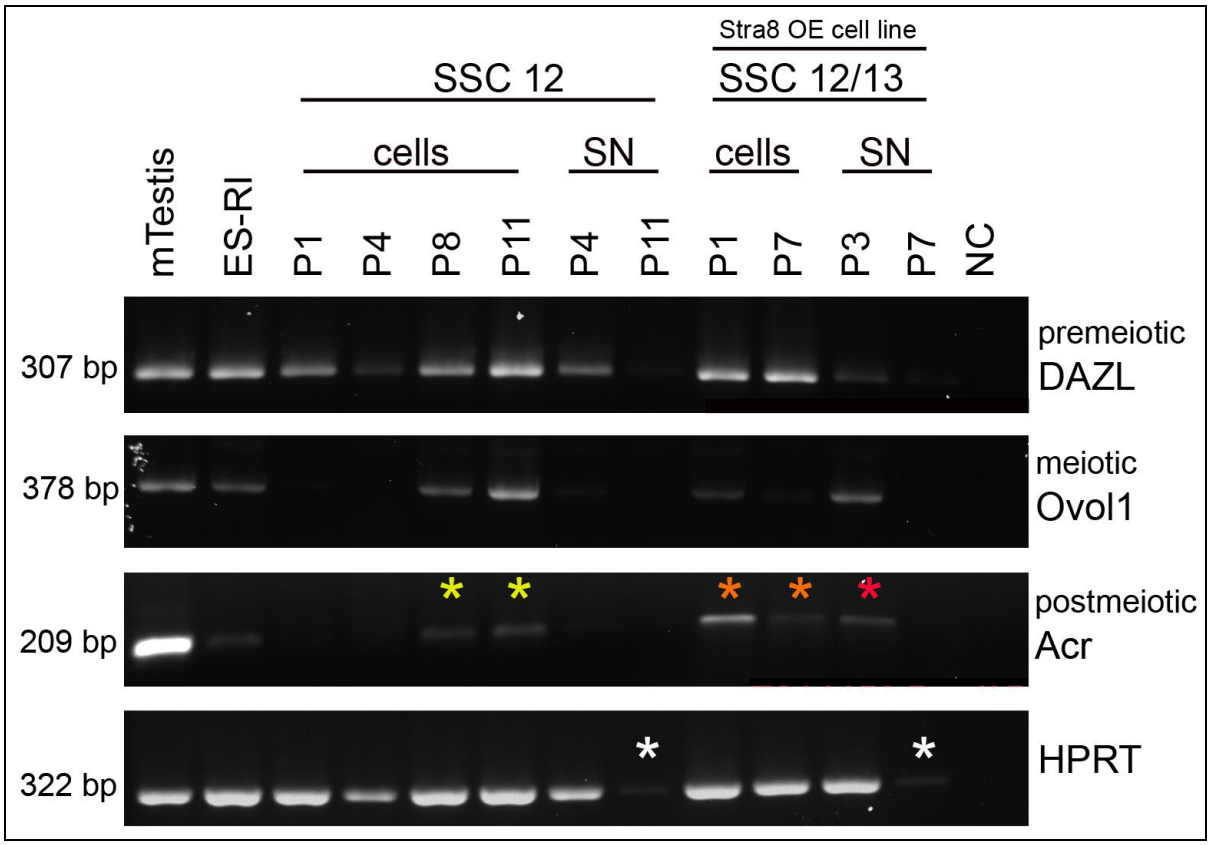


Fig. 59: RT-PCR expression analyses of spermatogenesis marker genes during *in vitro* differentiation without RA induction. RT-PCR results show the expression of premeiotic marker gene *DAZL*, meiotic marker gene *Ovol1* and the early postmeiotic gene *Acr* in SSC 12 and *Stra8* overexpressing cell line SSC 12/13 during *in vitro* differentitation without RA induction. Yellow, orange and red stars mark weak detectable PCR fragments in SSC 12 and SSC 12/13, respectively. White stars indicate samples without cDNA according to the *HPRT*-PCR result. Fragment sizes of PCR products are indicated within the figure. P: passage number; SN: supernatant-derived cells; Stra8 OE cell line: *Stra8* overexpressing cell line; NC: negative control with H_2O; HPRT: PCR for cDNA loading control; bp: base pairs.

3.4.4.3 Methylation profile during *in vitro* differentiation

The methylation profile of imprinted genes in cells undergoing *in vitro* differentiation was analyzed by bisulfite pyrosequencing. These analyses were performed in collaboration with Prof. Zechner (Institute of Human Genetics, University of Mainz). Genomic DNA was isolated at different time points of *in vitro* differentiation. Further bisulfite pyrosequencing of samples as well as data evaluation were performed by Prof. Zechner's group. Fig. 60 presents the methylation status of differentially methylated regions (DMRs) of the paternally imprinted *H19* and *Meg3* genes as well as the maternally imprinted *Igf2r* and *Snrpn* genes in SSC 12 and *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 during differentiation including RA application.

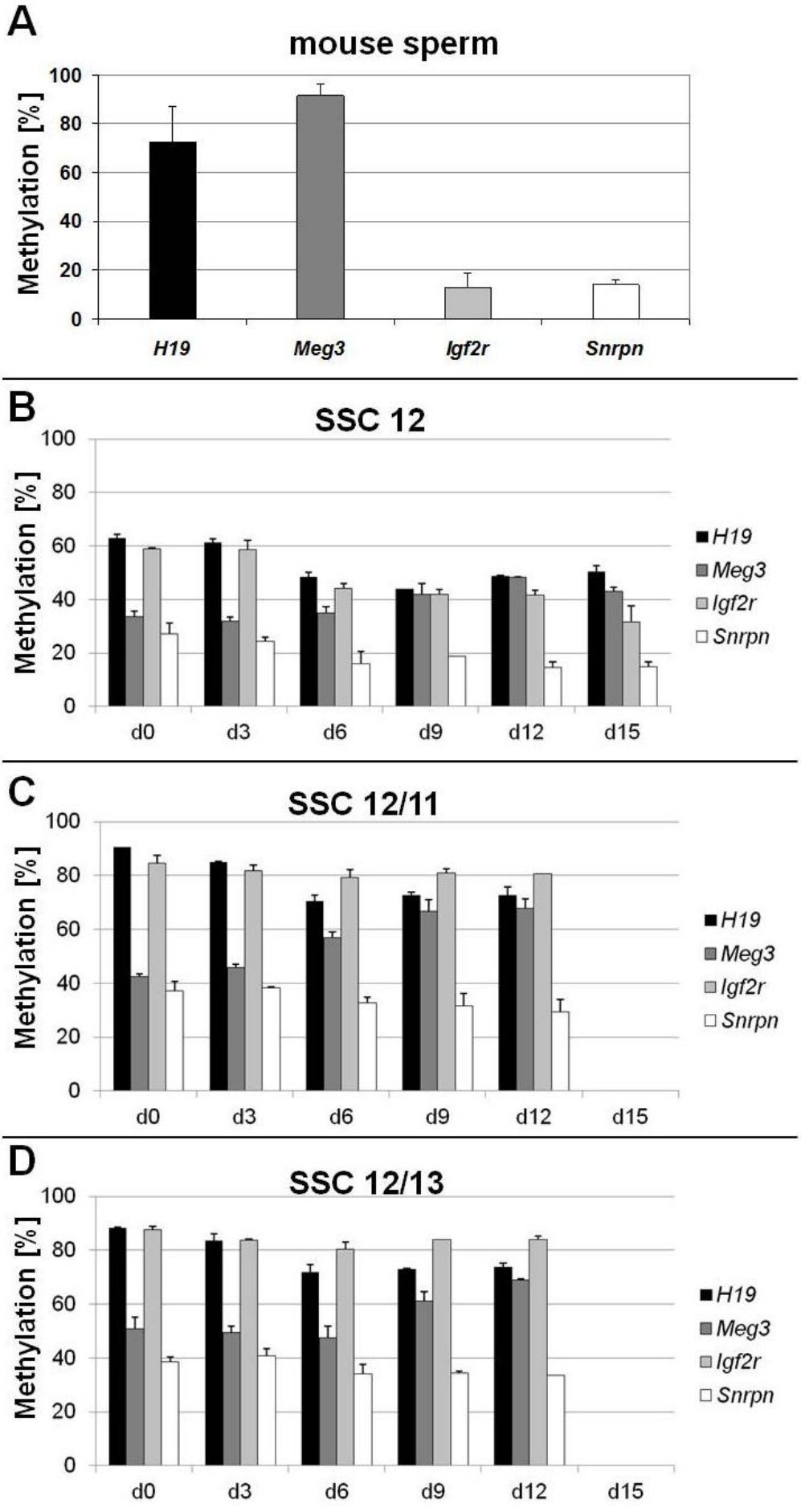
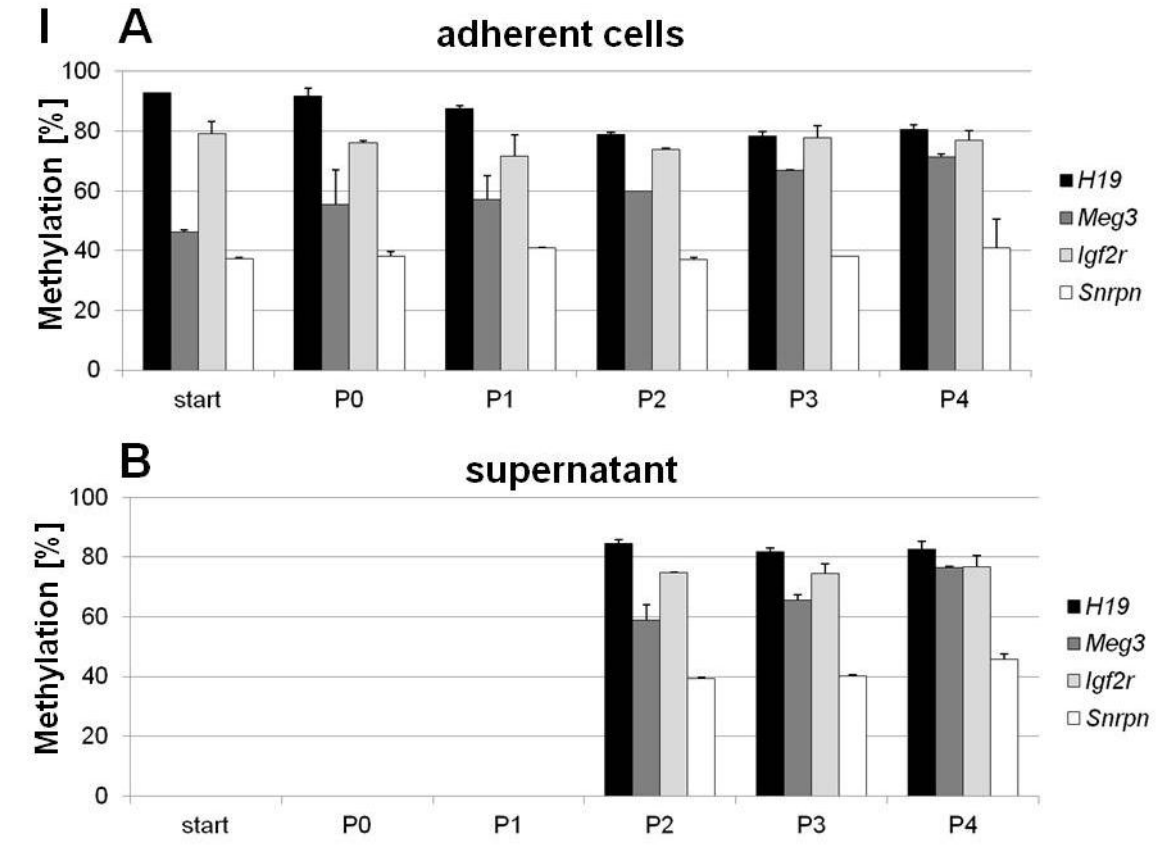

A
mouse sperm
Methylation [%]
100
80
60
40
20
0
H19
Meg3
Igf2r
Snrpn
B
SSC 12
Methylation [%]
d0
d3
d6
d9
d12
d15
H19
Meg3
Igf2r
Snrpn
C
SSC 12/11
Methylation [%]
d0
d3
d6
d9
d12
d15
H19
Meg3
Igf2r
Snrpn
D
SSC 12/13
Methylation [%]
d0
d3
d6
d9
d12
d15
H19
Meg3
Igf2r
Snrpn

Fig. 60: Methylation analysis of imprinted genes during *in vitro* differentiation of SSC 12 and *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 with RA induction. The methylation status of the paternally imprinted *H19* ICR and *Meg3* IG-DMR and the maternally imprinted *Igf2r* DMR2 and *Snrpn* DMR1 were analyzed by bisulfite pyrosequencing for **(A)** mouse sperm as positive control, **(B)** SSC 12 and the *Stra8* overexpressing cell lines **(C)** SSC 12/11 and **(D)** SSC 12/13 during *in vitro* differentiation with RA induction. Mean values with standard deviations are shown. Data comprised two biological replicates and were collected and analyzed by Prof. Zechner's group (Mainz). d: day of differentiation; ICR: Imprinting Control Region; DMRs: differentially methylated regions.

The methylation profile of mouse sperm as a positive control was kindly provided by Prof. Zechner's group and depicted the expected hypermethylation of the paternally imprinted *H19* and *Meg3* genes (70-90%) and a hypomethylation of the maternally imprinted *Igf2r* and *Snrpn* genes (~10%) (Fig. 60 A).

Neither SSC 12 nor the *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 displayed a methylation pattern comparable with mouse sperm. During the differentiation process SSC 12 displayed a slight decrease of *H19* methylation from 60% to 50% and *Meg3* a slight increase from 35% to 40%. In contrast, both maternally imprinted genes showed a loss of methylation (*Igf2r*: from 60% to 35%; *Snrpn*: from 30% to 15%), but only *Snrpn* nearly reached the strongly hypomethylated status present in sperm (Fig. 60 B). The *Stra8* overexpressing cell lines SSC 12/11 (Fig. 60 C) and SSC 12/13 (Fig. 60 D) displayed a very similar methylation pattern with an overall increased methylation status of imprinted genes compared to SSC 12. *H19* showed a loss of methylation from 90% to 70% during differentiation and *Meg3* an increase of methylation from 50% to 70%. The methylation status of maternally imprinted *Igf2r* (80%) and *Snrpn* (35%) genes remained almost unchanged during differentiation.


I
A
adherent cells
Methylation [%]
100
80
60
40
20
0
start
P0
P1
P2
P3
P4
H19
Meg3
Igf2r
Snrpn
B
supernatant
Methylation [%]
100
80
60
40
20
0
start
P0
P1
P2
P3
P4
H19
Meg3
Igf2r
Snrpn

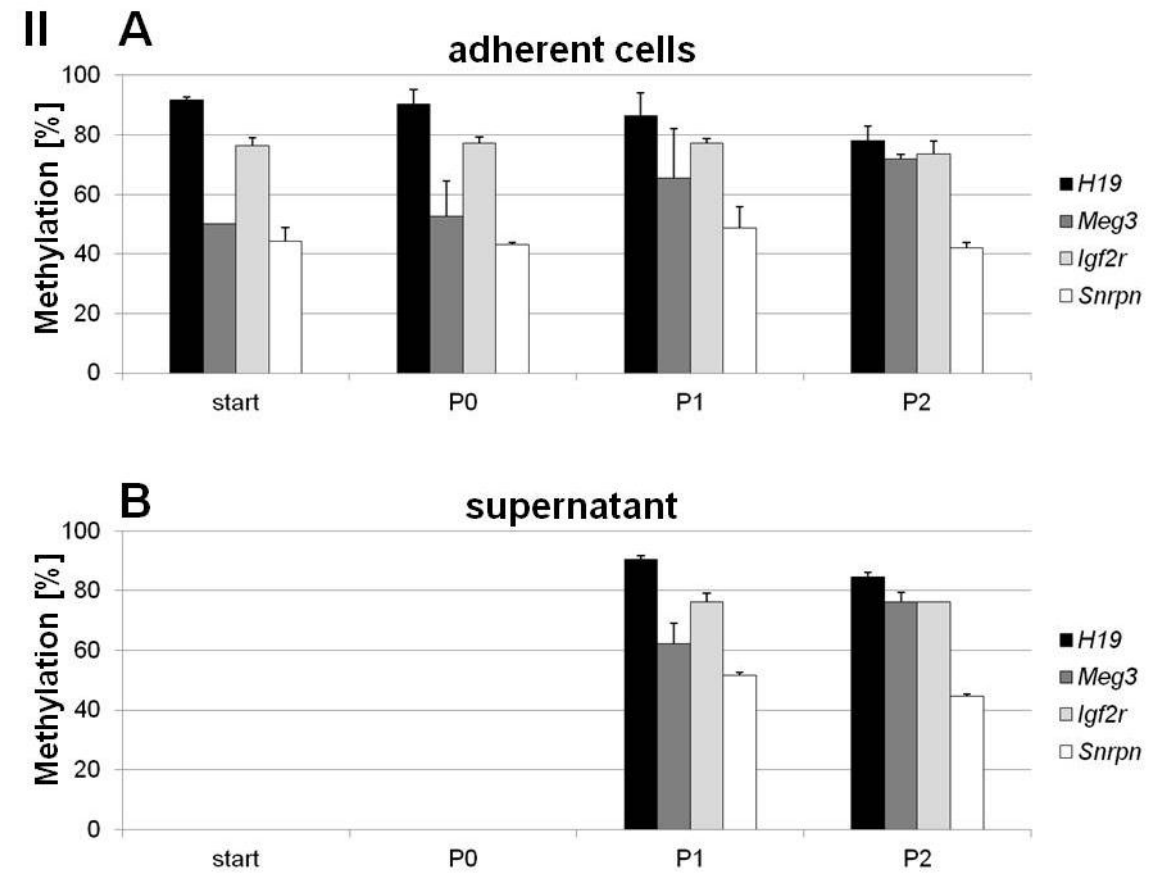

II
A
adherent cells
Methylation [%]
100
80
60
40
20
0
start
P0
P1
P2
H19
Meg3
Igf2r
Snrpn
B
supernatant
Methylation [%]
100
80
60
40
20
0
start
P0
P1
P2
H19
Meg3
Igf2r
Snrpn

Fig. 61: Methylation analysis of imprinted genes in *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 during *in vitro* differentiation without RA induction. The methylation status of the paternally imprinted *H19* ICR and *Meg3* IG-DMR and the maternally imprinted *Igf2r* DMR2 and *Snrpn* DMR1 were analyzed by bisulfite pyrosequencing for the *Stra8* overexpressing cell lines **(I)** SSC 12/11 and **(II)** SSC 12/13 during *in vitro* differentiation without RA induction. For each cell line **(A)** adherent (two biological replicates) and **(B)** supernatant-derived cells (one biological replicate) were analyzed. Mean values with standard deviations are shown. Data were collected and analyzed by Prof. Zechner's group (Mainz). start: before start of differentiation; P: passage number during differentiation.

Furthermore, the methylation profiles of SSC 12 and *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 without RA application during *in vitro* differentiation were studied. Here, the analyzed time points during differentiation are indicated in passage numbers, because the cell lines exhibited different proliferation rates and had to be passaged at different days. In addition to adherent cells, also supernatant-derived cells were analyzed. Taken together, the imprinted genes in *Stra8* overexpressing cell lines SSC 12/11 (Fig. 61 IA) and SSC 12/13 (Fig. 61 IIA) did not exhibit large differences in their methylation status compared to an *in vitro* differentiation including RA induction. The methylation pattern of supernatant-derived cells reflected the methylation status of their corresponding adherent cells (Fig. 61 IB + IIB).

Compared to the *in vitro* differentiation including RA induction, the adherent SSC 12 cells showed a remarkably increased methylation of the paternally methylated *H19* ICR (70%-80%) as well as the maternally methylated *Igf2r* DMR2 (60%) and *Snrpn* DMR1 (40%), whereas the *Meg3* IG-DMR displayed as similar methylation pattern (Fig. 62 A). As in *Stra8* overexpressing cell lines supernatant-derived SSC 12 cells reflected the methylation status of their corresponding adherent cells (Fig. 62 B).

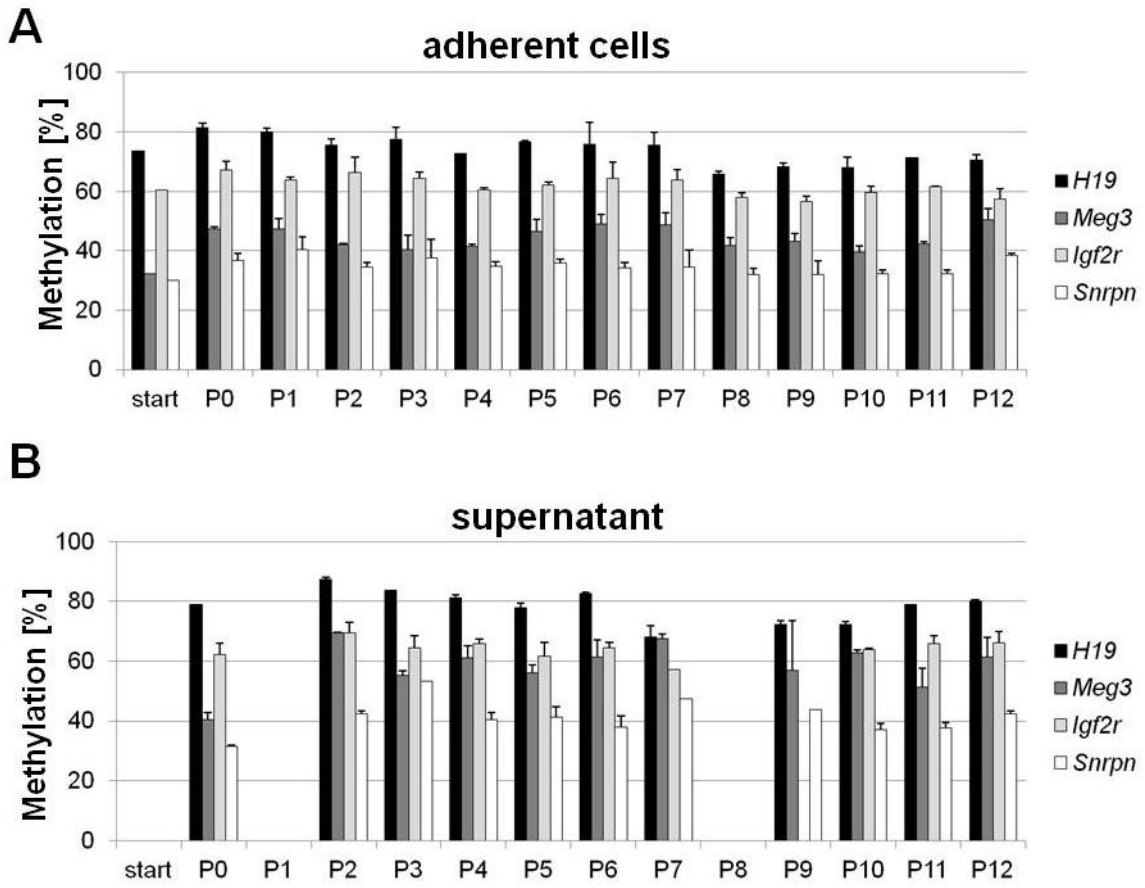


Fig. 62: Methylation analysis of imprinted genes in SSC 12 cells during *in vitro* differentiation without RA induction. The methylation status of the paternally imprinted *H19* ICR and *Meg3* IG-DMR and the maternally imprinted *Igf2r* DMR2 and *Snrpn* DMR1 were analyzed by bisulfite pyrosequencing for **(A)** adherent (two biological replicates) and **(B)** supernatant-derived SSC 12 cells (one biological replicate). Mean values with standard deviations are shown. Data were collected and analyzed by Prof. Zechner's group (Mainz). start: before start of differentiation; P: passage number during differentiation.

3.4.4.4 Emergence of blastocyst-like structures during *in vitro* differentiation

During induction of differentiation cells revealed peculiar morphological features, which resembled blastocyst-like structures (Fig. 63). These morphological changes were observed in *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 in a much greater extent than in SSC 12 cells. These cell structures appeared from day 7 of cell differentiation and reached their highest number at day 9 followed by detachment from the culture vessel. These cells exhibited a vaculated cytoplasm with a nucleus aside the cell membrane (Fig. 63 J-L).

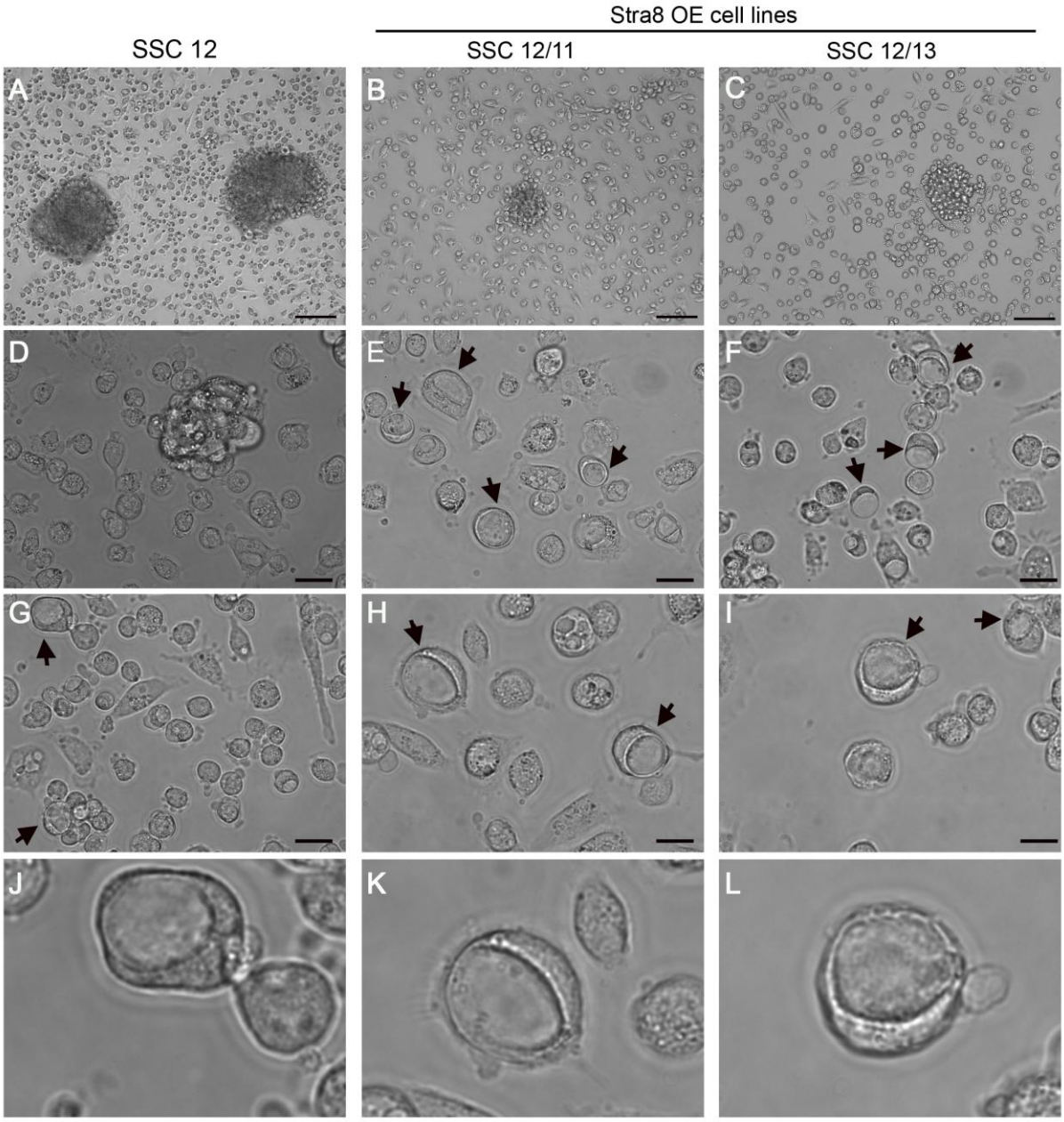


Fig. 63: Emergence of blastocyst-like structures at day 9 of *in vitro* differentiation. Brightfield images showing blastocyst-like structures, indicated with arrows, in SSC 12 and *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 at day 9 of *in vitro* differentiation. A vacuolated cytoplasm with a nucleus aside the cell membrane can be clearly identified in magnifications of single cells (J-L). Stra8 OE cell lines: *Stra8* overexpressing cell lines. Scale bars: A-C: 100 µm; D-G: 50 µm; H-I: 50 µm + 1.6 x; J-L: Magnifications of single cells in G-I.

DAPI staining of these cells revealed no apoptotic features such as membrane blebbing or nuclear fragmentation (Fig. 64). Also small sealed membrane vesicles representing apoptotic bodies could not be observed.

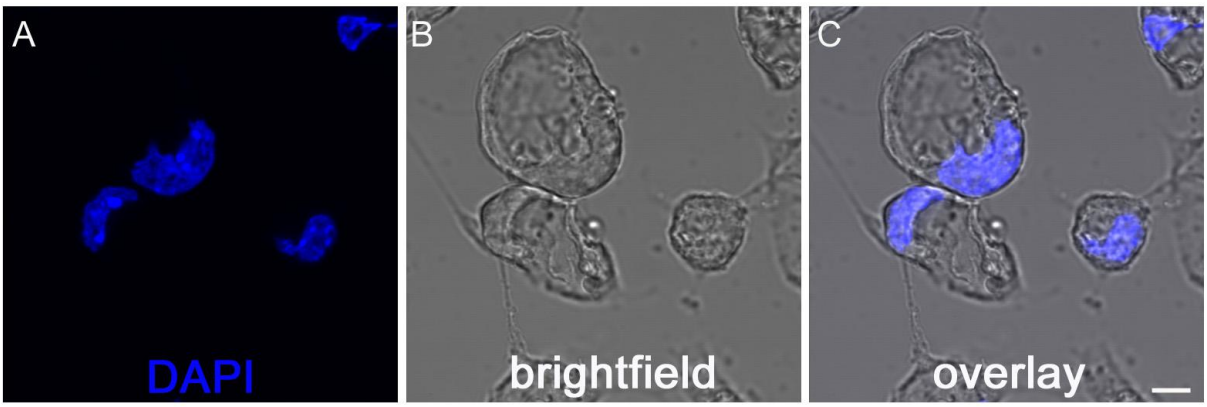


Fig. 64: DAPI-Staining of SSC 12/11 cells at day 9 of *in vitro* differentiation. DAPI staining of SSC 12/11 cells revealed a vacuolated cytoplasm with a nucleus aside the cell membrane without any nuclear fragmentation indicating no apoptotic processes at day 9 of *in vitro* differentiation. Scale bar: 10 µm.

An apoptosis assay as well as electron microscopy analysis was performed for further characterization of these cell types.

3.4.4.4.1 Apoptosis assay on SSC 12 cells and *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13

An Annexin V and 7-AAD based apoptosis assay (2.2.4.13; Khromov et al., 2012) was performed in order to exclude definitively apoptotic processes of these cells during differentiation (Fig. 65). Fig. 65 A confirmed the existence of blastocyst-like structures before SSC 12 and *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 were harvested at day 8 of differentiation induction and incubated with Annexin V and 7-AAD. While the Ca^{2+} dependent protein Annexin V binds to membrane phospholipid phosphatidylserine, which is exposed at the outer leaflet of the plasma membrane at the latest stage of apoptosis, represents 7-AAD a vital dye, which is excluded from viable cells because of their intact membrane. Flow cytometric analysis allows the determination of viable, early and late apoptotic as well as dead cell fractions. The evaluation of the apoptosis assay, shown in Fig. 65 B, revealed no fraction of Annexin V stained cells. Nevertheless, the detection of 7-AAD positive cells did not reveal an increased fraction of dead cells within *Stra8* overexpressing cells compared to SSC 12 at the analyzed time point of differentiation. While the initial cell line SSC 12 as well as the *Stra8* overexpressing cell line SSC 12/13 possessed a fraction of 22% dead cells, SSC 12/11 showed even a decreased proportion of 12% dead cells (Fig. 65 B, highlighted in red). Overall 76-86% of the analyzed cells were viable (Fig. 65 B, highlighted in green). Thus an increased apoptosis rate due to *Stra8* overexpression during *in vitro* differentiation could be excluded and consequently the blastocyst-like structures monitored in *Stra8* overexpressing cell lines did not represent dead cells.

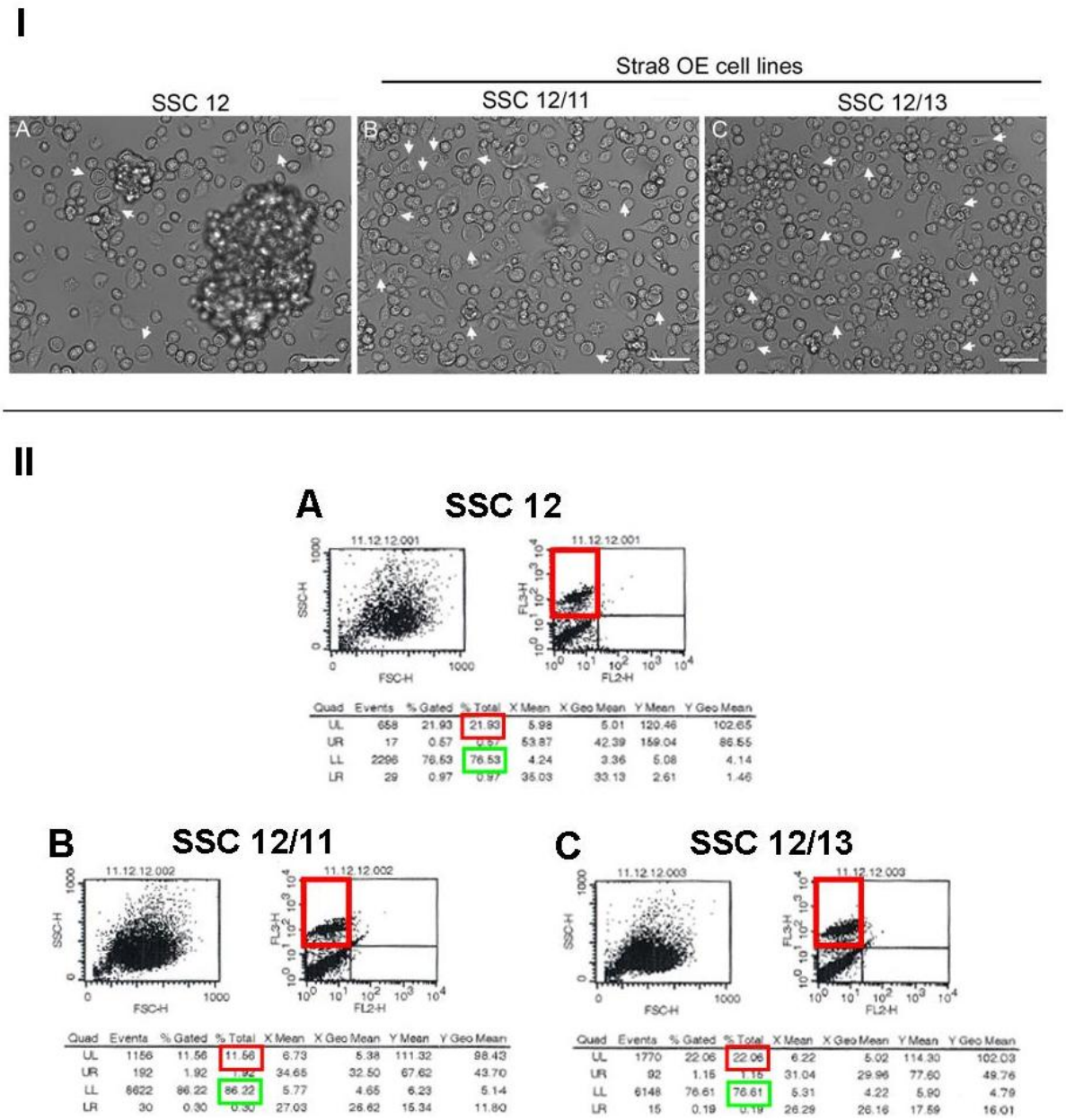


Fig. 65: Validation of cell viability of SSC 12 cells and *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 at day 8 of *in vitro* differentiation by apoptosis assay. (I) Brightfield images show blastocyst-like structures, indicated with white arrows, derived from SSC 12 and *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 at day 8 of *in vitro* differentiation. Scale bars: 50 µm. **(II)** Results of flow cytometric measurements after Annexin V and 7-AAD staining show the percentage of viable (highlighted in green) and dead (highlighted in red) cells in the analyzed cell lines at day 8 and confirmed the viability of the majority of the cells. Stra8 OE cell lines: *Stra8* overexpressing cell lines.

3.4.4.4.2 Electron microscopy analysis on *Stra8* overexpressing cell line SSC 12/13

For further characterization, electron microscopy analysis was performed exemplarily with the *Stra8* overexpressing cell line SSC 12/13 and should provide an indication of the cell type showing blastocyst-like structures.

After induction of differentiation SSC 12/13 cells were isolated from the supernatant at day 8 as well as adherent cells harvested at day 9 and fixed for electron microscopy analysis (2.2.3.7). Further processing, electron microscopy as well as data interpretation was performed by Prof. Dr. Andreas Meinhardt, Department of Anatomy and Cell Biology at the University of Giessen. Prof. Meinhardt interpreted the data as followed: all in all the cells did not show any features for meiotic or postmeiotic differentiation, whereas their existence could not be completely excluded, if their occurrence is less than 5% (Fig. 66 A-C; E-G). Semi thin cell sections analyzed by light microscopy did not reveal any sperm tail structures or acrosomes (Fig. 66 D+H). Cells isolated from the supernatant were smaller than the adhered cells and contained numerous small bodies with one quarter of the cell size. These bodies were not homogenous, but revealed bright and darker areas. Moreover, these cells exhibited several small vacuoles with or without a membrane defined content, whereas vacuoles with membranes resembled autophagosomes indicating cell stress. Cells with big vacuoles were predominantly visible in semi thin sections. In contrast, adherent cells were larger with a decreased number of vacuoles and autophagosomes. A very well distinct rough endoplasmic reticulum indicated an intensive protein translation in the cells, but no characteristic features of germ cells could be identified.

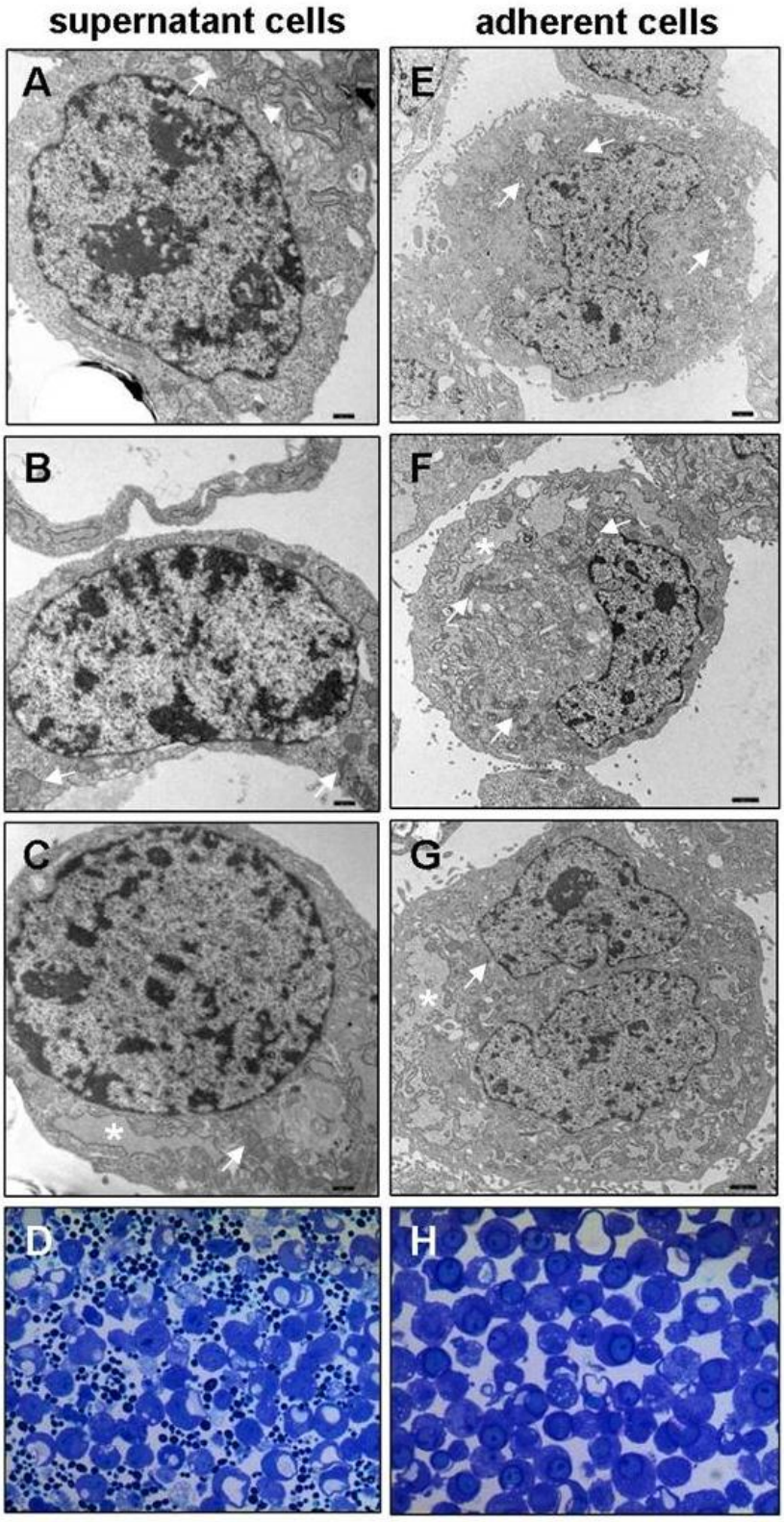


Fig. 66: Electron microscopy analysis of supernatant and adherent SSC 12/13 cells at day 8 and day 9 of *in vitro* differentiation. (A-C; E-G) Electron microscopy and **(D, H)** light microscopy images of semi thin sections of supernatant-derived and adherent SSC 12/13 cells. Cells shown in D and H were stained with Toluidine Blue. Processing of cells, electron microscopy as well as data interpretation was performed by Prof. Dr. Andreas Meinhardt, Department of Anatomy and Cell Biology at the University of Giessen. Arrows: mitochondria; arrow head: rough endoplasmic reticulum; star: dilated rough endoplasmic reticulum. Scale bars: A-C: 500 nm; E-G: 1000 nm.

4 Discussion

4.1 Restoration of male fertility

The treatment options for male infertility are crucial defined by the underlying cause of the impaired infertility (1.1). The current available reproductive therapies are not suitable to treat all infertility cases and require experimental approaches in the research, which may result in new fertility options (1.1; Fig. 2, bottom). The first part of the discussion focuses on two therapeutical approaches using patient-derived testicular material and iPSCs to treat male infertility, which were aimed at in the presented thesis. After a conclusion of the pros of these sought approaches, the discussion of this part will be closed with the description of an *in vitro* spermatogenesis approach using mouse embryonic stem cells (ESCs) in order to obtain important information for the improvement of therapeutical approaches to restore male infertility.

4.1.1 Usage of patient-derived testicular material

Several therapeutical approaches make use of testicular material from the patient himself (1.1; Fig. 2; orange and blue boxes). These approaches provide that the patient possess at least SSCs and the underlying cause of his infertility effects later stages of the spermatogenesis. Here the culture of patient-derived SSCs is one of the promising approach and offers various opportunities for the treatment of male infertility (1.1; Fig. 2; bue boxes). One idea behind is the isolation of the patient's SSCs, their expansion in culture and further *in vitro* stimulation to generate male germ cells followed by ICSI (1.1). In order to follow this therapeutical approach one aim of the presented thesis was the generation of proliferating human SSC cell lines from testicular biopsies derived from infertile men, who underwent a TESE. The established human SSCs should be further used for direct *in vitro* differentiation into haploid male germ cells. Although the isolation of SSCs and their long-term culture have already been shown in various species (1.3), the same procedures using human tissue is a well known issue and to date, there is no reliable protocol available for the establishment of *in vitro* proliferating human SSC cell lines. Unfortunately, this difficulty was also confirmed in the presented thesis (3.1). Despite several attempts, including testing of different combinations of feeder layers, culture media and conditions (3.1.3), an enrichment of putative human SSCs could not be exceeded and no stable proliferating cell line could be established. This failure is due to several factors. Although several phenotypic markers of mouse and human SSCs are described in the literature and also used for the isolation of these cells using different cell sorting approaches (Guo et al., 2014), there is still a lack of

reliable markers to exclusively detect undifferentiated spermatogonia. Here, the surface marker α6-Integrin was used for MACSorting procedures, which is described as a SSC surface protein and an accepted marker used for human SSC isolation by other groups (Shinohara et al., 1999; 2000; Valli et al., 2014). Nonetheless, α6-Integrin is not exclusively expressed by SSCs, but also detected in human sertoli and leydig cells (He et al., 2010). Thus, the usage of this marker can just lead to an enrichment and not to a pure SSC cell population. We suppose the successful enrichment of putative human SSCs in the performed experiments based on their grape-like structure (3.1.3.1, Fig. 5; 3.1.3.2, Fig. 6, Fig. 8), which is characteristic of SSCs, and their positive immunostaining for the described SSC marker PLZF (3.1.3.5, Fig. 10; He et al., 2010). Here, the best results were achieved by culturing isolated SSCs in StemPro+3 medium (Smorag et al., 2012) on gelatine coated plates at normoxia (3.1.3.2). In this context it is astonishing that the use of matrigel coated plates did not further improve the cultivation of human SSCs (3.1.3.3). Compared to gelatine, matrigel additionally provides several extracellular matrix derived factors to facilitate cell attachment and proliferation. But the question arises why the cultivation of human SSCs beyond their enrichment after MACSorting was not successful. Here passaging of putative human SSCs on new culture plates was the most critical step. The efficiency of cell attachment as well as further proliferation of the passaged SSCs was very low and finally resulted in the loss of isolated SSCs (3.1.3.4). In prospective attempts to establish proliferating human SSC cell lines an improvement might be achieved by culturing or passaging these cells on patient-derived testicular cells. As already seen in SSC cultures on gelatine coated plates, isolated human SSCs prefer growing on their natural testicular feeder developed during culture (3.1.3.2, Fig. **7**). The principle of MACSorting offers the opportunity to use the flow through fraction containing patient-derived testicular cells, which might provide appropriate natural factors to keep cells in culture. Further analyses of this so called Human Testicular Feeder (HTF) were performed in context of co-culture experiments and are discussed later in 4.2.2.

Additionally, the material delivery as well as the isolation procedure complicate the establishment of long-term human SSC cultures. Testicular biopsies are available limitedly and supplied in small fractions of tissue material for cell isolation approaches, whereat SSCs anyway represent just 0.03% of all germ cells (Tegelenbosch and de Rooij, 1993) and the isolation via MACSorting additionally implies a certain loss of cells by using columns. The lack of biopsy delivery also limits the opportunities to test different parameters. Furthermore, study-to-study variations in biopsy quantity and quality must not be undervalued and were also recognizable in the presented thesis (3.1.3.2, Fig. 6).

The efforts to establish proliferating human SSC cell lines were stopped after several unsuccessful attempts, so that the intended experiments for direct *in vitro* differentiation of

human SSCs into haploid male germ cells could not be performed. Here, it was originally planned to use protocols adopted from Eguizabal et al (2011) and Easley et al. (2012), who applied complex differentiation culture media to force the progression of human iPSCs through meiosis and to obtain haploid male germ cells.

In brief, to complete the list of experimental approaches to overcome male infertility by using patient-derived testicular material: *in vitro* expanded SSCs could also be used for autotransplantation into the patient's testes to achieve a repopulation and to recover spermatogenesis. Considering the various reports of successful SSC transplantations in several species (Schlatt et al., 2002; Honaramooz et al., 2003a, b; Izadyar et al., 2003b; Mikkola et al., 2006; Kim et al., 2008; Richardson et al., 2009), pioneered by Brinster and Zimmermann in 1994, a clinical translation of this approach should be feasible in the near future. Moreover, researchers intensively work on the reprogramming of human SSCs into pluripotent cell lines without any genetic modifications according to similar reports in mice (1.3). These human multipotent germline stem cells (hMGSCs) could be further used for *in vitro* differentiation of haploid germ cells followed by ICSI. The use of testicular tissue itself could offer the opportunities of xenotransplantations (Geens et al., 2006; Wyns et al., 2008), organ cultures (Sato et al., 2013) as well as autotransplantations (Schlatt et al., 1999; Hermann et al., 2012), whereat these experimental approaches are not discussed further in this context (1.1, Fig. 2, orange boxes).

4.1.2 Usage of patient-derived iPSCs

Pluripotent stem cells are an unique tool for the *in vitro* differentiation of SSCs for transplantations as well as haploid male germ cells for ICSI applications (1.1, Fig. 2, red and yellow boxes; 1.2). In general differentiated cells are able to regain a pluripotent cell status by somatic cell nuclear transfer, fusion with a pluripotent cell or reprogramming to iPSCs.

The generation of iPSCs was a groundbreaking achievement in the stem cell research and offers great potential for clinical applications in regenerative and reproductive medicine (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). iPSCs represent pluripotent stem cells, which are derived from non-pluripotent cells by artificial manipulation and resemble ESCs as natural pluripotent stem cells in many features including their indefinite self-renewal and differentiation potential, epigenetic pattern and expression of pluripotency marker genes. During the reprogramming process pluripotency related transcription factors like Oct3/4, Sox2, Klf4 and c-Myc, also known as Yamanaka factors according to the pioneer of the iPSC technology (Takahashi and Yamanaka, 2006), are forced expressed to induce pluripotency in the target cells. Initially integrative virus systems including retroviruses (Takahashi and Yamanaka, 2006; Park et al., 2008) and lentiviruses (Stadtfeld et al., 2008a) were used to achieve an ectopic overexpression of the mentioned factors. But since the use of integrative

methods bears the risk of genomic integration and tumorigenicity, researchers have intensively worked on the development of reprogramming methods, which allows theoretically clinical application of iPSCs without any safety concerns for the patient. Up to now the generation of iPSCs was reported using non-integrating vector systems including adenoviruses (Stadtfeld et al., 2008b) and non-viral plasmids (Okita et al., 2008) as well as non-DNA based approaches using recombinant proteins (Kim et al., 2009; Zhou et al., 2009) and RNAs including modified mRNAs (Warren et al., 2010) and miRNAs (Miyoshi et al., 2011). Nevertheless, most of these methods were not applicable to all target cells and revealed a lower efficiency and process compared to integrative viral systems.

Among these non-integrative approaches the mRNA based reprogramming is the most promising method, which bases on the repeated delivery of synthetic modified mRNA encoding for the pluripotency associated transcription factors and their translation in the cytoplasm of cells. This yields in the generation of iPSCs within nearly 20 days with high reprogramming kinetics and efficiencies, thereby avoiding any genomic integration or insertional mutagenesis. Only a physiological reprogramming method without the application of any compounds would be superior to this approach, which is still a challenge to be established.

A successful mRNA reprogramming is known to be technical complicated including the preparation of synthetic mRNAs and a precisely procedure of the mRNA transfection series. In the presented thesis patient-derived iPSCs were generated with the Stemgent® mRNA Reprogramming System, which provides a set of all components needed for the generation of hiPSCs from adult human fibroblasts by mRNA reprogramming. We aimed at the generation of male patient-specific hiPSCs, which revealed different underlying causes for the patient's infertility (3.2), and their further differentiation to human SSCs (1.1, Fig. 2, red boxes). Finally scrotal human fibroblast (SHF) cell lines derived from infertile men were established in culture and selected for reprogramming experiments, because of their marginal exposure to external factors (3.2.1). Unfortunately, the offered kit was not sufficient to generate hiPSCs from the chosen target cells, predominantly because of their moderate proliferation rate, and had to be adapted yielding in a "Feeder Free" mRNA/miRNA Reprogramming (3.2.2.2). Considering that there was no practical knowledge in generation and culturing of hiPSCs available in the institute, it took finally a total of four reprogramming trials including several modifications within the protocols in order to successful reprogram SHF cells from infertile men by mRNA applications (3.2.2; Tab. 5) and to establish their cultivation and cryopreservation (3.2.3, Tab. 7). Because of the quite late stage of the practical work for the presented thesis, the hiPSCs' property of low proliferation and the need to establish appropriate differentiation protocols, it was unlikely to achieve the final goal of *in vitro* differentiation of the generated iSHF cells into human SSCs in the remaining time.

Therefore, at this time the focus of the presented thesis was placed on the establishment and characterization of a co-culture system for the generation of putative SSCs from mouse ES cells (3.3; 4.2). Nevertheless, the further development of experimental approaches based on the use of patient-derived testicular tissue (4.1.1) and iPSCs is of great importance for treatment of male infertility, which is illustrated and discussed briefly in the following section.

4.1.2.1 The advantages of experimental approaches to treat male infertility

The currently available reproductive therapies do not offer any opportunity for infertile men to father children from their own genetic material, when they not produce any functional sperm caused by any reason, which could be extracted by different techniques for ART (1.1). This difficulty could be solved with the already described experimental approaches using patient-derived testicular material or iPSCs. If an arrest during spermatogenesis is the underlying cause of infertility, this cause might be overcome during *in vitro* differentiation of human SSCs or haploid male germ cells. Moreover, *in vitro* approaches offer the opportunity to correct a genetic defect in the father's genome, which might be a risk for the newborn. Genome engineering could be done by traditional homologous recombination or ZFN (Zinc-Finger Nucleases), TALEN (Transcription-Activator Like Effector Nuclease) and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas based methods (Richard, 2015). In contrast to approaches using patient-derived testicular material, the iPSC technology is also advantageous for men, who reveal no natural SSCs at all, because it is a promising method to differentiate from any cell type patient-specific SSCs for transplantation or haploid male germ cells for ART to father biological children.

4.1.3 *In vitro* approach of spermatogenesis using mouse ESCs

Important knowledge for the prospective derivation of male gametes of hiPSCs to treat male infertility derives from basic research of *in vitro* differentiation experiments using mouse ESCs, which can provide important information about *in vitro* gametogenesis and epigenetic reprogramming.

In our institute one experimental approach was established for the first time yielding in the production of living offspring using haploid male germ cells derived from mouse ESCs or maGSCs (Nayernia et al., 2006; Nolte et al., 2010). In brief, this established *in vitro* differentiation protocol bases on a two step selection system using each a premeiotic and postmeiotic reporter construct, called *Stra8-EGFP* and *Prm1-DsRed*, respectively. The retinoic acid (RA) inducible expression of EGFP under the promoter control of the premeiotic gene *Stra8* (Stimulated by Retinoic Acid Gene 8) was used to enrich a SSC resembling population by two FACSortings. The *in vitro* differentiation of these cells was induced by

familiar tools. After embryoid body (EB)- like structure formation upon cell culturing on gelatine coated dishes without LIF for several passages, cells were induced with RA for up to seven days. Differentiation of haploid male germ cells was detected by Ds-Red expression indicating an activation of the postmeiotic *Prm1* promoter. The authors assumed that the RA induced cells resembled PGCs (Primordial Germ Cells), while haploid male germ cells were released to the medium and collected within the supernatant for ICSI experiments, which resulted in offspring originated from *in vitro* generated male gametes. The growth abnormalities and the prematurely death within five days to five month after birth of the progeny reflected the result of improper epigenetic reprogramming in the *in vitro*-derived sperm. After a genome wide demethylation in PGCs, androgenic *de novo* methylation of paternal specific imprinting marks have to occur during spermatogenesis. This epigenetic reprogramming process mediated by methyltransferases is described in detail in the second part of the discussion (4.2.5). However, methylation analyses using bisulfite pyrosequencing revealed that the paternally imprinted *H19* gene was just partly re-established during *in vitro* differentiation. The authors hypothesized that the disturbed establishment of paternal-specific methylation pattern led to phenotypic abnormalities in the offspring, which revealed aberrant imprinting patterns in several analysed tissues (Nayernia et al., 2006; Nolte and Zechner, unpublished data).

For a successful application of *in vitro*-derived gametes to treat male infertility, it is of great importance to overcome the problem of improper epigenetic reprogramming during *in vitro* spermatogenesis. One idea bases on the overexpression of germ cell related genes, which are associated with the meiotic process and might have an impact on genomic imprinting (Yu et al., 2009; Medrano et al., 2011). The already mentioned premeiotic gene *Stra8* is a promising candidate gene to achieve the desired improvement and is further presented in the following section.

4.1.3.1 *Stra8*- a promising gene for improvement of *in vitro* spermatogenesis?

The RA responsive and premeiotic germ cell marker gene *Stra8*, which was first discovered in P19 embryonal carcinoma cells (Bouillet et al., 1995), is expressed in ESCs as well as in premeiotic spermatogonia of adult males and premeiotic follicles of adult females (Oulad-Abdelghani et al., 1996; Menke et al., 2003; Zhou et al., 2008a,b). Because of its limited expression in pluripotent cells and the downregulation during differentiation, *Stra8* is suggested to have a regulatory function in controlling the balance between pluripotency and differentiation of ESCs (Nolte, unpublished data). In ESCs STRA8 was shown to shuttle between the cytoplasm and the nucleus (Tedesco et al., 2009) suggesting a putative role for the regulation of protein activity important for cell proliferation and signal transduction

(Gama-Carvalho and Carmo-Fonseca, 2001; Fried and Kutay, 2003). The STRA8 protein possesses a highly evolutionary conserved helix-loop-helix domain localized at the N-terminus (Tedesco et al., 2009), which can be linked to protein-protein interactions (Norton, 2000) and may comprise functional sequences for the active shuttle of proteins between nucleus and cytoplasm (Black et al., 2001). Within its helix-loop-helix domain the STRA8 protein is endowed with a functional nuclear localization signal (NLS) indicating a putative binding of STRA8 to DNA and acting as a transcription factor or at least as a transcriptional co-regulator (Tedesco et al., 2009). Apart from its role in ESCs, *Stra8* is crucial for the initiation of meiosis of germ cells in both males and females (Bowles et al., 2006; Koubova et al., 2006; Anderson et al., 2008). Male and female *Stra8*-deficient mice are infertile with severe defects in gametogenesis leading to a meiotic arrest (Baltus et al., 2006; Koubova et al., 2006; Mark et al., 2008) indicating that the decision to enter meiosis is determined by RA induction of *Stra8* preceding premeiotic DNA replication. Moreover it is suggested that STRA8 interacts with chromatin remodelling proteins like SETD8 and ARID4B (Pantakani, unpublished data), which are regulators of imprinting control regions (ICRs; Wu et al., 2006; Pannetier et al., 2008). Concerning these functions of STRA8, it was hypothesized that an overexpression of *Stra8* might enhance the progression of meiosis and support correct imprinting during *in vitro* spermatogenesis.

These experiments were intended to be performed with the available cell line SSC 12 derived from the work of Nayernia and co-workers (2006), which was already endowed with both reporter constructs *Stra8-EGFP* and *Prm1-DsRed* (3.4.2.1). Additionally, for this purpose a new ESC line, Stra8-EGFP/Sycp3-DsRed, was generated (3.4.2.2), which derived from a double transgenic mouse containing premeiotic *Stra8-EGFP* and meiotic *Sycp3-DsRed* constructs (Smorag et al., 2012) and provides the advantage that all cells, which might undergo meiosis, are red fluorescent due to the activation of their *Sycp3* promoter. Because of time limitation, finally the *in vitro* spermatogenesis experiments were carried out with two stable *Stra8* overexpressing cell lines, SSC 12/11 and SSC 12/13, which derived from the transfection of SSC 12 with a *Stra8* overexpressing construct (3.4.3) and revealed a two times increased *Stra8* expression compared to SSC 12 (3.4.3.1, Fig. 54). The potential effect of *Stra8* overexpression on the process of *in vitro* spermatogenesis was analysed in comparison to the original cell line SSC 12.

4.1.3.2 The impact of *Stra8* on the process of meiosis and epigenetic reprogramming during *in vitro* spermatogenesis

A stable *Stra8* overexpression during *in vitro* differentiation, which was induced with generally used tools including cultivation of cells on gelatine coated dishes and medium

without LIF and supplemented with RA, was indeed accompanied by changes in cell morphology as well as in molecular and methylation profile (3.4.4).

The effect of *Stra8* overexpression comprised obviously morphology changes yielding in a major cell detachment within nine days and a prematurely end of induction in comparison to the original cell line SSC 12 (3.4.4.1, Fig. 55). An expression analysis of the postmeiotic marker gene *Gpx4*, which is known to be expressed in the mitochondria of sperm and indicates a possible tail formation (Nayernia et al., 2004; Nolte et al., 2010), indicated a more rapid and stronger increase of *Gpx4* mRNA expression in *Stra8* overexpressing cell lines normalized to their non-induced state (3.4.4.2.2, Fig. 58) and thereby their earlier entry into and progress through the meiotic process. This corresponded to the assumption that *Stra8* might have the potential to accelerate and enhance the progression of meiosis during *in vitro* differentiation. However, the initiation of haploid cell production could not be conclusively supported on protein level using immunostaining analyses (3.4.4.2.1, Fig. 56, Fig. 57). Also an electron microscopy analysis of one of the *Stra8* overexpressing cell lines did not reveal any evidence for meiotic or postmeiotic structures during *in vitro* differentiation (3.4.4.4.2, Fig. 66). Moreover, no Ds-Red positive cells were detected within emerged EB-like structures derived both from SSC 12 and *Stra8* overexpressing cell lines suggesting the absence of haploid cells due to the obvious lack of promoter activitation of the postmeiotic gene *Prm1*. The absence of Prm1-DsRed positive cells contradicts the outcome of the original *in vitro* differentiation protocol (Nayernia et al., 2006; Nolte et al., 2010), in which the authors detected Prm1-DsRed positive cells emerged from Stra8-EGFP positive cells within the EB-like structures, which were further released into the supernatant. Considering the immense number and size of EB-like structures also emerged in *Stra8* overexpressing cell lines, a DsRed detection was strongly expected. Former studies suggested that *Stra8* expression preceding premeiotic DNA replication defined the entry of male germ cells into meiosis (Baltus et al., 2006) and is just possible during a narrow time span strictly controlled by RA and thereby the *Stra8* expression considering the stage-specific expression of STRA8 protein in male mice localized in pre-leptotene as well as in early leptotene spermatocytes (Oulad-Abdelghani et al., 1996; Zhou et al., 2008a, b). Because this comprises spermatocytes in three different stages (VII-VIII), premeiotic DNA replication and entry into meiosis are thought to proceed more synchronously rather than over the three different stages during exogenous treatment with RA (Zhou et al., 2008b). Based on these findings it is possible that *Stra8* expression, which is essential for the onset of meiosis (Zhou et al., 2008b), was too strong in *Stra8* overexpressing cell lines during *in vitro* differentiation with RA induction resulting in an increased cell detachment after nine to twelve days of induction indicative of progressive apoptosis. However, it is worth to note that the viability of cells after eight days of induction was still validated using an apoptosis assay (3.4.4.4.1, Fig. 65).

Presumably after this time point the cells were overcharged by the expression of endogenous *Stra8* and stable transfected *Stra8*. *In vitro* experiments without additional RA induction further revealed an early onset of postmeiotic marker expression using RT-PCR experiments (3.4.4.2.3, Fig. 59). Considering that these results have to be confirmed by qRT-PCR experiments, they further indicated that already a two times increased *Stra8* expression in the *Stra8* overexpressing cell lines might lead to an overloading of differentiating cells.

The absence of Prm1-DsRed positive cells, representing haploid male germ cells, derived from *in vit*ro differentiated SSC 12 cells could be attributed to a slight, but required deviation from the original protocol. While the original protocol scheduled a cultivation of cells on gelatine coated vessels in medium without LIF for up to ten passages before RA induction (Nayernia et al., 2006; Nolte et al., 2010), this was not feasible with the *Stra8* overexpressing cell lines because of the already described cell detachment. Consequently, this hints at the importance of this step for a successful *in vitro* differentiation of haploid male gametes from SSC 12. This can be also one explanation for the fact that SSC 12 did not reveal a methylation profile of imprinted genes similar to that observed by Nayernia and colleagues (2006) during *in vitro* spermatogenesis with RA induction (3.4.4.3). The paternally imprinted *H19* and *Meg3* genes did not exhibit an androgenic hypermethylated pattern, *H19* even displayed a slight decrease of methylation. However, the methylation status of the maternally imprinted *Igf2r* and *Snrpn* genes tended to decrease over the course of *in vitro* differentiation and result in the hypomethylation expected for male germ cells.

Nevertheless, a comparison with the methylation patterns in *Stra8* overexpressing cells during *in vitro* spermatogenesis clearly indicated an impact of *Stra8* on the epigenotype, although it did not finally yield in a correct androgenic pattern of imprinted genes. The *Stra8* overexpression was accompanied by an overall increased methylation level in all analyzed genes compared to SSC 12 (3.4.4.3). *H19* was strongly hypermethylated and displayed a slight decrease over the course of differentiation, while *Meg3* showed a clear increase towards a hypermethylation. Both paternally imprinted genes clearly exhibited an androgenic epigenotype. However, the maternally imprinted genes did not undergo the essential demethylation and especially *Igf2r* rather showed a hypermethylation than the hypomethylation expected for sperm. The methylation patterns with or without RA induction were comparable indicating that the effect was due to the stable *Stra8* overexpression.

These data supported the hypothesis that a permanently increased expression of the pre-meiotic gene *Stra8* indeed had an effect on the genomic imprinting during *in vitro* spermatogenesis. However, it did not yield in a correct androgenic imprinting pattern, but the problem was rather shifted from the paternally to the maternally imprinted genes, which did not reveal the essential *in vitro* demethylation. But the *in vitro* reprogramming of imprinting

marks is a well-known problem, especially in the *in vitro* derivation of male gametes from ESCs yielding in sperm, which was not fertile and did not give rise to healthy offspring (Toyooka et al., 2003; Daley, 2007). Regarding the problematical *in vitro* epigenetic reprogramming, the group of Shinohara (2009) published an interesting study, in which they presented a GDNF-supported culture system for the development of mature SSCs from *in vitro* cultured fetal mouse germ cells (Lee et al., 2009). Although the methylation patterns of cultured SSCs were stable, the progeny derived from cultured cells revealed growth abnormalities and presumably irreversible genomic imprinting defects in male as well as female germ cells for at least four generations. The authors suggested that the functional SSCs did not gain the ability for a correct epigenetic reprogramming during germline development yielding in abnormal offspring. These data clearly illustrated that even a successful maintenance of a correct methylation pattern in *in vitro* cultured SSCs can not ensure a correct epigenetic reprogramming during germline development. Considering these findings it is highly questionable, if the process of a proper epigenetic reprogramming can be recapitulated completely at all in an *in vitro* system both using *in vivo* isolated germ cells as well as *in vitro* generated germ cells derived from pluripotent cells with the ability to give rise to healthy and fertile offspring without any heritable epigenetic defect. It is suggested that "subtle changes in the microenvironment of gonocytes interfered with the DNA methylation process, which coincided with the culture initiation" (Davis et al., 1999, 2000; Lees-Murdoch et al., 2003; Lee et al., 2009).

Despite these concerns, researchers will further work on the improvement of *in vitro* spermatogenesis with a special focus on the preservation of proper genomic imprinting persisting for subsequent generations. With regard to the appropriate approach in the presented thesis using the overexpression of a premeiotic gene, a temporary instead of a stable overexpression of proteins like STRA8 should be considered in order to improve this process *in vitro*. The doxycycline-inducible protein expression system could be used for the induction of STRA8 expression at certain time points during *in vitro* spermatogenesis experiments (Fig. 67 A). The generation of the required construct (Fig. 67 B), the validation of its functionality in transfected NIH 3T3 cells and identification of stable transfected SSC 12 clones were performed in cooperation with students during their practical courses, but the effect of inducible STRA8 expression during *in vitro* spermatogenesis could not be tested because of limited residual time of the presented thesis.

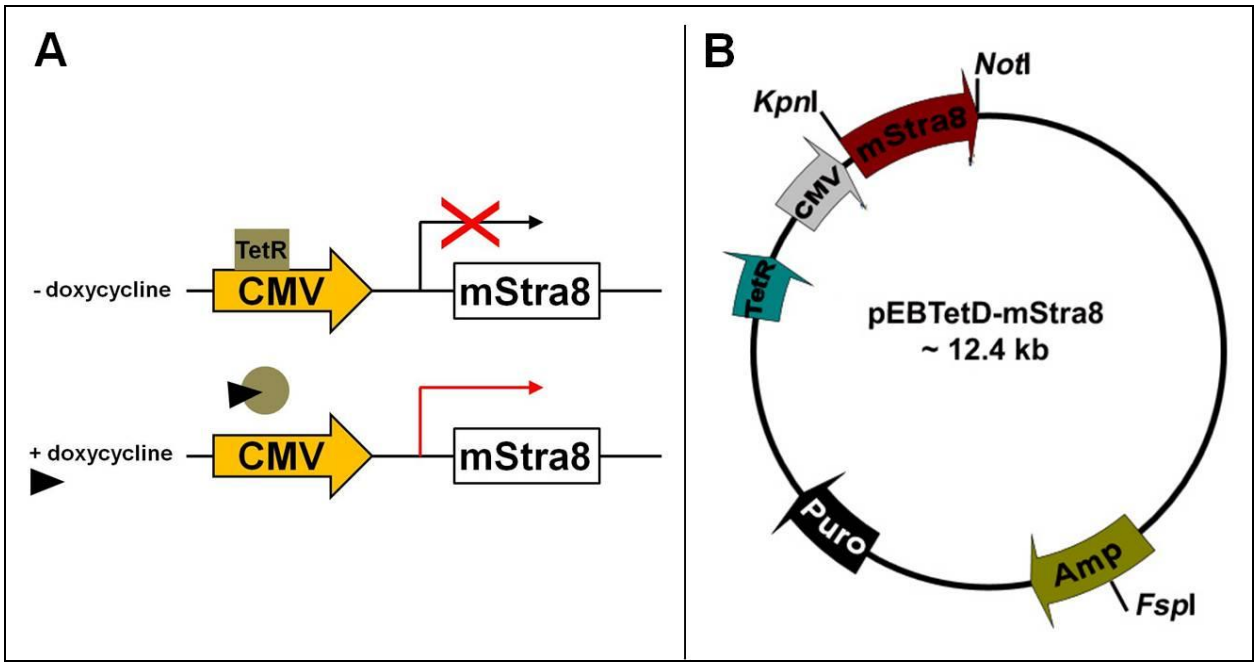


Fig. 67: Doxycycline-inducible protein expression system for time-specific induction of STRA8 expression. (A) Scheme of the doxycycline-inducible protein expression system. In the absence of doxycycline or tetracycline (Tet) a Tet repressor (TetR) binds to Tet operator binding sites, which are located downstream of the TATA box of the CMV promoter, and inhibits transcription of *Stra8*. Gene expression is activated by addition of doxycycline or tetracycline. The added reagent binds to TetR, which results in a confirmation change of TetR, its dissociation from the CMV promoter and allows *Stra8* expression. Modified, Stegmeier et al., 2005. **(B)** Plasmid map of generated pEBTetD-mStra8 construct, which provides the open reading frame of m*Stra8* driven by the CMV promoter, its tetracycline repressor (TetR) as well as a puromycin (Puro) and ampicillin (Amp) resistance cassette for cloning and bacteria selection, respectively. m*Stra8* was cloned at *Kpn*I/*Not*I restriction sites and linearized with *Fsp*I.

4.1.3.3 *Stra8* overexpression promotes the formation of blastocyst-like structures

The *in vitro* differentiation experiments were accompanied by the development of striking cell structures (3.4.4.4). They emerged predominantly in *Stra8* overexpressing cell lines both with and without RA induction, but were present in a greater extent in RA-induced cultures. These structures were also observed to a minor extent in the differentiating original cell line SSC 12 (3.4.4.4, Fig. 63). However, in the culture of SSC 12 these structures were first identified after their occurrence in the *Stra8* overexpressing cell lines, which caused an intend search in SSC 12 cultured cells. At the time point of *in vitro* differentiation revealing the hugest extent of these structures, the viability of these cells was proven by an apoptosis assay as well as no nuclear fragmentation as an indicator for apoptosis could be observed (3.4.4.4.1, 3.4.4.4, Fig. 64; 3.4.4.4.1, Fig. 65). An electron microscope analysis did not provide any detailed information about the cell type (3.4.4.4.2, Fig. 66). So the type of these viable structures remained unclear.

In the context of different experimental studies the researchers presented structures, which revealed a morphology closely resembling to the cells predominantly emerged during the *in vitro* differentiation of *Stra8* overexpressing cell lines (Fig. 68 A). The generation of these structures was predominately described as the formation of a blastocyst as the result of a fertilization of oocytes.

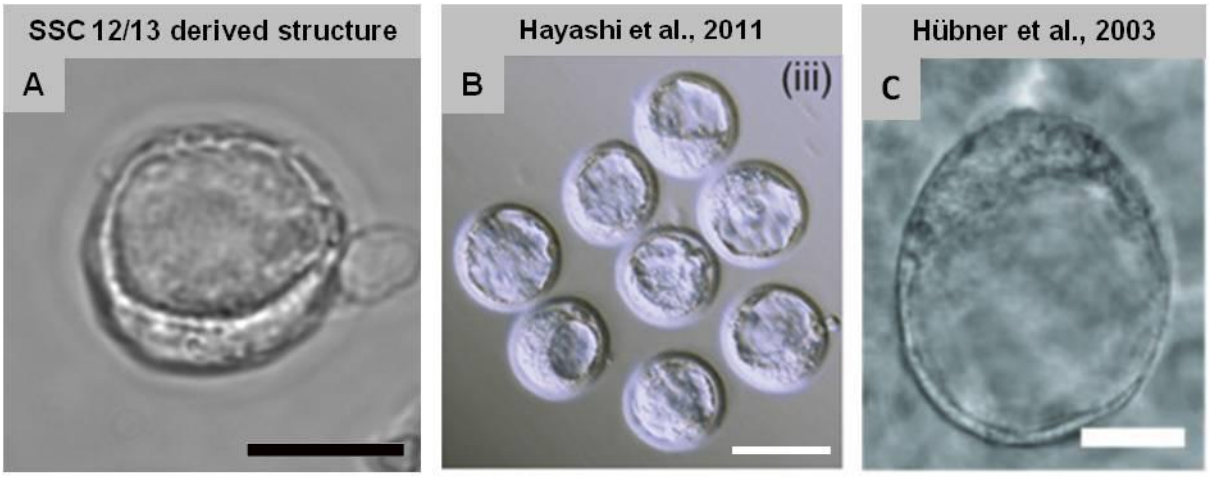


Fig. 68: Comparison of blastocyst-like structures. (A) *In vitro* differentiation of *Stra8* overexpressing cell lines gave rise to blastocyst-like structures. Here a SSC 12/13-derived blastocyst-like structure is shown. Scale bar: 50 µm. **(B)** Blastocysts derived from ICSI experiments. Scale bar: 100 µm. Hayashi et al., 2011. **(C)** Blastocyst-like structures *in vitro* derived from ESCs. Scale bar: 30 µm. Hübner et al., 2003.

Hayashi and colleagues (2011) generated mouse primordial germ cell-like cells from ESCs and iPSCs, transplanted them into germ cell depleted mice and used the produced mature sperm for ICSI experiments, which led to the formation of the mentioned blastocysts and finally gave rise to offspring (Fig. 68 B). But this experimental approach yielding in blastocyst-like structures completely differs from the *in vitro* spermatogenesis experiments carried out in the presented thesis. However, the group of Prof. Schöler (Max Planck Institute for Molecular Biomedicine, Münster) presented a protocol for the derivation of oocytes from mouse ESCs and found several structures resembling blastocysts after more than 40 days in culture (Fig. 68 C; Hübner et al., 2003). Provided that the structures emerged in *Stra8* overexpressing cells indeed reflect blastocysts, their development could be therefore due to processes of parthenogenesis or self-fertilization. The first alternative would require the derivation of oocytes, while the latter alternative would require the development of both male and female gametes during the performed *in vitro* spermatogenesis experiments. Generally, the male-to-female sex reversal could already be demonstrated in the *in vitro* derivation of sperm from female ESCs as well as of oocytes from male ESCs (Kerkis et al., 2007; Eguizabal et al., 2011), which is probably due to improper regulated expression of single genes (Colvin et al., 2001; Hübner et al., 2003). The derivation of oocytes from male ESCs can be caused by

an improper SRY expression leading to the entry of male PGCs into oogenesis (Tilmann and Capel, 2002).

At this point it is very important to emphasize that these possible explanations of parthenogenesis or self-fertilization for the development of blastocyst-like cells during the *in vitro* experiments are hypothetical and no data were collected, which provided clear evidence for these hypotheses, because the work of the presented thesis was further focused on a co-culture system for the generation of putative SSCs from mouse ESCs, which is discussed in the following second part.

4.2 A co-culture system using HTF cells for the generation of putative SSCs from mouse ESCs

The second part of the discussion will focus on the establishment of a co-culture system for the derivation of putative SSCs from co-culturing mouse ESCs and HTF cells. From a brief insight in the somatic cell signalling within the seminiferous tubules arises the characterization of the HTF cells, which is further discussed in regard to its features and potential to be used in co-culture systems. After a comparison of the here presented co-culture system with already described models, the characterization of the co-culture-derived SSC-like cells and its results are discussed based on currently available and appropriate analyses. Finally, hypotheses regarding the potential driving forces in the co-culture system will be presented.

4.2.1 Regulation of spermatogenesis by somatic cell signalling

The process of spermatogenesis within the seminiferous tubules of the testis is a highly regulated mechanism, which is mainly influenced by auto-, endo- and paracrine signalling of testicular cells. Especially the SSC niche, consisting of sertoli cells within the seminiferous tubules, the basement membrane and interstitial cells provide key factors for a growth factor milieu, which determines the fate of SSCs regarding their self-renewal, proliferation and differentiation into haploid male germ cells (1.4). Up to now the molecular mechanisms are not completely understood, but several studies in different species, especially in rodents, provide some insight into the somatic cell signalling within the testis.

The secreted products targeting SSCs, germ cells as well as other testicular cells can be roughly divided into self-renewal and proliferation as well as differentiation and spermiogenesis promoting factors (Huleihel et al., 2007; de Rooij, 2009). The outstanding role of sertoli cells, which represent the only somatic cell type within the seminiferous tubules, is clearly recognizable in the great number of their secreted factors: GDNF (Glial cell

line Derived Neurotrophic Factor), bFGF (basic Fibroblast Growth Factor) and LIF (Leukemia Inhibitory Factor) play an important role in the self-renewal and proliferation of SSCs, while SCF (Stem Cell Factor), activin A, BMP4 (Bone Morphogenetic Protein 4), IGF-I/II (Insulin-like Growth Factor-I/II), ABP (Androgen Binding Protein), inhibin and follistatin are involved in the differentiation process (Huleihel et al., 2007; de Rooij, 2009). Additionally, sertoli cells supply the testicular cells with binding and transport proteins (Sylvester, 1993), extracellular matrix and functional proteins (Russel and Peterson, 1985), proteases and their inhibitors (Fritz et al., 1993) and energy substrates (Groetegoed and Den Boer, 1987; Huleihel et al., 2007). Secretion of LIF and IGF-I/II was also proven for leydig cells, which further participate in the self-renewal and proliferation of SSCs by the production of CSF-1 (Colony-Stimulating Factor 1). Besides activin A, IGF-I/II and inhibin, leydig cells also secrete PDGF (Platelet-Derived Growth Factor) affecting the differentiation. However, the main task of leydig cells is the testosterone production in the testis, which is necessary for both self-renewal and differentiation of SSCs (Huleihel et al., 2007; de Rooij, 2009). The peritubular cells support the supply of GDNF (Spinnler, 2010), LIF and CSF-1 (Huleihel et al., 2007; de Rooij, 2009).
In several studies this knowledge was used for an *in vitro* recapitulation of the SSC microenvironment in order to maintain and expand SSCs under *in vit*ro culture conditions. The different approaches are based mostly either on the use of media supplemented with growth factors on feeder layer of non-testicular origin or on the SSC co-culture with testicular cells. Research groups developed defined culture systems using minimum essential media or advanced media like StemPro®-34 SFM, which are further supplemented with various growth factors such as GDNF, bFGF, LIF and others to provide comparable nourishment as in the *in vivo* niche (Sadri-Ardekani et al., 2009; Kanatsu-Shinohara et al., 2011). Some of these culture conditions were also used with non-testicular monolayers beneath the SSCs consisting of MEF (Murine Embryonic Fibroblasts), STO (SIM mouse embryo-derived thioguanine and oubain resistant) cells or Vero cells (Kanatsu-Shinohara et al., 2003; Kubota et al., 2004b). Another approach is based on the idea to utilize cells of testicular origin for a co-culture system to mimic the SSC niche more naturally by providing the cells' own produced factors. However, mostly the used media are also additionally supplemented with growth factors. Co-cultures mostly make use of sertoli cells, which is self-evident with regard to their outstanding role in production of SSC niche factors, as mentioned above (Huleihel and Lunenfeld, 2004; Baazm et al., 2013). Nevertheless, up to now a limited number of studies also used a mixture of somatic constituents of the SSC niche such as sertoli and peritubular cells (Khajavi et al., 2014; Mäkelä et al., 2014). In the presented thesis, human testicular feeder (HTF) cells were used for co-culture experiments, which were derived from MACSortings using human testicular biopsies from infertile men (3.3.1). Because the flow-through fraction of the MACSorting was used (3.1.2, Fig. 4), it was assumed that this

cell population included different testicular cells and its analysis is further discussed in the following section.

4.2.2 HTF cells reveal a peritubular cell character

For the determination of cell types supporting the used co-culture system, the HTF cells should be tested for the occurrence of the main somatic cells of the testis: the sertoli-, leydig- and peritubular cells, which were most likely present in the HTF originated from flow-through fractions of MACSortings with testicular biopsies (3.3.1). The characterization of HTF cells was complicated by the lack of specific markers for the identification of certain testicular somatic cells. To date, there are no markers available, which exclusively select one particular testicular somatic cell type. Nevertheless, in the literature there are accepted markers, whose detection using immunostaining or qRT-PCR experiments for expression analyses is acknowledged to prove the presence of sertoli-, leydig- as well as peritubular cells in different species. Sertoli cells are commonly proofed by the expression of FSHR (Follicle-Stimulating Hormone Receptor; Albert et al., 2012) or VIMENTIN (Langenstroth et al., 2014; Mäkelä, et al., 2014), whereas the latter protein is also an often used marker for peritubular cells (Langenstroth et al., 2014). Besides VIMENTIN, peritubular cells are usually described by the expression of SMA (Smooth Muscle Actin; Welsh et al., 2009; Albert et al., 2012; Kossack et al., 2013; Langenstroth et al., 2014), while leydig cells are often characterized by the presence of LHCGR (Luteinizin Hormone/Choriogonadotropin Receptor; Welsh et al., 2009; Kossack et al., 2013) or 3-β-HSD (3-β-Hydroxysteroid Dehydrogenase; Rebourcet et al., 2014). Thereby looking at several studies it is not unusual to use only one of these markers in order to verify the appropriate cell type. Because a mixture of testicular cells was expected, in the presented study several in the literature accepted testicular cell markers were used in order to ensure a more detailed molecular description of the HTF cells (3.3.1.1). Therefore, the list of analyzed markers was extended and additionally to the above mentioned markers the expression patterns of the sertoli cell-associated marker *INHIBIN*, the leydig cell-associated markers *P450* and *STAR* (Steroidogenic Acute Regulatory Protein; Landreh et al., 2014) as well as of the peritubular cell associated markers *DECORIN* (Adam et al., 2011), *PENTRAXIN 3* (Flenkenthaler et al., 2014), *CALPONIN* (Welter et al., 2013), *AR* (Androgen Receptor; Welsh et al., 2011), *GPER* (G-Protein Coupled Oestrogen Receptor; Sandner et al., 2014) and *AT1R* (angiotensin II type I receptor; Welter et al., 2014) were analysed by qRT-PCR experiments (3.3.1.1, Fig. 26, Fig. 27). Tab. 9 provides an overview of the analyzed markers for the characterization of the HTF cells and the corresponding results indicating a peritubular cell character.

Tab. 9: Results of the characterization of HTF cells using markers for sertoli-, leydig- and peritubular cells.

Analyzed marker	Expressed in…				
	Sertoli cells	Leydig cells	Peritubular Cells	HTF	
				qRT	IC
Lipid droplets	+	+	-	n.a.	- *
FSHR	+	-	-	-	n.a.
Inhibin	+	-	-	-	n.a.
Vim	+	-	+	++	+
3-β-HSD	-	+	-	-	n.a.
LHCGR	-	+	-	-	n.a.
STAR	-	+	-	++	n.a.
AR	-	-	+	+	n.a.
AT1R	-	-	+	++	n.a.
Calponin	-	-	+	++	n.a.
Decorin	-	-	+	+	n.a.
GPER	-	-	+	++	n.a.
Pentraxin 3	-	-	+	++	n.a.
SMA	-	-	+	+	+

FSHR: Follicle-Stimulating Hormone Receptor; Vim: Vimentin; 3-β-HSD: 3-β-Hydroxysteroid Dehydrogenase; LHCGR: Luteinizin Hormone/Choriogonadotropin Receptor; STAR: Steroidogenic Acute Regulatory Protein; AR: Androgen Receptor; AT1R: Angiotensin II Type I Receptor; GPER: G-Protein Coupled Oestrogen Receptor; SMA: Smooth Muscle Actin; HTF: Human Testicular Feeder; qRT: analyzed by qRT-PCR experiments; IC: analyzed by immunocytochemical staining; *: analyzed by Oil Red Staining performed by Farzaneh Rafi Manzelat (Master student); +: moderately expressed; ++; highly expressed, comparable to used positive control; -: not expressed; n.a.: not analyzed.

The expression pattern of the peritubular cell markers *PENTRAXIN 3*, *CALPONIN*, *GPER* and *AT1R* was comparable to *in vitro* cultured human testicular peritubular cells (HTPCs; Albrecht et al., 2006), which were used as positive control (3.3.1.1, Fig. 27). Moreover, *VIMENTIN* revealed an increased expression compared to human testis sample as a positive control (3.3.1.1, Fig. 26). Because also the other peritubular cell markers were at least moderately expressed in the HTF cells (3.3.1.1, Fig. 27), these data indicated a peritubular cell character of the HTF cells. Even if the mRNA of *SMA* was just moderately detected, the heterogeneous immunostaining for SMA and VIMENTIN in the immunocytochemical studies further support the peritubular cell background of the HTF cells (3.3.1.1, Fig. 27).

This assumption was further validated by the expression patterns of sertoli- and leydig cell markers, which, with exception of *STAR*, were not comparable with the human testicular cells used as a positive control (3.3.1.1, Fig. 26). Furthermore, an Oil Red Staining, which can be used to stain lipid droplets within the cytoplasm as characteristic features of sertoli- and leydig cells (Sonne et al., 2009; Riboldi et al., 2012), indicated the absence of these cells in the HTF cells. Fig. 69 shows exemplarily the staining reaction of HTF-2 cells with the Oil Red reagent as well as the negative control incubated with PBS performed by the master student

Farzaneh Rafi Manzelat ("Production of *in vitro* sperm as a new horizon in treating male infertility by reprogramming of human somatic cells derived from infertile men"; 2014).

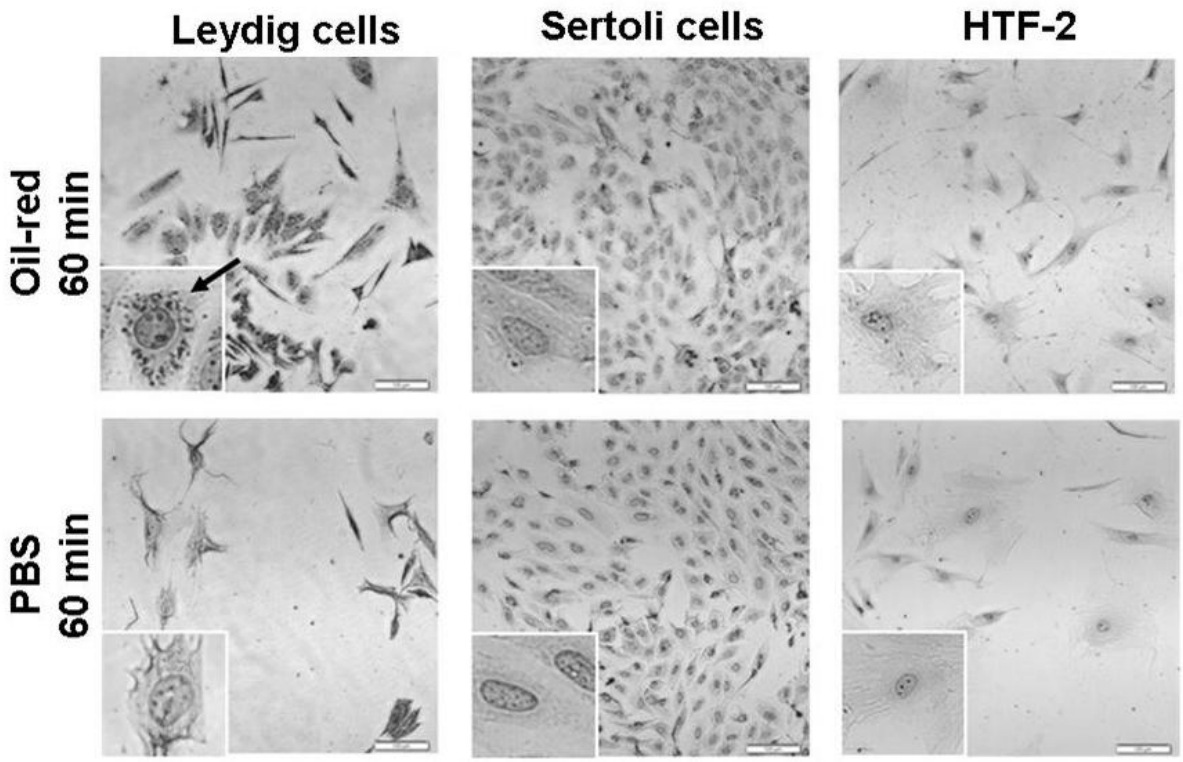


Fig. 69: Oil Red Staining of HTF-2 cells for the identification of lipid droplets in leydig- and sertoli cells. HTF-2 cells were stained with Oil Red to identify cytoplasmic lipid droplets as typical features for leydig- and sertoli cells. Mouse leydig cells (MA-10) and sertoli cells (15P-1) were used as positive controls, whereas here the lipid droplets could only be detected in leydig cells (arrow). However, HTF-2 cells did not reveal Oil Red stained lipid droplets and showed a different morphology than the positive controls. Incubation with PBS served as a negative control. The experiment was performed by the master student Farzaneh Rafi Manzelat ("Production of *in vitro* sperm as a new horizon in treating male infertility by reprogramming of human somatic cells derived from infertile men"; 2014). Scale bars: 100 µm.

Mouse leydig cells MA-10 and sertoli cells 15P-1 were used as positive controls. Cytoplasmic lipid droplets were observed in leydig cells, while no staining was monitored in sertoli cells (Fig. 69). Although both cell lines are immortalized, it is possible that the cell line 15P-1 reveals a weakened sertoli cell-like character after its transformation that may result in the lack of lipid droplets. Nevertheless, HTF-2 cells were not positively stained by Oil Red and revealed a different morphology than the positive controls. These finding supports the qRT-PCR data, which did not reveal an expression of sertoli- and leydig cell markers and indicate the absence of sertoli- and leydig cells in the HTF cells.

Because the data reveal a peritubular cell character of the HTF cells, it is worth to have a closer look on the role of peritubular cells, which have been underestimated for a long time (Mayerhofer, 2013). In men these thin, spindle-shaped cells form together with extracellular matrix proteins the wall of the seminiferous tubules consisting of several layers, whereat the

inner layers reveal a smooth muscle phenotype and the outer layers are characterized by a connective phenotype (Davidoff et al., 1990). Here SMA, VIMENTIN and CALPONIN, which have been found to be expressed in the HTF cells (Tab. 9), are important structural proteins. Besides CALPONIN, numerous factors such as endothelin 1 (Romano et al. 2007), PDGF (Romano et al., 2006), prostaglandins (Tripiciano et al., 1998), neurotransmitters (Miyake et al., 1986) and hormones (Yamamoto et al., 1989) regulate the contraction and relaxation of peritubular cells for the active transport of sperm. The maintenance of a stable wall is of great importance for fertility concerning the cases of infertile men with a remodelled and thickened wall of their seminiferous tubules (Schell et al., 2010; Welter et al., 2013). Besides proteins important for the skeletal system and vasculature development, human peritubular cells are thought to contribute to the inflammatory network and SSC signalling pathways (Flenkenthaler et al., 2014). The peritubular cells also influence other testicular cells by their production and secretion of extracellular matrix proteins, signalling factors and antioxidant proteins (Mayerhofer, 2013) and expression of different receptors, for example GPER (Sandner et al., 2014) and AT1R (Welter et al., 2014), which are also expressed in the HTF cells (Tab. 9). Angiotensin II, the agonist of AT1R, is suggested to be involved in sperm transport and inflammatory processes. Besides the nuclear receptors ESR1 and ESR2 (Estrogen Receptor 1 and 2, respectively), GPER is the third estrogen receptor expressed within the testis and has an inhibitory effect on the proliferation of peritubular cells (Sandner et al., 2014). Moreover, peritubular cells secrete the important neurotophic factors NGF (Nerve Growth Factor; Schell et al., 2008) and GDNF (Spinnler et al., 2010). While studies in rodents suggest an involvement of NGF in meiosis, its role in the human testis is not yet determined (Seidl et al., 1996; Perrard et al., 2007). GDNF is known to be a crucial factor for the self-renewal of SSCs and is expressed in rodents as well as in human testis (Oatley and Brinster, 2012).

Many insights into the role of peritubular cells within the SSC niche in men were and are still provided by an adequate primary *in vitro* culture system of adult human peritubular cells, which was developed by the group of Prof. Mayerhofer (Albrecht et al., 2006). They established a culture method for these cells derived from adult patients with obstructive azoospermia but normal spermatogenesis, termed as HTPCs, as well as with non-obstructive azoospermia with impaired spermatogenesis and testicular fibrosis, labelled as HTPCFs. Although such cellular models are not able to recapitulate the *in vivo* situation exactly, since their availability HTPCs are preferentially used to analyze human peritubular cells, because rodents do not represent the best model considering the morphological differences between the seminiferous tubules' wall of these species (Mayerhofer, 2013). Albrecht and colleagues (2006) cultured their established HTPCs in minimal medium consisting of DMEM supplemented with 10% FCS without any antibiotics and were able to

grow them for at least nine passages with proliferation at slow pace. These culture conditions and growing behaviour is comparable to the HTF cells, which were also cultured in minimal medium, but additionally contains the antibiotics penicillin/streptomycin (FB medium; 3.3.1). HTF cells revealed a moderate proliferation for at least 10 to 12 passages, but it decreased markedly with further increasing number of passages.

Based on experiments with HTPCs, Sandner and colleagues (2014) describe that the GPER, also expressed in the HTF cells (Tab. 9), is linked to the proliferation of peritubular cells and their apoptosis. They incubated HTPCs with a nonsteroidal agonist, which exclusively activates GPER when applied in certain concentrations and led to a significantly reduction of adherent HTPCs within 24 hours of incubation. Using life cell video imaging the stop of proliferation, morphology changes and the detachment of HTPCs were recorded indicating the initiation of apoptosis. This process could be inhibited by the prior addition of a GPER selective antagonist. Based on these results the application of a GPER selective antagonist in the culture medium for HTF could be one possibility to maintain a sufficient proliferation rate of HTF cells during the co-culture, which seemed to be an important parameter for a successful derivation of SSC-like cells from ESCs in the co-culture system (4.2.6). However, here it would be very important to exclude any effect from the inhibition of the GPER on the generated putative SSCs.

Moreover, using the HTPC *in vitro* model Spinnler et al. (2010) could show a constitutive GDNF production of HTPCs, which further support the GDNF level supplied by sertoli cells and thereby contribute to the growth factor milieu of the SSC niche. Considering the peritubular cell character of the HTF cells used in the co-culture system presented in this thesis, it is assumed that GDNF, probably secreted by the HTF cells, plays a decisive role, which is discussed later in more detail (4.2.6). Nevertheless, in further analyses it should be looked closely at the products secreted by the HTF. Based on the hypothesis that GDNF is the driving force in the presented co-culture system, immunofluorescence staining of GDNF in cultured HTF cells could be used to validate its presence in these cell lines. Moreover, the expression level of GDNF during the period of co-culture could be investigated using qRT-PCR analyses.

Based on the hypothesis that the production of defined factors for the SSC niche by peritubular cells is dependent on testosterone, Chen et al. (2014) showed that this hormone, *in vivo* produced by leydig cells (Quigley et al., 1995), regulates the GDNF expression in mouse peritubular cells. Using a primary culture of adult mouse peritubular cells they confirmed the induction of GDNF expression by testosterone on the mRNA and protein level. Culturing of mouse peritubular cells in serum free DMEM/12 medium supplemented with 10 ng/ml testosterone led to a maximal accumulation of GDNF in the medium after 48 hours of incubation. A halfvolume of medium was replaced every 12 hours in order to maintain a

constant supply of testosterone. Assuming that GDNF indeed has an effect in the co-culture system introduced in this thesis, application of testosterone to the medium during co-culture might further improve the efficiency of the generation of SSC-like cells from mouse ESCs. Up to now studies on the expression of AR for testosterone in germ cells are controversial and could not identify exactly the AR expressing germ cell types (Vornberger et al., 1994; Johnston et al., 2001), but the anti-androgen flutamide could be used in order to verify a possible testosterone-dependent effect on HTF cells' secretion pattern within the co-culture system.

Moreover, using a secretome analysis, the naturally secreted factors of the HTF cells could be determined and could reveal other crucial factors, which might have forced the generation of putative SSCs from mouse ESCs in the co-culture.

4.2.3 Co-culture system using mouse ESCs with HTF cells- about similarities and differences with other co-culture approaches

At this point a comparison of the here presented co-culture system with already described co-culture approaches is appropriate, but only conditionally feasible. In the presented thesis the usage of mouse ESCs cultured on HTF cells aimed at the generation of putative mouse SSCs, while other studies used co-culture approaches primarily for the *in vitro* culturing of isolated SSCs (1.4). The co-culture of mouse ESCs and HTF cells combines actually two different approaches for the *in vitro* generation of SSCs: the unique potential of ESCs to differentiate into any cell type including germ cells (1.2) as well as the co-culture with testicular cells to recapitulate the *in vivo* SSC niche (1.4; 4.2.1). The fact that this system included a co-culture of cells derived from two different species has to be also considered in comparing the co-culture approaches. In the following the comparison is focused on the different used cell monolayers beneath the germ cells as well as medium compositions used in *in vitro* co-cultures, which aimed at the self-renewal and survival of *in vivo* isolated SSCs and are mostly corresponding to the co-culture using mouse ESCs and HTF.

In the presented co-culture system the HTF was used as a feeder layer during the whole period of co-culture. Expression analyses revealed a peritubular cell character for these cells (4.2.2) and even an intensive database search did not yield in any already published studies, which exclusively used peritubular cells for co-culturing of SSCs. As already mentioned above co-culture systems make often use of non-testicular cells. Mitomycin C treated embryonic fibroblasts such as MEF and STO are prevalently used for the *in vitro* maintenance of *in vivo* isolated SSCs. Nagano et al. (1998) showed that the survival of mouse SSCs could be supported by their cultivation on STO feeders yielding in an *ex vivo* culture for nearly four month. The functionality of the *in vitro* cultured mouse SSCs was

proven by their contribution to the regeneration of spermatogenesis in transplantation assays. Using a 7-day culture system, Nagano and colleagues (2003a) revealed that the stem cell maintenance is dependent from the type of feeder layer, which is used. The study demonstrated that also mitomycin C treated NIH-3T3 fibroblasts or mutant embryonic fibroblasts expressing no or a reduced level of Steel factor, which has been shown to have an impact on the survival and proliferation of certain types of spermatogonia (Yoshinaga et al., 1991; de Rooij et al., 1999), yield in a survival of cultured SSCs comparable to STO feeder cultures finally examined in their colonization activity in transplantation assays. However, the cultivation of SSCs on certain fibroblasts had a significantly positive effect on the cell survival and the final outcome of colonized cells following transplantations (Nagano et al., 2003a). Interestingly, the group also tested different mitotically inactive sertoli cell lines as one testicular cell type for the *in vitro* maintenance of SSCs. While two sertoli cell lines, namely MSC-1 and 15P-1, yielded in SSC survival comparable to the use of STO feeder, other cell lines, such as SF7 and TM4, resulted even in a significantly decreased colonization activity. This result was surprisingly in regard to the hypothesis that sertoli cells, representing the most important contributor to the SSC niche (1.4; 4.2.1), should support the *in vitro* culture of SSCs. Nagano and colleagues (2003a) suggested that the sertoli cell lines SF7 and TM4 especially supported the differentiation of spermatogonia, which finally yielded in stem cell loss in the *in vitro* culture (Griswold, 1995; Nagano et al., 2003a).

In the last year, Mäkelä and colleagues published an interesting *in vitro* co-culture system using the proliferative and migratory properties of testicular cells in order to overcome the poor viability of SSC-like cells *ex vivo* (Mäkelä et al., 2014). This work revealed that the use of proliferating primary cells, like HTF cells, might be advantageous for the *in vitro* culture of SSCs compared to co-cultures using mitotically inactive feeder cells like STO, MEF or even sertoli cells presented by Nagano et al. (2003a). The following comparison highlights the overlapping and differences of culture conditions and cell development in the co-culture systems presented in this thesis and published by Mäkelä and co-workers (2014). They used small fragments of mouse seminiferous tubules, from which somatic cells spread out characterized as sertoli- and peritubular cells by marker expression analyses, whereas the presence of leydig cells was just provable within the first two weeks of culture. After one to two weeks of culture, spermatogonial cells were detected by the expression of the ubiquitous spermatogonial cell marker MAGE-B4 (Österlund et al., 2000). As Mäkelä and colleagues, the co-culture system presented in this thesis was also based on the use of primary and proliferative testicular cells characterized by a peritubular cell character, but the analyses did not reveal the presence of sertoli- and/or leydig cells (4.2.2). The use of a simple culture medium during the whole culture period is one more commonality of both co-culture systems, which forewent the addition of any growth factors to the culture medium just consisting of

essential medium supplemented with 10-15% FCS. The used culture medium contrasts with other media compositions used in sertoli free mouse and human SSC culture systems (Kanatsu-Shinohara et al., 2003; Kubota et al., 2004b; Kanatsu-Shinohara et al., 2005; Sadri-Ardekani et al., 2009; Kanatsu-Shinohara et al., 2011). Generally, in these studies the SSCs were cultured on mitomycin C treated feeder or different coated flasks with medium consisting of minimal essential medium or advanced medium such as StemPro®-34 SFM, which were further supplemented with various factors such as GDNF, bFGF, EFG, LIF and serum. These studies had the use of a very coordinated medium in common, whereas the compositions and concentrations of supplements could differ slightly among the studies. In comparison to these media, the culture medium used by Mäkelä and co-workers (2014) and in the here presented co-culture system was very simple and cost effective for the cultivation of mouse SSCs suggesting that the sertoli- and peritubular cells took over the supply with growth factors nutrients, which were provided by the supplemented media used in the other studies.

During their co-culture, Mäkelä et al. (2014) identified MAGE-B4 positive cells in areas with high cell densities. Also the formation of SSC-like cells in the co-culture using mouse ESCs and HTF cells was primarily observed in grape-like structures near local HTF cell aggregates. Moreover, Mäkelä and colleagues (2014) could finally prove the undifferentiated state of their co-culture-derived mouse SSCs in transplantation assays and confirmed that even after at least four weeks in *ex vivo* culture the SSCs remain their spermatogenic differentiation potential. This final proof could not be obtained using the co-culture-derived putative mouse SSCs in the presented thesis, whereat the possible reasons are discussed later in detail (4.2.5). Moreover, the use of sertoli- and peritubular cells were sufficient to culture spermatogonia for at least five weeks (Mäkelä et al., 2014). Using HTF cells as a nearly confluent monolayer putative mouse SSCs could be propagated at least three weeks with a maximal expansion to five weeks. This was strongly dependent on the proliferation rate of the HTF cells. A decreased proliferation rate was accompanied by a reduced formation of SSC-like cells, which finally yielded in the detachment of these cells. The extended culture observed by Mäkelä et al. (2014) could be attributed to their combined use of primary sertoli- and peritubular cells. From rodent studies it is known that these cell types form tight cell structures (Tung and Fritz, 1980; Skinner et al., 1985; Hadley et al., 1990), which were identified as cell clusters and cord-like structures with a diameter of 200-700 mm by Mäkelä et al. (2014) and grew during co-culture, while a stagnation of co-culture was observed when these formations were completed. Although also similar cell clusters, obviously consisting of HTF cells, were also observed in the here introduced co-culture system, these clusters did not reach a comparable diameter as observed by Mäkelä and co-workers (2014).

They hypothesized that GDNF secretion by sertoli cells was the driving force in their co-culture system. A GDNF concentration, comparable to those, which have been used in supplemented media of sertoli free mouse and human SSC cultures (Kanatsu-Shinohara et al., 2005; Sadri-Ardekani et al., 2009; Kanatsu-Shinohara et al., 2011), was detected by an ELISA assay and could be further stimulated by FSH treatment indicating the functionality of the primary sertoli cells. This corresponds to the findings of Nagano et al. (2003a), who demonstrated that the addition of GDNF to the culture medium significantly improves the SSC survival *in vitro*. Considering the peritubular cell character of the HTF (4.2.2) and the ability of HTPCs to secrete GDNF (Spinnler et al., 2010; 4.2.2), it is assumed that GDNF played also an important role for the generation of SSC-like cells during co-culture of mouse ESCs and HTF cells. The GDNF signalling pathways supporting SSC self-renewal and maintenance is discussed later in detail (4.2.6). Nevertheless, especially the work of Mäkelä and colleagues highlights the importance to clarify the presence of GDNF, its concentration and possible enhanced production stimulated by other reagents in the co-culture system using mouse ESCs and HTF. As already mentioned above immunfluorescence stainings and qRT-PCR expression analyses could be used to localize GDNF within the HTF and determine the GDNF levels, respectively (4.2.2). The usage of a GDNF enzyme linked immunosorbent assay (ELISA) according to Mäkelä and colleagues (2014) could be used to detect the course of GDNF concentration during the progress of co-culture. Moreover, FSH can stimulate the GDNF production of mouse sertoli cells (Mäkelä et al., 2014), but up to now this was not shown for HTPCs, particularly since the presence of FSH receptors in HPTCs could also not be proven to date (Spinnler et al., 2010). As already mentioned Chen et al. (2014) demonstrated the testosterone induced GDNF production by mouse peritubular cells. However, this could also not be proven in HTPCs until now, but it would be worth to test the influence of testosterone in HTF cells considering that HTPCs indeed possess the essential ARs (Mayerhofer, 2013). Considering this knowledge from the literature, the growth factor GDNF turns out to be one of the driving forces within the co-culture system using mouse ESCs and HTF. Before a more detailed overview about the possible driving forces is presented, the result of this co-culture system in form of putative SSCs derived from mouse ESCs should be discussed in the view of methods, which are in the literature accepted for the characterization of germ cells.

4.2.4 Characterization of co-culture-derived SSC-like cells based on currently available methods

In mice, SSCs are thought to be a subpopulation of the undifferentiated spermatogonia, which are classified into single (A_s), paired (A_{pr}) and aligned (A_{al}) A-type spermatogonia

according to the number of cells connected by cytoplasmic bridges (Fig. 70; Kanatsu-Shinohara and Takashi Shinohara, 2013). A_{pr} spermatogonia can divide into chains consisting of up to 16 A_{al} cells. These cells eventually differentiate within six divisions into spermatocytes and finally in mature spermatozoa, thereby passing the differentiating A_1 to A_4, intermediate and type B spermatogonial state. The naturally process of spermatogenesis in mice takes 35 days. The A_s cells are characterized by their ability to self-renew and to produce A_{pr} spermatogonia, which give rise to A_{al} cells. SSCs are considered to be the self-renewing A_s spermatogonia, but transplantation studies suggest that just 10% of these cells represent the SSC population in mice (Nagano et al., 1999).

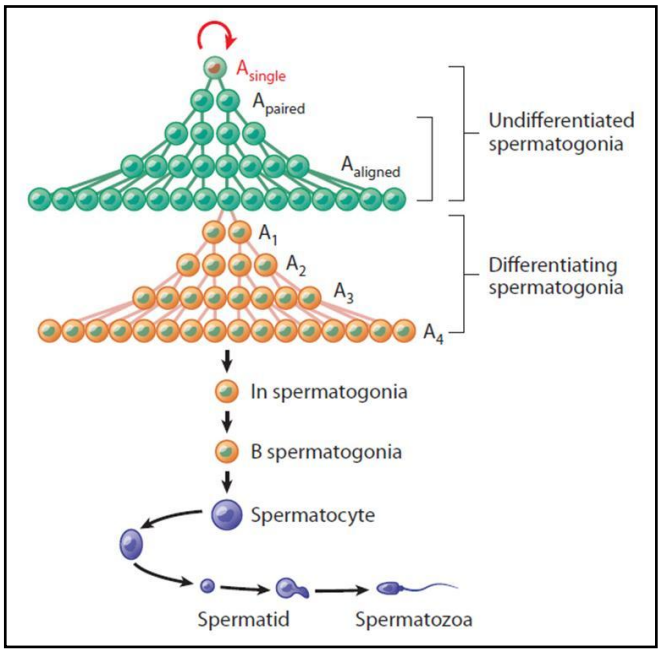


Fig. 70: Scheme of cell types during mouse spermatogenesis. In mice, undifferentiated spermatogonia consist of single (A_s), paired (A_{pr}) and aligned (A_{al}) A-type spermatogonia. A_{al} can enter the process of differentiation into male gametes, which comprises differentiated A_1-A_4, intermediate and B-type spermatogonia followed by maturation from spermatocytes to mature spermatozoa. Undifferentiated as well as differentiating spermatogonia are connected by cytoplasmic bridges. Kanatsu-Shinohara and Takashi Shinohara, 2013.

In the present study, during co-culture with HTF cells the ESCs underwent morphological changes, which finally yielded in grape-like structures (3.3.2, Fig. 28; Fig. 71 A). This compact cell morphology is described to be characteristic for *in vitro* cultured SSCs and is remarkably similar among rodent and non-rodent species (Fig. 71 B-F). In the literature the description of these *in vitro* cultured cells, isolated from the specie's testis using testicular cell suspensions or cell sorting procedures, is unfortunately not consistent and comprises designations such as SSCs, germline stem cells, germ cells or cells named according to the used sorting marker. Based on their characteristic shape the co-culture-derived cells were termed as putative SSCs or SSC-like cells in the presented thesis. The viable and

proliferating state of these cells was further proven by an apoptosis- and BrdU assay (3.3.3.1).

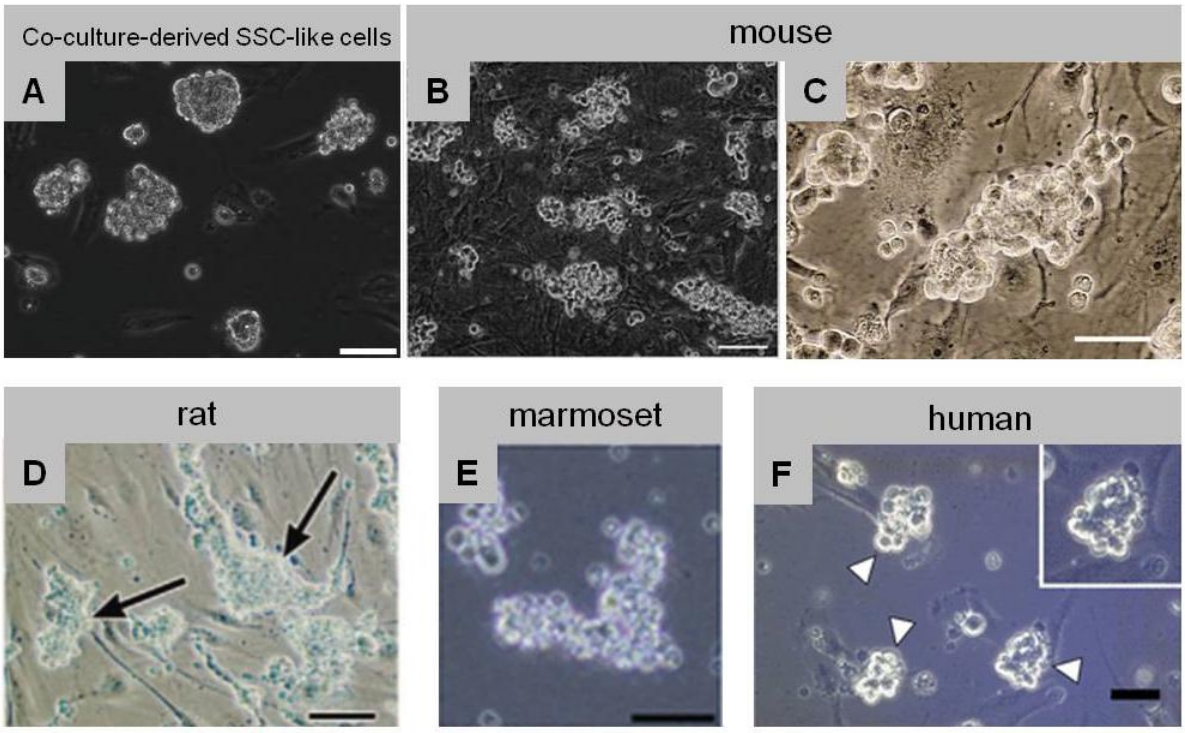


Fig. 71: : *In vitro* cultured germ cells reveal a similar morphology. (A) SSC-like cells derived from co-culture of mouse ESCs with HTF cells cultured in DMEM medium supplemented with 10% FCS and 1% penicillin/streptomycin. Scale bar: 40 µm. **(B)** Cultured mouse SSCs, named as germline stem cells. Scale bar: 50 µm. Sato et al., 2013. **(C)** Thy-1 positive cells cultured on STO feeder in serum-free medium supplemented with GDNF, soluble GFRα1 and bFGF. Thy-1, a marker for undifferentiated spermatogonia (Kubota et al., 2003), was used for enrichment by MACSorting. Scale bar: 50 µm. Kubota et al., 2004b. **(D)** Epcam (Epithelial Cell Adhesion Molecule) positive cells cultured on STO feeder in serum-free medium supplemented with GDNF, GFRα1, bFGF and/or LIF. Epcam was used for enrichment of A-type spermatogonia (van der Wee et al., 2001) Scale bar: 100 µm. Ryu et al., 2005. **(E)** Adult marmoset testicular cells cultured in MEMα medium containing 10% FCS and 1% penicillin/streptomycin. Authors detected the germ cell marker MAGE A4 (Melanoma-Associated Antigen 4; Aubry et al., 2001; Eildermann et al., 2012b) in the cultured cells. Scale bar: 50 µm. Langenstroth et al., 2014. **(F)** Human testicular cells cultured on uncoated dishes in MEMα medium containing 10% FCS and 1% penicillin/streptomycin. Cultured cells were positive for the germ cell markers VASA (Toyooka et al., 2000; 2003) and MAGE A4. Scale bar: 100 µm. Kossack et al., 2013.

Further characterization of co-culture-derived putative SSCs was complicated by the deficiency of markers, which exclusively detect SSCs and distinguish them from ESCs at the molecular level, representing the original cell type used in the here presented co-culture system. Although the origin of ESCs is not yet clearly determined, several studies suggest that ESCs may have a germ cell origin based on their common molecular properties with other pluripotent cells (for review: Zwaka and Thomson, 2005). Until now *in vitro*-derived germ cells can just be inadequately characterized by the expression analyses of marker genes, which are detectable in both pluripotent and germ cells. A comparative systematic

expression analysis of germ cell- and premeiotic markers revealed comparable expression levels of germ cell-associated genes such as *Stella* and *Fragilis* and premeiotic associated genes such as *Piwil2*, *Dazl* and *Vasa* on mRNA as well as on protein level in ESCs and various germline-derived pluripotent cells (Xu et al., 2011). The close relationship between ESCs and germ cells was also shown by several groups, which were able to convert ESCs into primordial germ cell (PGC) like cells as well as germ cell-like cells using certain *in vitro* culture conditions (Hübner et al., 2003; Toyooka et al., 2003; Geijsen et al., 2004). Also pluripotent cells can be derived from PGCs as well as SSCs indicating that germline cells have a pluripotency memory and the potential to regain this unique pluripotent state (Matsui et al., 1992; Kanatsu-Shinohara et al., 2004; Guan et al., 2006). The expression of several marker genes such as *Oct4*, *Stella*, *Fragilis*, *Vasa* and *Dazl* is shared by ESCs and germ cells including PGCs and SSCs (Toyooka et al., 2000; Geijsen et al., 2004; Kehler et al., 2004; Cauffmann et al., 2005). Therefore, a reliable molecular characterization of these cells derived from ESCs is still very challenging. However, because up to now researchers evaluate their *in vitro*-derived germ cells molecularly on the basis of the expression analyses of germ cell-associated genes, this strategy for characterization was also pursued in the presented thesis. The review by Phillips and co-authors (2010) provides an overview of general germ cell markers used for sorting procedures as well as characterization analyses of rodent A_s spermatogonia to elongated spermatids. The performed analyses for the characterization of the SSC-like cells and the corresponding results are summarized in Tab. 10. Preferentially, markers were used for analyses, which can be used to detect or enrich A_s spermatogonia, whose status was confirmed by spermatogonial stem cell transplantation assays (Phillips et al., 2010).

Tab. 10: Results of the characterization of SSC-like cells.

Analyzed marker	Expressed in…		
	in vivo SSCs	co-culture-derived SSC-like cells	
		IC	qRT
AP	+	+	n.a.
α-6-Integrin*	+	+	n.a.
Gfrα1*	+	n.a.	n.a.
Gpr125*	+	n.a.	+
Ngn3*	+	n.a.	n.a.
Oct4*	+	+	n.a.
Plzf*	+	+	n.a.
Ddx (Vasa)	+	+	n.a.
Sall4	+	+	n.a.

AP: Alkaline Phosphatase; Gfrα1: GDNF Familiy Receptor α1; Gpr125: G-Protein-coupled Receptor 125; Ngn3: Neurogenin 3; Oct4: Octamer-Binding Transcription Factor 4 ; Plzf: Promyelocytic Leukemia Zinc Finger Protein; Ddx (Vasa): DEAD (Asp-Glu-Ala-Asp) Box Polypeptide 4 (aliases: Vasa) ; Sall4: Sal-Like Protein 4; SSC: Spermatogonial Stem Cell; qRT: analyzed by qRT-PCR experiments; IC: analyzed by immunocytochemical staining; +: expressed; n.a.: not analyzed. *: detect or enrich A_s spermatogonia, confirmed by spermatogonial stem cell transplantation assays (Phillips et al., 2010).

Immunocytochemical analyses using different markers confirmed the germ cell character of the co-culture derived SSC-like cells (3.3.3.2; Tab. 10). The analysed cells expressed OCT4, which is known as a key regulator of pluripotency in ESCs (Niwa et al., 2000). In the testis this factor is preferentially expressed in A_s spermatogonia and essential for the self-renewal of SSCs (Pesce et al., 1998; Dann et al., 2008; He et al., 2009) suggesting an undifferentiated spermatogonial state of the SSC-like cells derived from the co-culture. This assumption was further supported by the positive staining for PLZF and SALL4. PLZF is expressed in undifferentiated spermatogonia A_s, A_{pr} and A_{al} and seems to play an important role in SSC self-renewal, because *Plzf* mutant mice revealed a progressive loss of germ cells yielding in male infertility (Buass et al., 2004; Costoya et al., 2004). PLZF can interact directly with SALL4 (Hobbs et al., 2012), which is consequently also expressed in A_s, A_{pr} and A_{al} spermatogonia (Gassei and Orwig, 2013). Additionally, the co-culture-derived SSC-like cells revealed a moderate expression of *Gpr125*, which is a further marker for undifferentiated spermatogonia within the seminiferous tubules (Seandel et al., 2007). The presence of undifferentiated spermatogonia within the co-culture-derived cell population was also supported by the performance of *in vitro* differentiation experiments (3.3.5), which revealed an upregulated expression of the postmeiotic markers *Gpx4* and *Acrosin* and thereby confirmed that at least some co-culture-derived cells can be induced to enter the differentiation process. However, this assumption bases on qRT-PCR experiments and the proof of appropriate postmeiotic markers on protein level was not provided within the presented thesis. Moreover, the methylation pattern of paternally and maternally imprinted genes *H19* and *Meg3* as well as *Snrpn* and *Igf2r* did not reveal any convincing trend towards an androgenic imprinting pattern (3.3.3.3), which would comprise methylated paternally and demethylated maternally imprinted genes (Kanatsu-Shinohara et al., 2004). Therefore, at this point a precise statement regarding a successful *in vitro* differentiation of postmeiotic cells from co-culture-derived cells is not possible.

Further analyses revealed an expression of the SSC surface marker α6-INTEGRIN and the ATP-dependent RNA helicase VASA. The expression of both germ cell-associated proteins is not restricted to undifferentiated spermatogonia, but also detectable to the level of spermatocytes and round spermatids, respectively (Shinohara et al., 1999, 2000; Toyooka et al., 2000; 2003), indicating the presence of differentiating spermatogonia within the co-culture-derived cell population. This assumption was further supported by the expression

pattern of molecular markers for germ cell development, which revealed a turning-on of early postmeiotic gene expression in the co-culture system without any exogenous induction of germ cell differentiation (3.3.5). However, the expression of the early postmeiotic genes *Gpx4* and *Acrosin* was just analyzed on the mRNA level by RT-PCR analysis.

These results indicated a co-culture-derived cell population consisting of undifferentiated and differentiating spermatogonia. But considering the already mentioned deficiency of definitive markers for the characterization of spermatogonia, SSCs can just be unequivocally identified using a transplantation assay developed by Brinster and Zimmermann (1994). This functional SSC assay bases on the microinjection of testicular cell suspensions containing the putative SSCs into the seminiferous tubules of germ cell depleted mice. These mice can be generated by busulfan treatment, which destroys the endogenous spermatogenetic activity (Bucci and Meistrich, 1987). The stem cell activity of transplanted SSCs is proven by the regeneration and maintenance of spermatogenesis within the transplanted testis (Brinster and Zimmermann, 1994). Generally, a transplantation is supposed to be accompanied by a rapid loss of SSCs and a colonization of just 10% of transplanted cells, which need up to one week to home within their niche and start propagation (Nagano, 2003b). In order to finally proof their SSC phenotype, co-culture-derived cells were used for transplantation assay (3.3.4). Because FACS- and MACSortings using appropriate surface markers are suitable approaches to enrich stem cells within a testicular cell suspension obtaining nearly one stem cell in 15 total cells (Kubota et al., 2003, 2004a), the co-culture-derived SSC-like cells underwent a MACSorting using the surface marker α6-Integrin before cell transplantation procedure. Simultaneously this sorting served for removal of HTF cells before transplantation to exclude any interference of these somatic cell types. Unfortunately, the transplanted mice did not regain fertility indicating the absence of functional male gametes. Because the naturally fertilization is a well-known problem because of immotile sperm produced during re-established spermatogenesis using *in vitro* generated SSCs (Nayernia et al., 2006; Nolte et al., 2010), the transplanted testes were checked for regenerated spermatogenesis by H&E staining finally confirming the absence of any sperm production (3.3.4, Fig. 37). This is in contrast to other studies, in which SSC-like cells derived from ESCs could successfully initiate and complete spermatogenesis in germ cell depleted mice upon transplantation providing the final proof of functional *in vitro* generated germ cells (Hayashi et al., 2011). These results clearly suggest that the co-culture-derived SSC-like cells did not represent functional SSCs, because they were not capable to regenerate spermatogenesis. However, using genotyping PCR analyses the presence of transplanted cells could be proven within the testis at least 15 days upon transplantation (3.3.4, Fig. 39), but these results did not allow any conclusion regarding the viability or colonization of transplanted cells within the tubules. A possible colonization of transplanted cells could be checked by immunohistochemical

analyses using markers for undifferentiated spermatogonia. At the moment appropriate analyses are performed using a SALL4 specific antibody to check for colonized spermatogonia within the seminiferous tubules up to 15 days upon transplantation. Up to the time of thesis submission, these analyses were not yet completed, but the so far examined stainings did not reveal any SALL4 positive cells on testis sections. Moreover, an EGFP-specific antibody could be used to check for the homing of transplanted cells within the seminiferous tubules, thereby taking advantage of the reporter construct *Stra8-EGFP* within the ESCs used for co-culture experiments.

Nevertheless, it is worth to note that no teratoma formation was observed upon transplantation using co-culture-derived cells, although mice were partially analyzed two month after transplantation prior a fruitless phase of test mating (3.3.4). Considering the ESC based origin of co-culture-derived cells used for transplantation assays, this fact indicated that the ESCs experienced some kind of development during co-culture. If cells had been still exhibited ESC character, they would have initiated teratoma formation according to their unique differentiation potential. Using comparative transplantation experiments, Brinster and Avarbrock (1994) observed no sign of spermatogenesis, but testicular and/or abdominal tumor formation within 30 days upon injections of ESCs into mouse seminiferous tubules. Transplantation of co-culture-derived SSC-like cells did not regenerate spermatogenesis indicating that these cells were not able to home within the SSC niche in germ cell depleted mice, but they also did not initiate teratoma formation suggesting that ESCs experienced a differentiation process in the co-culture. Here the assumption arose that the co-culture-derived cells resembled germ cell precursor cells, which will be further discussed in the following section.

4.2.5 The co-culture-derived cell population reveals a predominantly primordial germ cell character

In mice, pluripotent cells of the proximal epiblast are the origin of germ cells, which arise from founder cells at embryonic day E6.0-6.5 forming a distinct cell cluster at E7.25 known as primordial germ cells (PGCs). These PGCs further migrate to the developing hindgut endoderm, reach the mesentery at E9.5 and colonize at the genital ridges of the developing gonads at E11.5, where the sexually bipotential cells experience sex-determination and enter either the spermatogenic or oogenic pathway for production of gametes (Fig. 72; Saitou and Yamaji, 2012).

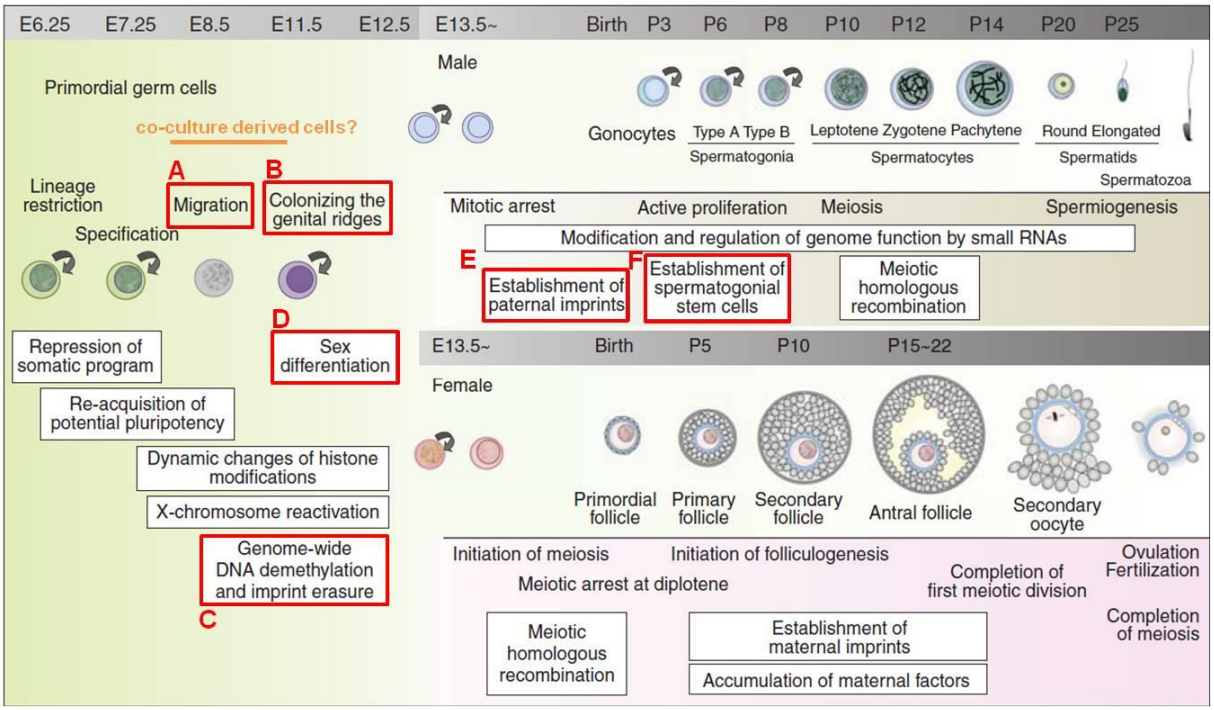


Fig. 72: Scheme of germ cell development in mice. The major events during male germ cell development are highlighted in red. The primordial germ cells (PGCs) represent the precursors of male and female germ cells. They start migrating at around embryonic day E7.5 from the proximal epiblast **(A)** and colonize at E11.5 in the genital ridges **(B)**. After an erasure of their genomic imprints **(C)**, PGCs experience a sex-determination **(D)**. Germ cells with a male cell fate regain paternally imprints during their differentiation into gonocytes **(E)**. E: embryonic day; P: postnatal day. Modified, Saitou and Yamaji, 2012.

During their migration and proliferation in the gonad the PGCs undergo a comprehensive epigenetic reprogramming including an erasure of somatic genomic imprinting by DNA demethylation (Yamahazi et al., 2003). Imprinting represents a monoallelic expression status of a subset of genes and is essential for a normal embryonic development. The parental-specific allele expression is defined by epigenetic modifications such as DNA methylation in the differentially methylated region (DMR) of the imprinting control region (ICR) of the gene. The gene transcription is influenced by nucleotide sequences enriched with 5-methylcytosines at CpG dinucleotides termed as CpG islands, which are transferred by DNA methyltransferases. Mostly the repressed allele is methylated, while the active allele is unmethylated at its DMRs (Bartolomei et al., 1993; Ferguson-Smith et al., 1993; Stöger et al., 1993; Bird et al., 2002). Somatic DMRs acquire their methylation status after fertilization, while germline DMRs gain this status during gametogenesis. During further germline development the demethylated PGCs undergo a sex-specific *de novo* DNA methylation of ICRs of maternally and paternally inherited chromosomes, which is triggered by the *de novo* methyltransferase DNMT3A and its stimulatory protein DNMT3L (Bourc'his et al., 2001; Kaneda et al., 2004; Jia et al., 2007). This establishment of parental-specific imprints occurs during late fetal development in the male and in early postnatal phase of females (Hajkova et

al., 2002, 2008; Lucifero et al., 2004). Around E12.5-13.5 male PGCs exhibit the onset of resetting of the paternal imprinting marks, which is completed at the level of gonocytes. Consequently, functional SSCs reveal a hypermethylated status of paternally imprinted genes such as *H19* and *Meg3*, while maternally imprinted genes such as *Snrpn* and *Igf2r* exhibit a hypomethylated pattern.

An analysis of the methylation status of imprinted genes using bisulfite pyrosequencing did not reveal such an androgenic and SSC characteristic methylation pattern in co-culture-derived cells (3.3.3.3). Moreover, these cells did not show significant differences in their methylation levels compared to the starting ESCs, while the latter one conformed approximately to already described methylation patterns of undifferentiated male ESCs (Zechner et al., 2009). ESCs have been shown to reveal methylation levels comparable to somatic cells (Geijsen et al., 2004; Zechner et al., 2009). Furthermore, Hajkova and colleagues (2002) confirmed a somatic methylation pattern for PGCs during their migration phase. Considering these findings, the results of the methylation studies suggest a PGC status of co-culture-derived cells before the onset of epigenetic reprogramming and the beginning of imprinting erasure. This assumption can also be linked to the already described positive immunostaining results (4.2.4, Tab. 10): OCT4 was shown to be expressed in PGC and is necessary for their survival (Schöler et al., 1989; Kehler et al., 2004). Additionally SALL4, VASA and α6-INTEGRIN are also expressed in PGCs (Toyooka et al., 2000; Durcova-Hills et al., 2008; Morita-Fujimura et al., 2009; Eildermann et al., 2012). Moreover, the PGC character of co-culture-derived cells was substantiated by significantly increased expression of *Fkbp6* and *Mov10l1* (3.3.3.2, Fig. 34), which are supposed to be “the long-missing tool for the *in vitro* derivation of functional gametes from pluripotent stem cells“ (Sabour et al., 2011). Using gene expression microarrays with male and female PGCs of E11.5 to E18.5, the group of Prof. Schöler identified these genes among 11 germ cell-related genes to be highly expressed in PGCs, while ESCs revealed a low expression level. In contrast to other markers these germ cell-associated genes allow a discrimination between germ cells and pluripotent cells. The low or absent expression in somatic cells indicated a role of these genes in reproduction and fertility. Nevertheless, it has to be noted that the authors also detected high expression levels of these genes in mouse spermatogonial cells (Sabour et al., 2011).

Based on these data it is hypothesized that the cells, derived from the co-culture of mouse ESCs with HTF cells, represent a mixed cell population with a predominantly PGC character and presumably a small fraction of undifferentiated and differentiating spermatogonia. A final characterization of the co-culture-derived cells could be done by a comparative single cell transcriptome analyses in order to assess the gene regulating network, which determines the phenotype, physiology and behaviour of cells (Tang et al., 2011). For a definitive cell

classification, the co-culture-derived cells should be compared to naturally PGCs and SSCs, the co-culture starting ESC cell line, wild type ESCs as well as somatic cells.

However, the assumption that undifferentiated spermatogonia represented a relatively small proportion within the co-culture-derived cell population offers a possible explanation for the unsuccessful transplantation assays. According to Nagano (2003b) germ cell transplantation is anyway accompanied by a major loss of transplanted SSCs during their homing process, which requires their attachment to the recipient's sertoli cells, the migration to their final position at the basement membrane of the seminiferous tubules and their local survival. The migrating process takes approximately seven days and implies a loss of 75% of transplanted SSCs within the first day and finally just 12% SSCs reach the basement membrane and initiate self-renewal. The author mentioned inefficient adhesion of transplanted cells to sertoli cells, their low survival rate during migration and an eventually inappropriate SSC niche as possible reasons for this SSC degradation upon transplantation (Nagano, 2003b). Considering this additional tremendous loss of cells after transplantation, it is assumed that the possibility was very low that the minor fraction of undifferentiated spermatogonia in the co-culture-derived cells survived in an appropriate amount in order to repopulate the seminiferous tubules.

4.2.6 What are the driving forces in the co-culture system?

In the presented thesis the co-culture of mouse ESCs and HTF cells turned out to be a robust and reproducible system for the derivation of a cell population revealing a primordial- and spermatogonial cell character, what might be attributed to different factors.

Certain growth factors such as GDNF, bFGF and CSF-1 were shown to be key regulators for the self-renewal and proliferation of SSCs and progenitor spermatogonia. Among the essential factors within the SSC niche GDNF is the most considered cytokine, which was recently shown to be constitutively released by *in vitro* cultured human peritubular cells (Spinnler et al., 2010). Based on the predominantly peritubular cell character of the HTF cells (4.2.2), it is hypothesized that GDNF is one crucial cue within the here presented co-culture system. GDNF is a member of the TGF-ß (Transforming Growth Factor-β) superfamily binding to receptor complexes consisting of GFRα1 (GDNF Familiy Receptor α1) and c-Ret proto-oncogene, a transmembrane tyrosine kinase receptor (Sariola and Saarma, 2003; Buageaw et al., 2005). The receptor is expressed in undifferentiated A_s, A_{pr} and A_{al} spermatogonia of mice (Hofmann, 2008; Grisanti et al., 2009), but also in SSCs from primates and humans (Maki et al., 2009; Wu et al., 2009) and indicates that GDNF signalling is of great importance for the preservation of SSCs in various mammalian species. Upon GDNF binding, SSCs respond by the activation of different pathways including phosphatidylinositol 3-kinase/serine-threonine kinase AKT family (PI3K/AKT), Src family

kinase (SFK) and Ras/Erk 1/2 signaling mechanisms (Braydich-Stolle et al., 2007; Oatley et al., 2007; He et al., 2008; Phillips et al., 2010). Oatley and co-authors (2007) suggested that PI3K/Akt is involved in SSC survival, while genes for SSC self-renewal are regulated by SFK signalling. Using microarray analyses in GDNF deficient cultures of self-renewing mouse SSCs, six target genes of the GDNF signalling pathways could be identified including the genes *Bcl6b* (B Cell CLL/Lymphoma 6 Member B), *Egr2* (Early Growth Response 2), *Egr3* (Early Growth Response 3), *Etv5* (Ets Variant 5), *Lhx1* (LIM Homeobox 1) and *Tspan8* (Testraspanin 8) (Oatley et al., 2006). Up to now the reduction or even loss of the transcriptional repressor BCL6b could be linked to an impaired self-renewal potential of SSCs and spermatogenesis using mouse *in vitro* and *in vivo* experiments, respectively (Oatley et al., 2006). Considering that several "*in vivo* and *in vitro* studies clearly indicate that GDNF is a key player responsible for maintaining SSCs by regulating their self-renewal" (Meng et al., 2000; Naughton et al., 2006; Oatley and Brinster, 2008; Spinnler, 2010), it is not unreasonable to conclude a major role of GDNF presumably secreted by the HTF cells in the here introduced co-culture system. The required preconditions for a functional GDNF signalling in the co-culture are fulfilled: (1) GDNF is known to be constitutively released by *in vitro* HTPCs (Spinnler et al., 2010) and is presumably secreted by the HTF cells representing predominantly human peritubular cells in the co-culture, (2) GDNF can have an effect on mouse ESCs (Reyes et al., 2008) and (3) mature GDNF is highly evolutionally conserved indicating that human GDNF can mediate GDNF signalling in mouse cells (Grimm et al., 1998; Gao et al., 2011).

Further studies showed that *in vitro* culturing of SSCs can be greatly enhanced by the addition of the growth factors bFGF and CSF-1, but in contrast to GDNF a supplementation of culture medium with just one of these both factors was not sufficient to achieve a comparable support of SSC maintenance in culture and consequently these factors are thought to act in combination with GDNF (Kubota et al., 2004b; Oatley et al., 2009). However, occurrence of bFGF in the co-culture system is likely unreasonable, because up to now its production is attributed to sertoli cells (Mullaney and Skinner, 1992), which are very likely not present in the HTF cells used in co-culture experiments (4.2.2). In contrast to this, an influence of CSF-1, which was shown to be localized in mouse leydig (Ryan et al., 2001) and peritubular cells (Shima et al., 2004), is possible by supporting the maintenance of the SSC phenotype *in vitro* (Oatley et al., 2009). An impact of CSF-1 on co-culture requires the production of this growth factor by the HTF cells, but to date a CSF-1 secretion could not be confirmed yet for human peritubular cells, so that a possible impact of CSF-1 in the co-culture system can just be assumed.

Last year the group of Prof. Mayerhofer published their interesting results of a secretome analysis using their established *in vitro* HTPC culture (4.2.2; Albrecht et al., 2006;

Flenkenthaler et al., 2014), in which they identified various secreted proteins contributing to the skeletal system and vasculature development, inflammatory network and SSC fate. Interestingly, they found the TGF-β signalling pathway is significantly enriched in the analyzed secretome and hypothesized that HTPCs play an important role "in the development and maintenance of testicular functions by secreting several proteins that act through the multistage TGF-β signal transduction pathway in close interplay with surrounding Sertoli and Leydig cells" (Flenkenthaler et al., 2014). Although the bone morphogenetic protein 4 (BMP4) was not found in this published secretome analysis, it is worth to have short look at this ligand of the TGF-β family, which is known to be crucial for the transcriptional regulation of PGC specification (Lawson et al., 1999) and differentiation of spermatogonia (Pellegrini et al., 2003). These mouse studies revealed the expression of BMP4 receptors in proliferating and migrating PGCs as well as postnatal dividing spermatogonia suggesting an important role of BMP4 signalling in the production of gametes throughout male life. The PGC fate concerning their formation, survival and differentiation is determined by BMP4 downstream targets such as Smad1 and Smad5 (Okamura et al., 2005). In mice, BMP4 is produced by sertoli cells (Pellegrini et al., 2003). Considering the assumption that the co-culture-derived cell population revealed a predominantly PGC character containing a small fraction of undifferentiated and differentiating spermatogonia (4.2.5), BMP4 can hypothetically act as a PGC and spermatogonia inducing factor in the co-culture system. This hypothesis presumes a secretion of BMP4 by human peritubular cells, and then also by HTF cells. Although it should be recalled that BMP4 was not found in the secretome analysis by Flenkenthaler and colleagues (2014), this concern is slightly relativized by the fact that the group also did not mention any GDNF supply in the analyzed secretome, although HTPCs have shown to be a constitutively producer of this growth factor also belonging to the TGF-β family (Spinnler et al., 2010). A potential effect of GDNF on PGCs within the co-culture-derived cell population cannot be discussed as no literature is currently available, which document any GDNF-specific receptors on the surface of PGCs.

The HTF cells themselves seem to be a considerable factor within the co-culture system and are necessary for both the ESC attachment at the start and for the supply of essential factors during the co-culture. The so called SSC-like cells could not be generated by using gelatine or MEF feeder coated culture vessels without or in combination with conditioned culture medium derived from the culture of pure HTF cells providing secreted factors (3.3.6). This indicates that the derivation of SSC-like cells did not need any cell line for cell attachment as well as not just the HTF released factors within the conditioned medium, but was dependent on the presence of HTF cells as well as its secreted factors. Attempts to perform equivalent co-cultures using mouse ESCs with mouse testicular feeder (MTF) cells were unsuccessful and failed presumably because of the non-proliferating cells derived from testicular cell

suspensions. MTF cells could not be propagated beyond passage number two. The rapidly decreasing proliferation of primary rodent testicular cells is a well known issue (Mäkelä et al., 2014). In contrast to HTF cells, the MTF cells did not derive from MACSorting using mouse testis tissue. However, it can be assumed that these cells would not be more proliferative.

The long-term culture of co-culture-derived SSC-like cells seemed to be limited by the slowly decreasing proliferation of the HTF cells during proceeding co-culture (4.2.3), which was accompanied by a reduced occurrence of SSC-like cells. This could be attributed to the less supply of cells for cell attachment as well as of secreted nutrients and factors. Considering that HTF cells represented primary cell lines, the decline in proliferation was not surprising. This limiting factor at advanced co-culture might be overcome by seeding co-culture-derived cells on HTF cells from lower passages. It is assumed that the usage of primary testicular cells resembles mostly the *in vivo* condition thereby providing natural secreted factors. Considering that the *in vivo* SSC niche is not a rigid environment, but rather a dynamical and flexible system providing thereby an accurately controlled growth factor milieu (1.4; 4.2.1), it could be advantageous to avoid any external influence on this more or less natural environment in the co-culture provided by predominantly peritubular cells of the HTF.

4.3 Future perspectives

Pluripotent stem cells and their unique potential to differentiate into any cell type including germ cells determine the research field of reproductive biology aiming at the establishment of reproducible and reliable protocols for the *in vitro* derivation of male and female germ cells. But why are so many researchers interested in the *in vitro* generation of germ cells? The availability of such protocols will offer the general opportunity to study the process of gametogenesis including meiosis and to use this knowledge for the development of approaches to treat infertility. Moreover, *in vitro* spermatogenesis experiments can provide some insight into the intracellular mechanisms of SSCs, their self-renewal, survival and differentiation into haploid male germ cells, which might further contribute to a better understanding of underlying self-renewal mechanisms of other stem cell populations and general stem cell biology.

The reproducible and robust co-culture system, established in the presented thesis, might provide a good model for basic research in regard to the mechanisms regulating PGCs, SSCs and their entry into the differentiation process as well as the capability of pluripotent cells to generate germ cells *in vitro*. Because it is assumed that the co-culture-derived cells represent a mixed population of PGCs, undifferentiated and differentiating spermatogonia, a transcriptome analysis might provide some insight into the regulatory network of germ cells. The unlimited access to these cell types derived from the co-culture system allows the

investigation of molecular cell processes in more detail. Even if this system is characterized by its high reproducibility, there are potential options to further optimize its efficiency. Because the HTF cells presumably possess a significant role in this system, the preservation of its sufficient proliferation rate during co-culture would be surely beneficial for a prolonged maintenance of the co-culture-derived cells in culture. This could be mediated by a selective inhibition of GPER, which is linked to the proliferation of HTPCs and their apoptosis (Sandner et al., 2014). A prolonged culture might be accompanied by an increased differentiation of mouse SSCs, whose fraction within the co-culture-derived cell population is supposed to be rather small. This differentiation could be further supported by the use of a modified culture medium. The factors bFGF and CSF-1 are described as important regulators for the *in vitro* culture of SSCs acting in combination with GDNF (Kubota et al., 2004b; Oatley et al., 2009), which is assumed to be the driving force in the presented co-culture system. The addition of bFGF and CSF-1 to the culture medium might support the derivation and maintenance of SSCs from ESCs.

The transplantation assay remains the final proof of the existence of SSCs derived from co-culture experiments. The transplantations carried out within the presented thesis did not result in repopulation of the seminiferous tubules, but genotyping PCR analyses for the reporter construct *Stra8-EGFP* contained in the ESCs used for co-culture experiments indicated the presence of transplanted cells within the testis at least 15 days post transplantation. An EGFP-antibody staining on testis sections could be used in order to validate a colonization of transplanted cells in the seminiferous tubules at least in the indicated time frame. However, considering the suggested minor fraction of undifferentiated spermatogonia within the co-culture-derived cells as well as the major loss of cells during germ cell transplantation and the homing process of SSCs (Nagano, 2003b), it would be worth to test further a more accurate transplantation technique in order to ensure a better and increased cell delivery and thereby cell number to colonize in the testis. This could eventually lead to regeneration of spermatogenesis in at least some seminiferous tubules and would represent the functional proof of co-culture-derived SSCs.

Knowledge of important intracellular signalling pathways of SSCs obtained by such *in vitro* culture systems will further help to develop culture systems, which are suitable for the *in vitro* culture, promotion of self-renewal and rapid expansion of isolated SSCs. Although the cultivation of SSCs derived from various species has been already successfully performed, the absence of a reliable protocol for cultivation and expansion of human SSCs illustrates the difficulty to transfer this knowledge to human, which was also present in this thesis. Here, the researchers are faced with several problems including the lack of specific markers for human SSCs, the limited availability of human testis material for SSC isolation and study-to-study variations in biopsy quantity and quality. Considering that only the first mentioned difficulty

might be solved in the next years, the establishment of a human SSC long term culture system will remain challenging. Consequently, its intended use to treat male infertility using cultured human SSCs for repopulation of the patient's testis or for their *in vitro* differentiation into male gametes for assisted reproduction treatments (ART) will require tremendous load of experimental work to evaluate proper culture conditions. However, using the MACSorting approach, cultivation of isolated human SSCs on the flow-through fraction derived from the sorting procedure representing predominantly peritubular cells might support their maintenance and proliferation in culture. As observed in the presented thesis, isolated human SSCs preferred growing on their natural testicular feeder developed during culture, which might provide appropriate natural factors and be more beneficial compared to the use of medium supplemented with various growth factors. The separation of somatic and germ cells displays a contrary approach for the establishment of human SSC cultures. As recently shown for the isolation and cultivation of putative marmoset SSCs, this separation might be crucial for the establishment of SSC cultures (Langenstroth et al., 2014). Considering the similarities of marmosets' and humans' testis, this approach might be also promising.

The prospective successful application of human SSCs for treatment of male infertility is additionally faced with the finding in mouse studies that SSCs under *in vitro* culture conditions fail to undergo proper epigenetic reprogramming indicated by abnormalities in their genomic imprinting, which can result in abnormal offspring development. Male germ cells could be also derived from different pluripotent cells, but normal and healthy offspring revealing proper methylation patterns of their imprinted genes could just be obtained when the process of gametogenesis was resumed *in vivo*. Consequently, beside the establishment of stable human SSC cultures, the setting of a proper epigenetic reprogramming under *ex vivo* conditions reflects an additional major challenge on the way to their application in reproductive medicine. Generally, it is highly questionable, if the complex process of epigenetic reprogramming can be completed properly under *in vitro* conditions. The attempt for an improvement of this process during mouse *in vitro* spermatogenesis presented in this thesis illustrated that the methylation pattern can indeed be influenced by the overexpression of a putative imprinting control gene such as *Stra8*. Here a temporary instead of a stable overexpression of STRA8 using a doxycycline-inducible protein expression system should be tested next in order to improve epigenetic reprogramming *in vitro*. Further the identification of imprinting control genes and their regulation might help to improve the establishment of correct imprinting marks during *in vitro* spermatogenesis, which is a mandatory precondition for the application of *in vitro* derived gametes in ART.

The use of iPSCs for the *in vitro* generation of germ cells offers infertile men the opportunity to father biological children independent from the underlying cause of their infertility and circumvent simultaneously the ethical and immunological limitations regarding the use of

ESCs. A potential application of human iPSCs in reproductive medicine requires iPSC generation methods without any persisting risk of cancer development. These non-integrative reprogramming methods are often accompanied by reduced efficiencies of human iPSC generation. Currently, the mRNA-based reprogramming, which was utilized in the presented thesis, represents the most promising method. Nevertheless, also their application in treatment of infertility and in general regenerative approaches is faced with problems considering tumorigenicity and epigenetic defects. For these reasons the development of reliable pre-transplant screenings are indispensable for the application of human iPSCs in ART. However, it is worth to look at the differentiation potential of the iSHF cell lines generated in the presented thesis and their ability to differentiate into germ cells using either a medium or a two step selection-based strategy. Using media-based differentiation approaches, cultivation of iSHF cells in complex culture media adopted from Eguizabal et al. (2011) and Easley et al. (2012) might lead to their progression through meiosis and gamete formation. The two step selection system based on premeiotic and postmeiotic transgenes for mouse *in vitro* spermatogenesis according to Nayernia et al. (2006) could be also applied to hiPSCs. Considering a putative limited transfection efficiency of hiPSCs with the reporter constructs, the chances of success of this differentiation protocol could be maybe reduced. Provided a successful application of one of these approaches with iSHF cells derived from infertile men, it is interesting to look, if the underlying cause of patient's infertility can be overcome during the *in vitro* differentiation process.

Moreover, the co-culture system established in the presented thesis could be applied for the differentiation of human germ cells. As the suitability of the co-culture system with different mouse pluripotent stem cells could be shown in the presented thesis, it is worth to test if co-culturing of iSHF cells with HTF cells in normal fibroblast medium could lead to similar morphology changes resembling SSCs. Here the low proliferation rate of hiPSCs has to be considered as a potential limiting factor for the success of this approach.

In summary, the recapitulation of spermatogenesis *in vitro* and its application in ART is a promising method to treat male infertility, but is still faced with major problems. Some problems might be solved by intensive basic research. Considering the huge amount of data for long term culture of SSCs derived from various species, sooner or later a protocol for the long term culture of human SSCs will surely be available. In contrast, it is highly questionable if problems concerning the risk of tumorigenicity of pluripotent cells and the improper epigenetic reprogramming during *in vitro* spermatogenesis can be completely solved to ensure a riskless application in men.

5 Summary

Currently different *in vitro* experimental approaches make use of the unique potential of pluripotent cells in order to produce functional sperm aiming at the establishment of new treatment opportunities for male infertility.

The establishment of proliferating human spermatogonial stem cell (SSC) lines and their *in vitro* differentiation into haploid male germ cells represents one of these approaches and was tried to succeed in the presented thesis. The isolation of human SSCs from testicular biopsies from infertile men was performed using the Magnetic Activated Cell Sorting (MACSorting) technique for the SSC surface marker α6-Integrin and succeeded several times in the enrichment of putative SSCs, which were characterized by their grape-like structures characteristically for SSCs and a positive staining for the germ cell marker PLZF. In a total of 15 attempts, culturing of isolated human SSCs was tried to be improved by testing different media compositions, seeding on MEF, gelatine or matrigel coated culture vessels as wells as culturing under normoxia or hypoxia. The cultivation in StemPro+3 medium on gelatine coated vessels under normoxia turned out to provide appropriate culture conditions for the enrichment of human SSCs after MACSorting, but unfortunately a stable and proliferating human SSC line could not be established for further *in vitro* differentiation experiments.

Because other studies also revealed great difficulties in the establishment of long term human SSC cultures, a further experimental approach aimed at using human pluripotent stem cells for *in vitro* differentiation of human SSCs. For the derivation of patient-derived human induced pluripotent stem cell (hiPSC) lines, 25 scrotal human fibroblast (SHF) cell lines derived from infertile men with different spermatogenesis phenotypes were collected in a cell bank. For the generation of hiPSCs from moderate proliferating human fibroblasts a modified so called “Feeder/Free” mRNA/miRNA reprogramming was successfully developed and finally yielded in the derivation and establishment of three iSHF cell lines iSHF-3, iSHF-6 and iSHF-13 derived from infertile men revealing different spermatogenesis phenotypes. These iSHFs were characterized by common tools to proof their pluripotency. iSHFs were positively stained for the Alkaline Phosphatase and at least for two pluripotency markers OCT4 and SSEA-4, revealed no karyotypic abnormalities and are now suitable for the start of *in vitro* differentiation experiments.

Various mouse studies proved the derivation of haploid male germ cells from pluripotent stem cells, but revealed especially improper genomic imprinting often yielding in unhealthy offspring. Here, the *in vitro* differentiation strategy, which was established in this Institute (Nayernia et al., 2006), should be improved by the overexpression of the premeiotic gene *Stra8* (Stimulated by Retinoic Acid Gene 8). These experiments were planned to be carried

out with the original cell line SSC 12 representing an embryonic stem cell (ESC) line derived from transfection of the wild type ES cell line ES-RI with a *Stra8-EGFP* reporter construct followed by an enrichment of *Stra8* overexpressing cells by two Fluorescence-Activated Cell Sortings (FACSorting) (Nayernia et al., 2006) as well as with the ESC line Stra8-EGFP/Sycp3-DsRed-2. The latter one was newly generated from double transgenic Stra8-EGFP/Sycp3-DsRed mice in the presented thesis and was shown to reveal a typical ES cell morphology, a male genotype and an OCT4 expression on protein level. An enrichment of *Stra8* expressing cells was also performed by twice FACSortings. Two *Stra8* overexpressing cell lines SSC 12/11 and SSC 12/13 were generated by stable transfection of SSC 12 with the generated *Stra8*-overexpressing construct phEF1α-puro-mStra8 and possessed a two times increased stable *Stra8* expression compared to SSC 12. Finally, *in vitro* spermatogenesis experiments using SSC 12/11 and SSC 12/13 cells indicated an earlier entry into and progress through the meiotic process analyzed on mRNA level of the postmeiotic marker *Gpx4*, whereas no meiotic or postmeiotic structures could be detected on the protein level using immunostaining analyses and electron microscopy. *Stra8* overexpressing cells revealed an overall increased methylation level of imprinting genes indicating that *Stra8* indeed has an effect on epigenetic reprogramming. Paternally imprinted *H19* and *Meg3* genes tend to an androgenic imprinting pattern, while the maternally imprinted *Igf2r* and *Srnpn* genes failed to undergo the essential demethylation in differentiating *Stra8* overexpressing cells.

A further part of the presented thesis focused on the development of a reproducible and robust co-culture system for the derivation of mouse SSCs. For this purpose the transgenic mouse ES cell line SSC 12 was co-cultured with mitotically active human testicular feeder (HTF) cells in normal fibroblast culture medium without any growth factors. HTF cells were characterized for testicular somatic cells on mRNA and partially on protein level, and did express neither the sertoli cell markers *FSHR* and *INHIBIN* nor leydig cell markers such as *3-β-HSD* and *LHCGR*. Because they revealed a moderate to high expression of peritubular cell markers *AR*, *AT1R*, *CALPONIN*, *DECORIN*, *GPER*, *PENTRAXIN 3*, *SMA* and *VIMENTIN*, HTF cells possessed a predominant human peritubular cell character. The co-culture-derived cells were called putative mouse SSCs because of their grape-like cell morphology, which is characteristic for SSCs according to the literature. The appearance of these structures was very efficient as well as robustly reproducible and could also be generated using mouse wild type ES-RI cells and wild type iPSCs suggesting a general suitability of the co-culture system for the derivation of putative SSCs. The generation of SSC-like cells failed, if MEF feeder layer, gelatine coated vessels or HTF-conditioned medium was used for co-culture experiments indicating a key role of the HTF cells.

The viability and proliferation of putative mouse SSCs was proven by apoptosis and BrdU assays. The SSC-like cells were positively stained for Alkaline Phosphatase and the germ cell markers α6-INTEGRIN, OCT4, PLZF, DDX4 (aliases: VASA) and SALL4. Moreover, putative mSSCs revealed a moderate expression of the later germ cell marker *Gpr125* and an increased expression of the primordial germ cell (PGC) marker *Fkbp6* and *Mov10l1* on mRNA level. The induction of differentiation of co-culture-derived cells revealed partly an up-regulation of postmeiotic markers *Gpx4* and *Acrosin* indicating the onset of the meiotic process detectable on the mRNA level. The analyzed imprinted genes *H19*, *Meg3*, *Igf2r* and *Snrpn* revealed rather a somatic than androgenic methylation pattern, which was not significantly different from the starting ES cell line indicating that the imprint erasure had not yet occurred. Transplantations of cells into germ cell depleted testes of busulfan treated mice did not result in a repopulation of the seminiferous tubules, but the detection of *Stra8-EGFP*-PCR constructs, contained in SSC 12 used for co-culture experiments, indicated the presence of transplanted cells for at least 15 days within the testis post transplantation. These whole data lead to the hypothesis that the co-culture of mouse ESCs with HTF cells yielded in a mixed cell population with a predominantly PGC character and presumably a small fraction of undifferentiated and differentiating spermatogonia.

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Annotations

- Parts of this PhD thesis are summarized in a manuscript, which is submitted in *Molecular Human Reproduction* (IF= 3.483).
  "Efficient derivation of male germ cells from mouse embryonic stem cells by co-culture with human testicular cells"
  Authors: **Mellies** N., Manzelat F.R., Fitzner A., Neuhaus N., Welter H., Mayerhofer A., Dressel R., Engel W., Zechner U., Nolte J.

- Parts of this PhD thesis were presented at the following meetings:
  - 3rd Workshop of the Research Unit 'Germ Cell Potential' FOR 1041, December 04-05, 2013, Münster:
    "Efficient generation of SSCs from mouse ESCs through co-culture with human testicular stromal cells" (talk)

  - 5th DVR-Kongress, December 04-07, 2013,Münster:
    "Efficient generation of SSCs from mouse ESCs through co-culture with human testicular stromal cells" (poster presentation)

- Contribution to the work within the frame of SPP1356 "Pluripotency and Cellular Reprogramming":
  - Lührig S., Kolb S., **Mellies** N., Nolte J. (2013): The novel BTB-kelch protein, KBTBD8, is located in the Golgi apparatus and translocates to the spindle apparatus during mitosis. *Cell Div*. 8 (1): 3.

  - Nolte N., Engel W., Zechner U.: "1st Funding period: Defining the pluripotency of Spermatogonial stem cells from adult testis; 2nd Funding period: Characterization of the putative pluripotency-regulating genes Lrrc34 and Zfp819", poster presentation at the International Symposium Reprogrammed Stem Cells, April 22-23, 2014, Berlin

  - Lührig S., Siamishi I., **Mellies** N., Wolf M., Zechner U., Engel W., Nolte J.: „Lrrc34, a novel nucleolar protein, is involved in ribosome biogenesis of pluripotent stem cells", poster presentation at the 7th International Meeting of the Stem Cell Network NRW, April 23-24, 2013, Cologne

- Lührig S., Kolb S., Adamczyk J., **Mellies** N., Nolte J.: “The novel BTB-kelch protein, KBTBD8, is ubiquitously expressed but specifically localized in the Cis-Golgi-Apparatus”, poster presentation at the European Human Genetics Conference 2012, June 23-26, 2012, Nuremberg

8 Acknowledgments

I want to express my deep gratitude to Prof. Dr. Sigrid Hoyer-Fender for being my referee and her valuable input during our meetings. I am very grateful for her readiness to take over the task of the first referee in the last year of my PhD study.

I sincerely thank Prof. Dr. Peter Burfeind for being my second referee and his instantaneously readiness to take over this task in the last year. I also thank him for the support and helpful suggestions during writing my thesis.

I would like to thank Prof. Dr. med. Dr. h.c. Wolfgang Engel for his guidance and scientific suggestions during the first three years of my PhD study.

Moreover, I would like to thank Prof. Dr. Rüdiger Behr, PD Dr. Michael Hoppert, Prof. Dr. Michael Kessel and Prof. Dr. Ulrich Zechner, who kindly agreed to evaluate my dissertation and participate in the examination.

I thank also our collaboration partners for their supportive studies as well as the Deutsche Forschungsgemeinschaft (DFG) for financing my PhD study.

I would like to express my deep gratitude to my supervisor Dr. Jessica Nolte-Kaitschick for the opportunity to compile my PhD study in her working group. Thank you for your guidance, support and friendly relationship during the entire process of my PhD project.
It was an exciting time for both of us!

I would like to thank my colleagues Priya Raju, Johanna Mänz, Luisa Freese, Manar Elkenani, Eva Stetter, Joanna Jakubiczka-Smorag and Dr. Lukasz Smorag as well as my former colleagues Sandra Lührig, Susanne Kolb and Diana Riethmüller. I thank you and all other members of the Institute of Human Genetics for their advice and support as well as the friendly working atmosphere.

Herewith, I express my heartfelt gratitude to my parents as well as to my sister and her family, who always believe in me. You supported me unconditionally with your encouragement and patience throughout my PhD time. Thanks for you trust and love! Together we completed this part of my live journey and therefore, I dedicate this thesis to you!