TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 1020

MEDICA - ODONTOLOGICA

IDENTIFICATION AND CHARACTERIZATION OF NEW GENES AND PATHWAYS REGULATING SPERMATOGONIAL CELL FATE

by

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TURUN YLIOPISTO
UNIVERSITY OF TURKU
Turku 2012

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ISBN 978-951-29-5049-2 (PRINT)
ISBN 978-951-29-5050-8 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2012

To my family

ABSTRACT

Juho-Antti Mäkelä

Identification and characterization of new genes and pathways regulating spermatogonial cell fate

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Spermatogenesis, i.e sperm production in the seminiferous tubules of the testis, is a complex process that takes over one month to complete. Life-long ability of sperm production ultimately lies in a small population of undifferentiated cells, called spermatogonial stem cells (SSCs). These cells give rise to differentiating spermatogonia, which are committed to mature into spermatozoa. SSCs represent a heterogeneous population of cells and many aspects of their basic biology are still unknown. Understanding the mechanisms behind the cell fate decision of these cells is important to gain more insights into the causes of infertility and testis cancer. In addition, an interesting new aspect is the use of testis-derived stem cells in regenerative medicine.

Our data demonstrated that adult mouse testis houses a population of Nanog-expressing spermatogonia. Based on mRNA and protein analysis these cells are enriched in stage XII of the mouse seminiferous epithelial cycle. The cells derived from this stage have the highest capacity to give rise to ES cell-like cells which express Oct4 and Nanog. These cells are under tight non-GDNF regulation but their fate can be dictated by activating p21 signalling. Comparative studies suggested that these cells are regulated like ES cells. Taken together these data imply that pluripotent cells are present in the adult mammalian testis.

CIP2A (cancerous inhibitor of PP2A) has been associated with tumour aggressiveness and poor prognosis. In the testis it is expressed by the descendants of stem cells, i.e. the spermatogonial progenitor cells. Our data suggest that CIP2A acts upstream of PLZF and is needed for quantitatively normal spermatogenesis. Classification of CIP2A as a cancer/testis gene makes it an attractive target for cancer therapy. Study on the CIP2A deficient mouse model demonstrates that systemic inhibition of CIP2A does not severely interfere with growth and development or tissue or organ function, except for the spermatogenic output. These data demonstrate that CIP2A is required for quantitatively normal spermatogenesis.

Hedgehog (Hh) signalling is involved in the development and maintenance of many different tissues and organs. According to our data, Hh signalling is active at many different levels during rat spermatogenesis: in spermatogonia, spermatocytes and late elongating spermatids. Localization of Suppressor of Fused (SuFu), the negative regulator of the pathway, specifically in early elongating spermatids suggests that Hh signalling needs to be shut down in these cells. Introduction of Hh signalling inhibitor resulted in an increase in germ cell apoptosis. Follicle-stimulating hormone (FSH) and inhibition of receptor tyrosine kinases resulted in down-regulation of Hh signalling. These data show that Hh signalling is under endocrine and paracrine control and it promotes germ cell survival.

Key words: testis, spermatogenesis, Hedgehog, Nanog, CIP2A, follicle-stimulating hormone, seminiferous tubule, stem cell

TIIVISTELMÄ

Juho-Antti Mäkelä

Spermatogonioita säätelevien uusien geenien ja solusignalointireittien tunnistaminen ja kuvaaminen

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Siittiöiden tuotanto eli spermatogeneesi tapahtuu kiveksen siementiehyissä. Se on monimutkainen, yli kuukauden kestävä tapahtumasarja. Elinikäinen siittiöntuotantokyky riippuu pienestä populaatiosta erilaistumattomia soluja, joita kutsutaan spermatogoniaalisiksi kantasoluiksi (SSC). Näistä soluista kehittyy erilaistuvia spermatogoniota, jotka edelleen kehittyvät siittiöiksi. SSC:t ovat heterogeeninen solupopulaatio. Tämän solujoukon perusbiologian ymmärtäminen olisi tärkeää miehen hedelmättömyyden ja kivessyövän synnyn sekä regeneratiivisen lääketieteen kannalta.

Tulostemme mukaan aikuisen hiiren kiveksessä on pieni kantasoluille tyypillistä Nanog-proteiinia ilmentävä spermatogoniopopulaatio, joka lähetti-RNA- ja proteiinimittausten perusteella on rikastunut vaiheeseen XII hiiren siemenepiteelin kehityksen aikana. Näiden solujen lukumäärä on tarkkaan säädelty. GDNF ei osallistu näiden solujen säätelyyn, mutta p21-signalointireitin aktivaatio johtaa niiden erilaistumiseen. Havaintomme tukevat käsitystä, että näitä soluja säädellään alkion kantasolujen (ES) tavoin, ja että aikuisen hiiren kiveksessä on monikykyisiä soluja.

CIP2A-proteiinia (cancerous inhibitor of PP2A) esiintyy monissa aggressiivisissa kasvaimissa, ja sen on havaittu olevan yhteydessä potilaan huonoon ennusteeseen. CIP2A:ta tavataan korkeina pitoisuuksina myös kiveksessä. Tästä syystä *CIP2A* luetaan syöpä/kives-geenien joukkoon. Tulostemme mukaan CIP2A on tärkeä määrällisesti normaalille spermatogeneesille. Se vaikuttaa spermatogoniaalisissa progenitor-soluissa, jotka ovat syntyneet SSC:ista. CIP2A:n puuttuminen ei vaikuta hiiren normaaliin kehitykseen ja kasvuun. Nämä löydökset rohkaisevat kehittämään CIP2A:han kohdistuvia syöpähoitoja.

Hedgehog-signalointi (Hh) osallistuu monien kudosten ja elinten kehitykseen ja ylläpitoon. Tutkimuksemme osoittavat, että Hh-signalointi vaikuttaa spermatogeneesissä kolmella tasolla: mitoottisissa, meioottisissa ja myöhäisissä post-meioottisissa soluissa. Hh-signalointi on estynyt varhaisissa post-meiottisissa soluissa, jotka ilmentävät Suppressor of Fused -proteiinia (SuFu). Sukusolujen apoptoottisten solukuolemien määrä kasvaa, jos Hh-signalointi estetään kemiallisesti. Follikkelia stimuloivan hormonin läsnäolo ja tyrosiinikinaasien tekeminen toimintakyvyttömäksi vähentää Hh-signalointia. Nämä tulokset osoittavat, että Hh-signalointia säädellään sekä endokriinisesti että parakriinisesti, ja että se edistää sukusolujen säilymistä elossa.

Avainsanat: kives, spermatogeneesi, Hedgehog, Nanog, CIP2A, follikkelia stimuloiva hormoni, siementiehyt, kantasolu

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ABBREVIATIONS

A_{al}	type A-aligned spermatogonium
Abp	androgen-binding protein
Acta2	smooth muscle actin
A_{dark}	type A-dark spermatogonium
ANOVA	analysis of variance
AP1	activator protein 1
A_{pale}	type A-pale spermatogonium
A_{pr}	type A-paired spermatogonium
AR	androgen receptor
A_{s}	type A-single spermatogonium
Atm	ataxia telangiectasia mutated
A_{undiff}	undifferentiated spermatogonia
B	type B spermatogonium
Bcl6b	B-cell CLL/lymphoma 6, member B
bFGF	basic FGF
BrdU	bromodeoxyuridine (5'-bromo-2'-deoxyuridine)
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CIP2A	cancerous inhibitor of PP2A
CO_2	carbon dioxide
CREB	cAMP response element-binding
CSF-1	colony-stimulating factor 1
CSF-1R	CSF-1 receptor
Cyp17a1	cytochrome P450 17a1
DAPK	death-associated protein kinase
DHH	desert hedgehog
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DSB	double-strand break
ED	embryonic day
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
eGFP	enhanced GFP
Epcam	epithelial cell adhesion molecule
ERK	extracellular signal-regulated kinase
Erm	Ets-related molecule
ES	embryonic stem
ESC	ES cell
EtOH	ethanol
FACS	fluorescence-activated cell sorting
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FSH	follicle-stimulating hormone
FSHR	FSH receptor
Gapdh	glyceraldehyde 3-phosphate dehydrogenase
GDNF	glial cell line-derived neurotrophic factor
GFP	green fluorescent protein
GFR1A	GDNF family receptor 1 alpha
GJA1	gap junction protein alpha 1
GLI	glioma-associated oncogene
GPR125	G protein-coupled receptor 125
GS	germ-line stem
Hh	hedgehog
Hprt1	hypoxanthine phosphoribosyltransferase 1
IHC	immunohistochemistry
IHH	indian hedgehog
iFCS	inactivated fetal calf serum
Il-1β	interleukin-1beta

In	intermediate-type spermatogonium
iPS	induced pluripotent stem
Itga6	α6-integrin
Klf4	Krueppel-like factor 4
L19	ribosomal protein L19
LIF	leukaemia inhibitory factor
LH	luteinizing hormone
Lhx1	LIM homeobox 1
KO	knock-out
MACS	magnetic-activated cell sorting
MAPK	mitogen-activated protein kinase
MDM2	murine double minute 2
MIX	1-methyl 3-isobutyl xanthine
mRNA	messenger RNA
NCAM	neural cell adhesion molecule
NCBI	National Center for Biotechnology Information
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	normal goat serum
OCT4	octamer-binding transcription factor 4
PCR	polymerase chain reaction
PBS	phosphate-buffered saline
PI3-K	phosphatidylinositol 3-kinase
PFA	paraformaldehyde
PGC	primordial germ cell
PKA	protein kinase A
PKB	protein kinase B
PLZF	promyelocytic leukaemia zinc finger
Ppia	cyclophilin A1
PP2A	protein phosphatase 2A
PTCH	patched
qRT-PCR	quantitative RT-PCR
RA	retinoic acid
Rac1	Ras-related C3 botulinum toxin substrate 1
RET	rearranged during transfection
RIA	radio-immuno assay
RNA	ribonucleic acid
RT	room temperature
RTK	receptor tyrosine kinase
RT-PCR	reverse-transcription PCR
SCF	stem cell factor
SCR	scramble
SEM	standard error of the mean
SHH	sonic hedgehog
siRNA	short-interfering RNA
SMO	smoothened
Sox2	SRY box 2
SRY	sex-determining region of the Y chromosome
SSC	spermatogonial stem cell
STRA8	stimulated by retinoic acid gene 8
SUFU	suppressor of Fused
Sycp3	synaptonemal complex protein 3
S26	ribosomal protein S26
TAF4B	TATA box-binding protein associated factor 4B
TBS	Tris-buffered saline
TCDM	Turku Centre for Disease Modelling
TGFβ	transforming growth factor beta
Thy-1	thymus cell antigen 1
TNFα	tumour necrosis factor alpha
UV	ultra-violet
WT	wild-type
WT1	Wilm's tumour antigen 1

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals I-IV.

I. Mäkelä J-A, Saario V, Bourguiba-Hachemi S, Nurmio M, Jahnukainen K, Parvinen M and Toppari J (2011). Hedgehog signalling promotes germ cell survival in the rat testis. *Reproduction*. 142(5): 711-721.

II. Ventelä S, Comé C, Mäkelä J-A, Hobbs RM, Mannermaa L, Kallajoki M, Chan EK, Pandolfi PP, Toppari J and Westermarck J (2012). CIP2A promotes proliferation of spermatogonial progenitor cells and spermatogenesis in mice. *PLoSONE.* 7(3): e33209.

III. Ventelä S*, Mäkelä J-A*, Kulmala J, Westermarck J and Toppari J (2012). Identification and regulation of a stage-specific stem cell niche enriched by Nanog positive spermatogonial stem cells in the mouse testis. *Stem Cells*. 30(5): 1008-1020. (*equal contribution)

IV. Mäkelä J-A, Toppari J and Ventelä S. Developing a culture system for murine Nanog-expressing spermatogonia. *Submitted.*

1. INTRODUCTION

Life-long ability to produce sperm, i.e. spermatogenesis, is an intricate process. For spermatogenesis to succeed a delicate balance has to prevail between germ cell proliferation and differentiation, and multiple steps of differentiation. The main regulators of these cellular events are two pituitary gland-derived hormones: luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These hormones exert their effects on Leydig cells and Sertoli cells, respectively, and orchestrate the expression of numerous paracrine growth factors that are crucial for spermatogenesis. A rat spermatogonial stem cell (SSC) has to go through at least 10 mitotic and two meiotic divisions and a complex process of differentiation to give rise to spermatozoa. Along the way, in theory, 4096-fold increase in cell number takes place. This process guarantees a continuous supply of differentiated germ cells at a rate of approximately 1000 cells per heart beat in man.

The incidence of testicular tumours and subfertility associated with low sperm counts have been on the rise globally during the modern era. The reasons behind these phenomena are largely unknown. This emphasizes the importance of basic research on testis for there is hardly a greater threat for the future of a species than loss of reproductive capacity. Spermatogonial stem cell (SSC) has been regarded the most primitive germ cell type within the adult mammalian testis. However, the term SSC has been used loosely in the literature and it actually refers to a heterogeneous population of cells. Despite their heterogeneity and lack of unique molecular marker to differentiate them from other germ cells, they have been considered to have one thing in common: ability to reconstruct spermatogenesis to an infertile recipient. Based on a recent report (Barroca et al. 2009) this definition of SSCs can no longer be regarded exclusive and SSCs need to be re-defined either functionally or molecularly. Spontaneous derivation of embryonic stem (ES) cell-like cells from testicular cells (Matsui et al. 1992, Kanatsu-Shinohara et al. 2004B, Guan et al. 2006, Seandel et al. 2007, Kossack et al. 2009) has increased the interest towards the biology of SSCs. The testis may provide an ethically-sustainable and immunologically competent source of pluripotent cells for regenerative medicine in the future.

Due to the rarity of SSCs *in vivo* and the complex architecture of the seminiferous tubules, the interaction of SSCs with their stem cell niche could be studied more thoroughly *in vitro*. Many different groups have succeeded in establishing long-term SSC cultures. However, none of them has managed to set-up a long-term co-culture for the constituents of the testis stem cell niche: Sertoli cells, peritubular myoid cells and SSCs. It would be, however, the most physiologically relevant *in vitro* environment to study the dynamics of the testis stem cell niche and SSCs. Besides this, reconstruction of the testis stem cell niche *in vitro* might enable certain aspects of SSC biology to be uniquely studied. It is after all the stem cell niche that dictates the fate of the stem cell.

The massive expansion in cell number during spermatogenesis is partially dependent on a group of proteins that are encoded by the so called cancer/testis (C/T) genes. These genes are highly expressed in malignancies and among normal tissues in the testis. *CIP2A* (cancerous inhibitor of PP2A) belongs to this group of genes (Junttila et al. 2007), which makes it an attractive target for pharmacological cancer therapy. A fundamental criterion that has to be met by such a target

is that systemic inhibition of its function should not have (severe) side effects. Additionally, the physiological role of CIP2A in spermatogenesis is interesting.

Hedgehog (Hh) signalling participates in the development of numerous tissues and organs, including the testis. It is also needed to maintain the stem and progenitor cell populations in many adult tissues. Despite a handful a reports about the expression of Hh pathway components in mammalian testis no physiological task during spermatogenesis has been assigned to it. Environmental factors have been suggested to impinge on Hh signalling in the testis (Fowler et al. 2008, Brokken et al. 2009). However, the physiological regulation of Hh signalling in the testis is unknown.

In the present study, we conducted a series of experiments to approach the spermatogenic process at different levels using *in vivo* and *in vitro* models and methods. Additionally, we developed a novel *in vitro* method that allows spermatogonial stem cells to be studied in an environment that mimics their *in vivo* surroundings as closely as possible. Data of the present study give new insights into the stem and progenitor cell biology of the mouse testis, and elucidates the regulation and function of Hedgehog signalling in rat spermatogenesis.

2. REVIEW OF THE LITERATURE

2.1 Postnatal testis development

At birth testicular tissue can be divided into testis cords and the surrounding interstitium. The testis cords, which later develop into seminiferous tubules, consist of primitive germ cells, called gonocytes, and somatic Sertoli and peritubular myoid cells. Androgen-producing Leydig cells, cells belonging to the immune system, fibroblasts, blood and lymph vessels, and nerves can be found in the interstitium. Mitotically quiescent gonocytes start to proliferate shortly after birth. This coincides with migration of these centrally located cells to the basement membrane of the seminiferous tubule (Clermont & Perey 1957). Getting in contact with the basement membrane is presumably crucial for their survival since the cells that remain in the centre degenerate (Roosen-Runge & Leik 1968). By postnatal day 7 the spermatogonial stem cell pool of the rat testis has formed. As determined by flow cytometry, on day 13-14 it gives rise to the first meiotic cells, preleptotene spermatocytes, and the first pachytene spermatocytes are seen between days 19-20. The first round of meiotic divisions is completed around day 24 and spermatid elongation starts by day 30-31 finally forming mature spermatozoa on day 36 (Clermont & Perey 1957, Malkov et al. 1998). The same events take place 4-6 days earlier during the mouse postnatal testis development (Bellvé et al. 1977).

The gradual appearance of different germ cell populations leads to an increase in length and diameter of the seminiferous tubules and requires thus proliferation of the somatic cells of the seminiferous tubules. Sertoli cells cease to proliferate at about 12 (Kluin et al. 1984) and 15 days of age (Bortolussi et al. 1990, Yang et al. 1990) in mouse and rat, respectively. Thereafter they start to mature and specifically express proteins that are seen exclusively in the Sertoli cells of adult mammals. Simultaneously the relative volume of the interstitium decreases. This process, and spermatogenesis in general, is dependent on the ambient temperature and requires an environment which is a couple degrees cooler than body temperature to succeed. Therefore the testis is situated in the scrotum and the temperature difference is created by heat loss through the scrotal skin and maintained by the countercurrent heat exchange system between the main testicular veins and arteries. Testis descent occurs by postnatal day 26 in rats (Odum & Ashby 2000). In the interstitium fetal-type Leydig cells are replaced by adult-type cells, the absolute number of which increases until 60 days of age (Bortolussi et al. 1990). Also the vascular architecture of the testis changes over time and the adult-pattern is established in around 40-day-old rat (Kormano 1967).

2.2 Spermatogenesis

Differentiation of diploid germ-line stem cells all the way to haploid spermatozoa, i.e. spermatogenesis, is an intricate process and takes 35 and 52 days to complete in the mouse and the rat, respectively (Clermont 1972). During the course of differentiation male germ cells go through a succession of cytological events and dramatic changes in morphology and biochemistry. Spermatogonial cells divide first mitotically to eventually give rise to spermatocytes that undergo meiosis (Clermont 1962). Two sequential meiotic divisions produce haploid spermatids (Stern 1993) that undergo a differentiation program in which the volume of cytoplasm reduces, the chromatin condenses and the flagellum and acrosome are formed (Clermont et al. 1993). For spermatogenesis to succeed a delicate balance has to prevail between many different

cellular processes, such as self-renewal versus differentiation of stem cells and various steps of differentiation. Any error during the process is multiplied in sperm production capacity since one spermatogonial stem cell once having entered the differentiation pathway is in theory able to give rise to 4096 sperm cells. In practice this huge expansion is smaller due to apoptosis taking place at different levels of maturation.

2.2.1 Cellular organization and cycle of the seminiferous epithelium

The testis is encapsulated by tunica albuginea, a tough fibrous layer of connective tissue. Spermatogenesis takes place in the seminiferous tubules of the testis. They are convoluted occasionally branching loops that are connected at their both ends to the rete testis. Rete testis is the beginning of the excurrent duct system that transports the fully differentiated spermatozoa to the epididymis. Besides seminiferous tubules, the adult mammalian testis is composed of supporting interstitial tissue where the vasculature, lymphatic vessels, nerves and testosterone-producing Leydig cells reside. Contractile peritubular myoid cells limit the seminiferous tubules towards the interstitium (Figure 1).

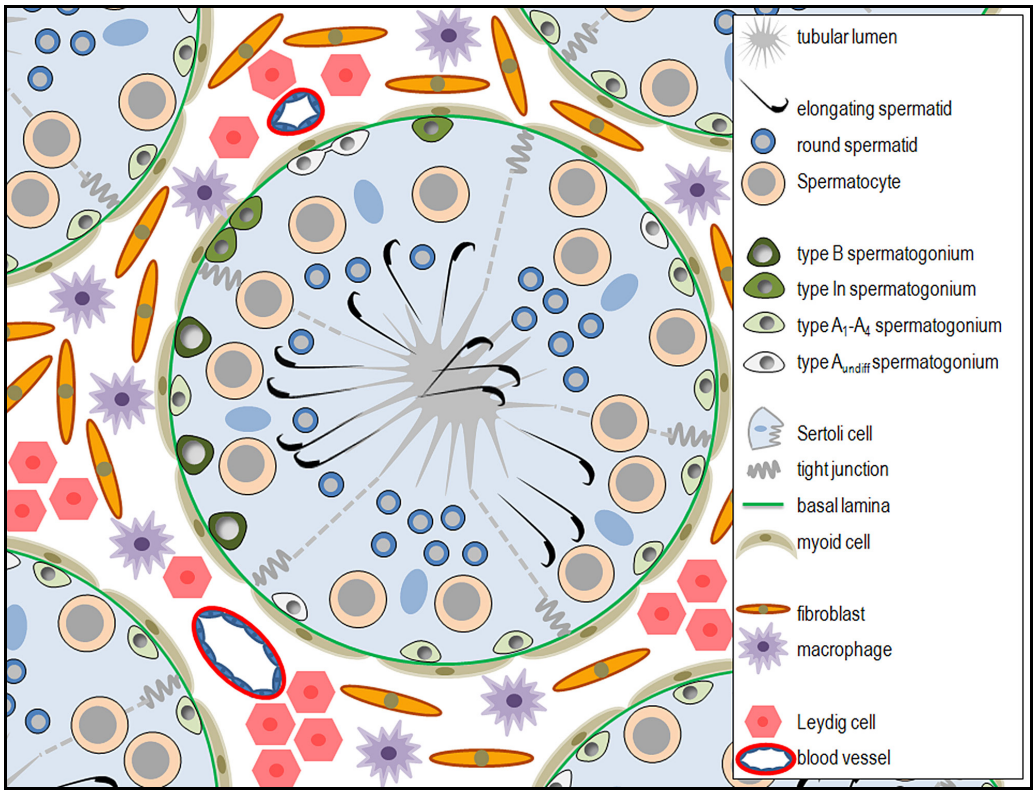

Figure 1. *Illustration of the architecture of a cross-section of testicular tissue in a sexually mature mammalian. Blood vessels, Leydig cells, macrophages and fibroblasts reside in the intertubular space, or interstitium. Peritubular myoid cells limit the seminiferous tubules towards the interstitium. Spermatogonia are situated on the basement membrane of the seminiferous epithelium. Sertoli cells are connected to each other by tight junctions at their basolateral parts. Sertoli cells extend from the basal lamina to the lumen. Meiotic and postmeiotic germ cells are engulfed by the Sertoli cells and released at the end of spermatogenic differentiation to the tubular lumen. In reality in mouse and rat all the different germ cell types do not exist within one cross-section.*

In the seminiferous tubules there are two sorts of cells: somatic Sertoli cells and germ cells that together form the seminiferous epithelium. The basement membrane, or basal lamina, of the seminiferous epithelium is generated from contributions of Sertoli cells and outlying peritubular myoid cells (Clermont 1972, Russell 1993). Tight junctions between Sertoli cells, called the blood-testis barrier, divide the seminiferous epithelium to two compartments: basal and adluminal (Dym & Fawcett 1970). Spermatogonia reside in the basal compartment in close association with the basal lamina, whereas meiotic cells mature in the adluminal compartment. Sertoli cells extend from the basal lamina to the tubular lumen. During the lengthy process of differentiation germ cells are in contact with Sertoli cell that have a crucial role in developing a microenvironment that supports germ cell maturation and survival (Griswold 1998). This is particularly true for meiotic germ cells which after having crossed the blood-testis barrier are metabolically dependent on nutrient supply by Sertoli cells (Mruk & Cheng 2004). Spermatids after having undergone complex morphological changes that result in formation of spermatozoa are released in the lumen of the seminiferous epithelium and carried to the rete testis by the flow of the luminal fluid and active tubule contractions generated by peritubular myoid cells.

Spermatogenesis is divided into 12 and 14 defined stages in mouse (marked with Roman numerals I-XII) and rat (I-XIV), respectively (Leblond & Clermont 1952, Oakberg 1956A) (Figures 2A and 2B). A stage is defined by the existence of specific types of germ cells at specific phase of differentiation in a cross-section of the seminiferous epithelium. It is worth mentioning that stages of the seminiferous epithelium are defined by the observer, and they have no relevance to the progression of the biological process *per se*. Stage VIII in both rat and mouse, for instance, is composed of diploid undifferentiated type A and differentiating type A1 spermatogonia, tetraploid preleptotene and pachytene spermatocytes, haploid step 8 round spermatids and haploid fully matured spermatids that are released as spermatozoa at the end of this stage. Distinction of the stages is based on the morphological features of spermatids, namely the formation and orientation of the acrosomal system and the shape and condensation of the nucleus. Differentiation of germ cells in a specific stage takes place in a synchronized manner and the cells develop from one stage to the next according to regular time intervals (Leblond & Clermont 1952). Undifferentiated spermatogonia can move longitudinally along the seminiferous epithelium, but once they become committed to further development, differentiation of the germ cells takes place in one region of the seminiferous tubule in a cyclic fashion thus going through step-wise all the stages of the seminiferous epithelial cycle. A differentiating spermatogonium has to complete four full cycles to mature into a spermatozoon that is finally detached from the seminiferous epithelium.

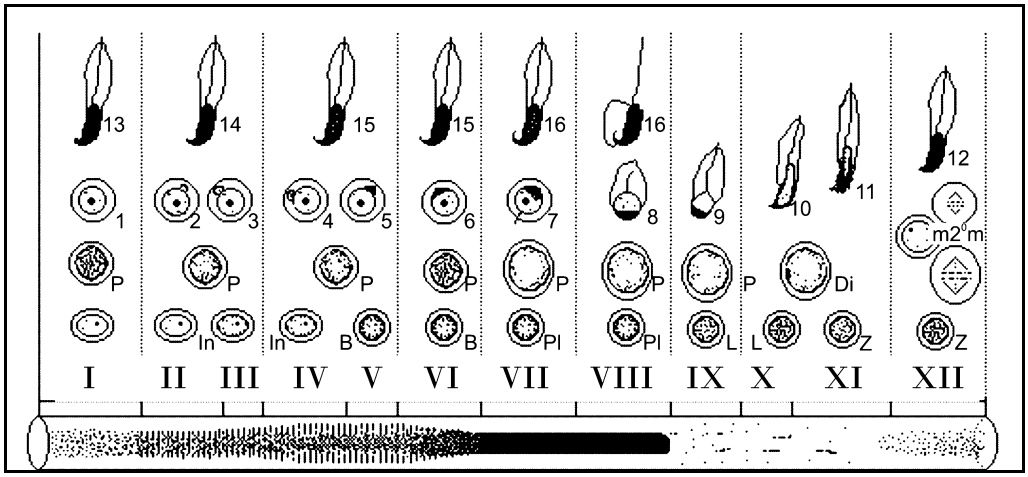

Figure 2A. *The cycle of the seminiferous epithelium in the mouse. The specific cell associations in the vertical columns represent specific stages (Roman numerals) of the mouse seminiferous epithelial cycle. Arabic numerals refer to different steps of postmeiotic germ cell differentiation. In, intermediate spermatogonia; B, type B spermatogonia; Pl, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; Di, diplotene spermatocytes; m2°m, meiotic divisions. Different stages can be visualized by transillumination-assisted microscopy where stages differ from each other by their light absorption characteristics. Type A spermatogonia and Sertoli cells are not included in the figure.*

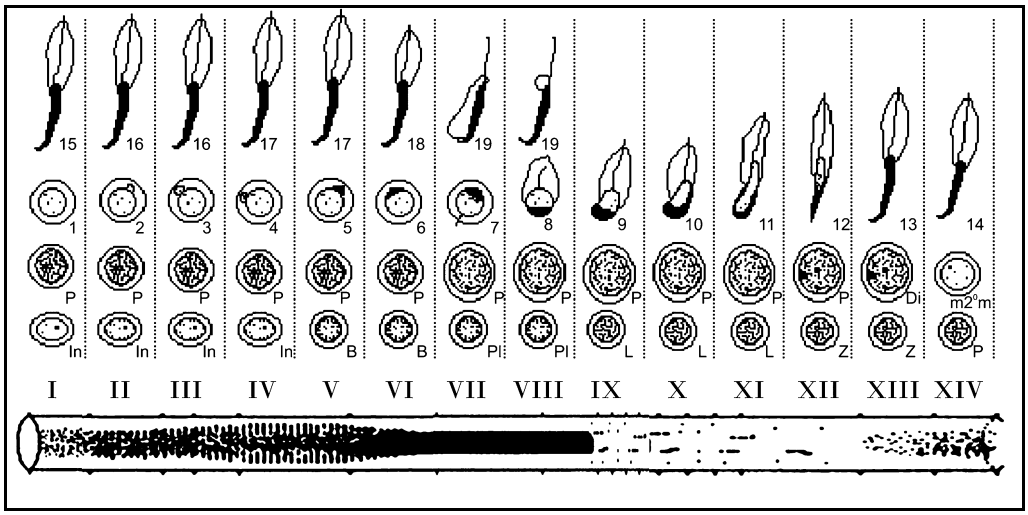

Figure 2B. *The cycle of the seminiferous epithelium in the rat. The specific cell associations in the vertical columns represent specific stages (Roman numerals) of the rat seminiferous epithelial cycle. Arabic numerals refer to different steps of postmeiotic germ cell differentiation. In, intermediate spermatogonia; B, type B spermatogonia; Pl, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; Di, diplotene spermatocyte; m2°m, meiotic divisions. Different stages can be visualized by transillumination-assisted microscopy where stages differ from each other by their light absorption characteristics. Type A spermatogonia and Sertoli cells are not included in the figure.*

The duration of one cycle of the seminiferous epithelium (from stage I through all the stages and back to stage I) takes 8.6 days in the mouse and 12.9 days in the rat, in C3H and Sprague-Dawley strains, respectively (Leblond & Clermont 1952, Oakberg 1956B). Stages that constitute the cycle have different durations which is reflected directly in the frequency at which they occur in a histological specimen. Longitudinal counterpart of a stage is a segment meaning a stretch of seminiferous tubule where the cells are at a specific stage of differentiation. Along the longitudinal axis of the seminiferous tubule different stages follow each other in a numerical order. They can be identified by their light absorption characteristics under a stereomicroscope. Differences in chromatin condensation and cell localization within the seminiferous epithelium can be observed by trans-illuminating the seminiferous tubule, which reveals that there are darker and paler zones (Parvinen 1982, Toppari & Parvinen 1985, Kotaja et al. 2004).

2.2.2 Germ cell differentiation during the spermatogenic cycle

Mammalian spermatogenesis is classically divided into three phases: 1. mitotic (proliferative), 2. meiotic and 3. spermiogenic phase. In the first, spermatogonia (stem cells and their differentiating progeny) undergo several mitotic divisions. In the second, descendants of spermatogonia, spermatocytes, give rise to four-fold number of spermatids by undergoing two consecutive meiotic divisions. In the third, spermatids go through a succession of cytological events to differentiate into spermatozoa.

2.2.2.1 Mitotic phase

Spermatogonium is the type of a germ cell that divides mitotically. There are many different types of spermatogonia that can be differentiated based on their shape and nuclear morphology, association with other cells of the same type, time of appearance during the seminiferous cycle and protein marker expression. The prevailing model for the expansion of spermatogonial population was originally proposed 1971 by Huckins (1971) and Oakberg (1971) and it has been refined more recently (de Rooij & Russell 2000). It assumes that the propagation of the spermatogonia originates from a small population of solitary undifferentiated type A spermatogonia, called A-single (A_s) cells. An A_s cell can divide into either two new A_s cells or two type A-paired (A_{pr}) spermatogonia that are committed to the differentiation pathway (Figure 3). A_{pr} cells connected by an intercellular bridge then give rise to A-aligned (A_{al}) spermatogonia that form chains of 4, 8 or 16 aligned and connected cells. Recently, it has been suggested that A_{pr} and A_{al} cells can dedifferentiate into A_s cells and reverse the commitment to differentiate (Nakagawa et al. 2010). A_s, A_{pr} and A_{al} cells are commonly referred to as undifferentiated spermatogonia. A_{al} spermatogonia without undergoing cell division differentiate into A_1 spermatogonia that go through six mitotic divisions to become primary spermatocytes that enter meiotic differentiation. A_1 cells and their premeiotic descendants (A_2, A_3, A_4, intermediate (In) and B spermatogonia) are known as differentiating spermatogonia (Figure 4) (Huckins 1971, Oakberg 1971).

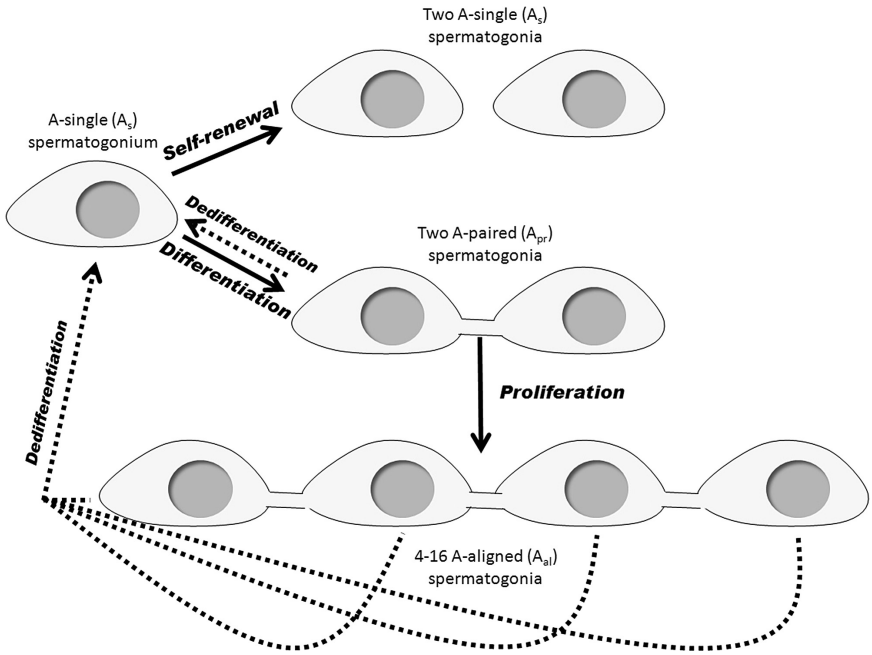

Figure 3. *Stem cell dynamics in the testis. Solid arrows represent the traditional view: An A-single spermatogonium is able to undergo either self-renewal division, that gives rise to two new A-single spermatogonia, or differentiation division. In the latter the progeny is committed to spermatogenesis as described in Figure 4. However, according to Nakagawa et al. (2010) A_{pr} and A_{al} are able dedifferentiate into A_s cells (dashed arrows).*

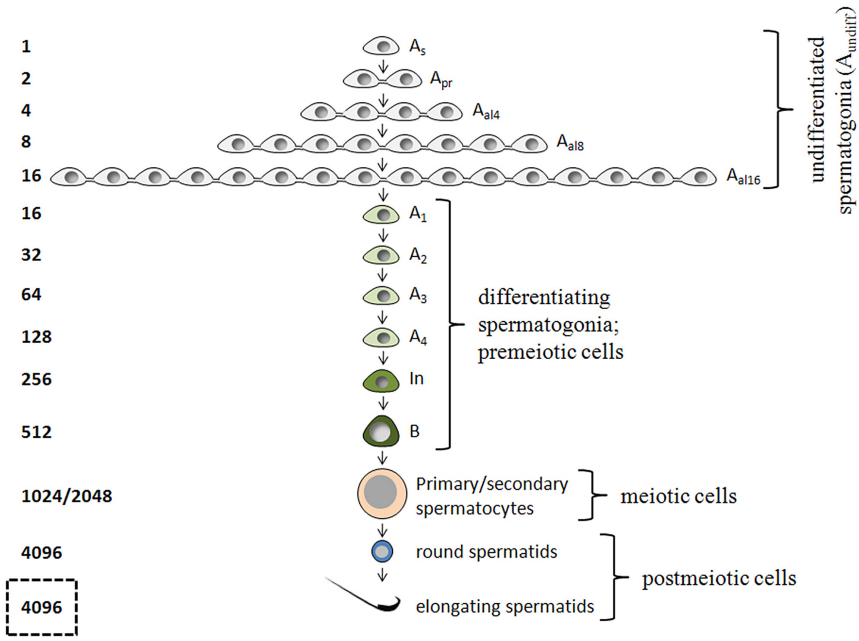

Figure 4. *Spermatogenesis. In theory one A-single spermatogonium having undergone differentiation division can give rise to 4096 spermatozoa. In reality this number is lower due to apoptosis. On the left the value in the column refers to the number of a specific germ cell type at the same level.*

Regulation and life-long maintenance of the spermatogonial population have been the focus of many studies over the past decades and many different spermatogonia-intrinsic and -extrinsic factors have been characterized. Although the morphological differences of undifferentiated and differentiating spermatogonia are subtle they diverge in gene expression. The expression of some of these factors is confined to certain spermatogonial populations, which can be exploited in antibody-based cell separation. In sorting of cells by their surface antigen expression fluorophore- or magnetic nanoparticle-conjugated antibodies are used. Cell suspension is first incubated with the conjugated antibodies and then either applied in the centre of a narrow, rapidly flowing stream of liquid (FACS) or transferred on a column in a strong magnetic field (MACS). In FACS the cells in a suspension flow one-by-one passing a fluorescence measuring station where the fluorescent properties of each cell are measured. Having passed this part of the device the stream breaks into droplets with solitary cells that can be given an electric charge based on their fluorescent character. Then depending on the electric charge (FACS) or attachment to the magnetic column (MACS) the desired cells can be separated.

One of the proteins that is differentially expressed in undifferentiated and differentiating spermatogonia is c-KIT. Gonocytes express c-KIT in the postnatal testis (Orth et al. 1996), while in the adult testis c-KIT is detected in type A_1-A_4 differentiating spermatogonia (Dym et al. 1995), spermatocytes and in a truncated form in spermatids and spermatozoa (Albanesi et al. 1996, Schrans-Stassen et al. 1999). *c-Kit* is also present in Leydig cells independent of the age (Manova et al. 1990). Due to its expression pattern negative selection for c-KIT has been employed in the sorting of SSCs (Shinohara et al. 1999, Kubota et al. 2003, Kanatsu-Shinohara et al. 2004A) and c-KIT is regarded a differentiating non-stem germ cell marker (Ohta et al. 2000, Rossi et al. 2003). GFR1α (GDNF family receptor 1 alpha) is a part of GDNF (glial cell line-derived neurotrophic factor) receptor complex and is expressed specifically by the undifferentiated spermatogonia at least some of which are SSCs (Meng et al. 2000, Hofmann et al. 2005, Kanatsu-Shinohara et al. 2003, Kubota et al. 2004). PLZF (promyelocytic leukaemia zinc finger) is expressed exclusively by undifferentiated spermatogonia, but not by differentiating spermatogonia (Buaas et al. 2004, Costoya et al. 2004). The up-stream regulators of PLZF are largely unknown but it has been shown that PLZF directly represses transcription of *c-Kit* in spermatogonia thus impeding their entry into differentiation (Filipponi et al. 2007). Gpr125 (G protein-coupled receptor 125) expression in the testis is associated with undifferentiated spermatogonia markers even though it has not been precisely characterized which germ cell types express the protein (Seandel et al. 2007). CD9 and Stra8 (stimulated by retinoic acid gene 8) are examples of genes that are expressed in both cell populations (Giuili et al. 2002, Kanatsu-Shinohara et al. 2004A). Testicular CD9 expression is not confined to the stem cells since a significant proportion of CD9-selected cells also express c-KIT (Kanatsu-Shinohara et al. 2004A). Although Stra8 is a putative stem cell marker in the testis (Giuili et al. 2002, Guan et al. 2006), *in vivo* most of the Stra8 expression stems from the cells entering meiotic differentiation due to their relatively high number and level of expression (Zhou et al. 2008). Indeed, retinoic acid-induced expression of Stra8 has been shown to regulate meiotic initiation in spermatogenesis (Anderson et al. 2008).

2.2.2.2 Meiotic phase

Type B spermatogonia undergo the final mitotic division to give rise to preleptotene spermatocytes in stage VI/VII of the mouse and rat seminiferous epithelial cycle. Preleptotene spermatocytes, the first cell type of the meiotic phase, penetrate the Sertoli cell tight junctions and enter the adluminal side of the blood-testis barrier. They replicate their DNA and proceed

to the prophase I of meiosis which can be subdivided to five different phases based on nuclear morphology: leptotene, zygotene, pachytene, diplotene (also known as leptonema, zygonema, pachynema, diplonema) and diakinesis. During the more than two-week-long meiotic prophase the spermatocyte itself and its nucleus grow in size. Preleptotene spermatocytes transit to leptotene spermatocytes. In leptotene spermatocytes, individual chromosomes that consist of two sister chromatids, condense into visible strands and synaptonemal complex starts to assemble. In zygonema, synaptonemal complex facilitates the pairing (synapsis) of homologous chromosomes. In pachynema, chromosomal crossing-over takes place and non-sister chromatids of homologous chromosomes exchange stretches of DNA in a random fashion. The pachynema is by far the longest phase lasting around 12 days in rat while the other phases take from a couple of hours to 3-4 days to complete. In diplotene spermatocytes, the synaptonemal complex holding the chromosomes together degrades and the chromosomes, that are a held together at the sites where crossing-over has occurred (chiasmata), separate from each other. In diakinesis, the nuclear membrane disintegrates and the meiotic spindle starts to form. The rest of the phases of the first meiotic division (metaphase, anaphase and telophase) take place rather quickly and two short-lived secondary spermatocytes are formed as the result. The phases of the second meiotic division are completed rapidly and each secondary spermatocyte produces two haploid round spermatids (Stern 1993).

2.2.2.3 Spermiogenic phase

During spermiogenic phase, or spermiogenesis, the newly-formed round spermatids become remarkably smaller by going through a succession of cytological events, losing their round appearance and cytoplasm, tightly packing DNA, and developing an acrosome and a flagellum in a process that is divided into 16 and 19 steps in mouse and rat, respectively. This phase takes about two weeks in mouse and three weeks in rat to be completed and results in release of fully elongated spermatids into the lumen of the seminiferous tubule (Clermont et al. 1993).

2.2.3 Spermatogonial physiology and expansion in primates

In primates, including man, two populations of type A spermatogonia exist: the A_{dark} and the A_{pale} spermatogonia (Clermont & Leblond 1959). A_{dark} spermatogonia are recognized in tissue sections by their dark haematoxylin staining, whereas the A_{pale} spermatogonia stain less. Besides the staining pattern their identification is based on recognition of delicate morphological characteristics. This classification of type A spermatogonia has been widely used in the literature even though up to 50 % of spermatogonia in adult primate tissue and even more in the developing testis cannot be unequivocally classified into either of the categories (Ehmcke & Schlatt 2006). A population of type A spermatogonia undergoing the A_{dark} to A_{pale} transition has been proposed in a number of studies (reviewed in Ehmcke & Schlatt 2006). However, lack of specific markers has hampered the development of a more valid scheme.

In the primate testis A_{dark} spermatogonia are thought to present the stem cell population, whereas A_{pale} spermatogonia are transit amplifying cells or progenitors, the functional counterpart of which does not exist in the rodent testis (Ehmcke et al. 2006). During pre-pubertal testis development A_{dark} spermatogonia are highly proliferative and contribute to the formation of the spermatogonial (stem) cell pool (Simorangkir et al. 2005). In a healthy, sexually mature rhesus monkey (*Macaca mulatta*) A_{dark} spermatogonia proliferate only rarely (Ehmcke et al. 2005A,B). However, X-irradiation-inflicted damage on A_{pale} and type B spermatogonia

induces their proliferation and leads thus to regeneration of the seminiferous epithelium and spermatogenic recovery (van Alphen et al. 1988). The proliferation of A_{pale} spermatogonia is bound to the progress of the seminiferous epithelial cycle and during every cycle they self-renew and give rise to differentiating progeny, i.e. type B spermatogonia (Ehmcke et al. 2006 and references therein). Despite their capacity to self-renew the A_{pale} spermatogonia cannot be considered true stem cells but amplifying progenitors that are able to undergo a limited though undefined number of cell cycles. According to a clonal model of spermatogonial expansion in the monkey testis, A_{pale} spermatogonia divide mitotically in stage VII of the seminiferous epithelial cycle. Two thirds of the progeny undergo a differentiating cell division in stage IX, whereas one third replenishes the A_{pale} cell population by undergoing self-renewal division (Ehmcke & Schlatt 2006). Since there are four different populations of type B spermatogonia in the rhesus monkey (B_{1-4}), and only one in man, one A_{pale} spermatogonium gives rise to 32 and 4 spermatocytes in monkey and man, respectively (Ehmcke et al. 2006). However, if the clonal expansion model holds true and only two thirds of the clones enter the differentiation pathway, a 48-fold increase in germ cell number takes place during the rhesus monkey pre-meiotic cell expansion (Ehmcke & Schlatt 2006). Based on the existing data, Ehmcke and Schlatt have also proposed a scheme for spermatogonial proliferation in the human testis, and it is quite similar to their clonal model in the monkey (Ehmcke & Schlatt 2006).

As described in chapter 2.2.1 stages of the seminiferous epithelial cycle are arranged longitudinally in the rodent seminiferous tubules. This is also true for macaques, whereas in man and new world monkeys more than one stage of spermatogenesis is observed in every cross-section of the seminiferous tubule. This is probably due to the asynchronized cell division and relatively small clonal expansion in the latter (Ehmcke & Schlatt 2006).

2.2.4 The Sertoli cell

Sertoli cells are the only somatic cells that exist inside the seminiferous tubules. The number of Sertoli cells determines testicular size, the number of germ cells the testis can support and thereby spermatogenic potential (Orth et al. 1988, Oatley et al. 2010). Sertoli cells are proliferating during the first two weeks of postnatal life in rodents but the Sertoli cells in the adult are thought to be terminally differentiated quiescent cells (Kluin et al. 1984, Bortolussi et al. 1990). However, they can resume cell cycle progression *in vitro* (Eddy & Kahri 1976, Ahmed et al. 2009). Several functions have been assigned to the Sertoli cells. These include: maintenance of the integrity and compartmentalization of the seminiferous epithelium, secretion of luminal fluid, participation in spermiation, phagocytosis of germ cell remnants, delivery of nutrients to the germ cells, steroidogenesis and steroid metabolism, movement of cells within the seminiferous epithelium, secretion of proteins and other products, regulation of the spermatogenesis, and being both target and mediator of hormone effects in the testis (Russell et al. 1990). The last two of these deserve further discussion.

2.2.5 Endocrine regulation of spermatogenesis

Testis is an endocrine organ. Hypothalamus controls gonadotropin release in the pituitary gland by secreting gonadotropin-releasing hormone (GnRH). Pituitary-derived glycoprotein gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) travel to the testis by blood stream and exert their effects directly on testicular somatic cells. Receptors for

gonadotropins are cell surface proteins. LH receptor is expressed by Leydig cells in the testicular interstitial tissue (Zhang et al. 1994), whereas Sertoli cells bind FSH (Heckert & Griswold 1991, Heckert & Griswold 1993). It is noteworthy that germ cells are not under the direct influence of the gonadotropins but are affected by these hormones in an indirect way. LH stimulates testosterone production from cholesterol in Leydig cells (Moyle & Armstrong 1970). FSH regulates the expression of multiple genes in the Sertoli cells (McLean et al. 2002). Testis-originating testosterone and inhibin act negatively on LH and FSH release from the pituitary, respectively, thus forming a feedback system characteristic of hypothalamus-pituitary-endocrine organ axis.

FSH effect on Sertoli cells is age-dependent and in puberty the dominant function of FSH on the Sertoli cells shifts from driving their proliferation to stimulating growth factor production that is needed to nurture germ cells (Meachem et al. 2005 and references therein). In adult mice, Sertoli cells are quiescent *in vivo* and their main function is to support spermatogenic differentiation of germ cells. Serum FSH levels are 7-fold higher in adult than in juvenile mice, as measured using radioimmunoassay (RIA) (Barakat et al. 2008). The highest steady state levels of *FSH receptor* (*Fshr*) mRNA (messenger ribonucleic acid) are recorded in stages XII-II of the rat seminiferous epithelial cycle and the lowest levels are observed in stages VII-VIII (Heckert & Griswold 1991). Additionally, FSH stimulates cAMP (cyclic adenosine monophosphate) production in a stage-dependent manner preferring stages XIV-VI (Kangasniemi et al. 1990). These data suggest that FSH exhibits its regulatory function mainly in the early stages of the seminiferous epithelial cycle. The signalling pathways downstream of FSHR are relatively well characterized. FSH has been shown activate signalling via cAMP/PKA (protein kinase A), MAPK (mitogen-activated protein kinase) and PI3-K (phosphatidylinositol 3-kinase) pathways and induce calcium influx and production of prostaglandins. It has to be mentioned that cAMP/PKA pathway is the most significant of these and the data supporting activation of it by FSH is solid (Walker & Cheng 2005). Signalling via cAMP/PKA directly contributes to the activation of PI3-K signalling and elevation of intracellular calcium, a multipotent intracellular signalling molecule. In response to different signalling pathway activation CREB (cAMP response element-binding), NF-κB (nuclear factor kappa B) and AP1 (activator protein 1) transcription factors take FSH effects to the transcriptional level (Walker & Cheng 2005). Individual genes whose expression has been shown to be affected by FSH include among others *Gdnf* (Tadokoro et al. 2002) – a crucial regulator of SSCs self-renewal *in vivo* and *in vitro* (Meng et al. 2000, Kubota et al. 2004), *Scf* (stem cell factor; Yan et al. 1999) – a germ cell survival factor (Hakovirta et al. 1999, Yan et al. 2000C), *FSH receptor* (Maguire et al. 1997), *transferrin* (Skinner at al. 1989) – a protein needed in iron transportation to germ cells (Sylvester & Griswold 1984), and *Abp* (androgen-binding protein) that binds and stores androgens within the seminiferous epithelium (Skinner at al. 1989). Interestingly, also AR (androgen receptor) expression is stimulated by FSH (Verhoeven & Cailleau 1988, Blok et al. 1989).

Despite its role as one of the most important regulators of Sertoli cell function, FSH is dispensable for qualitatively but not quantitatively normal sperm production in rodents. Mutations of the FSH receptor in men result in a severe decrease in sperm counts and subfertility (Tapanainen et al. 1997). Male mice lacking either *Fshb* gene encoding the β subunit of the dimeric FSH or FSHR are fertile but the females of the same genotype are infertile (Kumar et al. 1997, Dierich et al. 1998, Abel et al. 2000). However, both *Fshb* and *Fshr* KO (knock-out) male mice have small testes with small tubule diameter and reduced number of sperm with compromised motility but they were still able to sire normal litters (Kumar et al. 1997, Dierich et al. 1998). Interestingly, the *FSH*β mutations in men are associated with azoospermia (Lindstedt et al. 1998, Phillip et al. 1998, Layman et al. 2002). The reasons for this discrepancy between observations in man and

mouse, and in men deficient of FSH or FSHR function, are unclear. However, in man FSH might play a more crucial role in spermatogenesis than in mouse and the reported cases carrying a FSHR mutation might still possess residual activity of the receptor. Though highly unlikely, it is also possible that the azoospermic men with *FSHβ* mutation might have other functional defects explaining the inability for spermatogenesis (Meduri et al. 2008).

Interestingly, in mice lacking GnRH (and thus FSH and LH) testosterone alone was able to restore spermatogenesis (Singh et al. 1995). Exogenous FSH increased the size of the premeiotic cell population but was not able to support the completion of meiosis if the mice also lacked AR (O'Shaughnessy et al. 2010). In summary, the current data indicates that both FSH and testosterone alone are able to support the meiotic germ cells to some extent but germ cells cannot enter spermiogenesis in the absence of androgens (O'Shaughnessy et al. 2009).

2.2.6 Paracrine regulation of spermatogenesis

Spermatogenesis is regulated by a complex interplay of endocrine and paracrine signals. Most of the endocrine effects on testis are mediated indirectly by paracrine signalling. This stems from the fact that the production and secretion of many paracrine factors is under endocrine control by FSH and LH. FSH has been shown to control the expression of hundreds of genes in Sertoli cells (McLean et al. 2002). Among these are paracrine growth factors GDNF (Tadokoro et al. 2002), that is needed to maintain the spermatogonial cell population (Meng et al. 2000), and SCF (Yan et al. 1999), a survival factor for germ cells (Hakovirta et al. 1999, Yan et al. 2000C).

GDNF is a distant member of the transforming growth factor beta (TGF-β) superfamily (Lin et al. 1993). It signals through a receptor complex that consists of a glycosylphosphatidylinositol-anchored cell surface molecule GFR-1α (GDNF family receptor 1-alpha) and the RET (rearranged during transfection) transmembrane tyrosine kinase protein (Jing et al. 1996, Treanor et al. 1996). The binding of GDNF and consecutive activation of the receptor complex affects many different signalling pathways (Airaksinen & Saarma 2002). Signalling via SRC family kinases and activation of PI3-K/PKB, Smad2/3 and Ras signalling pathways have been shown to promote SSC proliferation (He et al. 2009). The role of GDNF in the maintenance of SSCs *in vivo* and *in vitro* is crucial (Meng et al. 2000, Kubota et al. 2004). In the absence of GDNF SSCs escape self-renewal and start to differentiate resulting in infertility in the long run, whereas GDNF overexpression leads to accumulation of SSCs and a seminomatous tumour formation (Meng et al. 2000, Creemers et al.2002, Yomogida et al. 2003). Interestingly, transplantation of SSCs with activated Ras signalling, one of the pathways stimulated by GDNF in SSCs, to the testes of an infertile recipient leads to seminomatous tumour formation as well (Lee et al. 2009).

Transcriptional targets of GDNF signalling include factors that are needed in the maintenance of SSCs: *Erm* (Ets-related molecule; Chen et al.2005, Oatley et al. 2007), *Bcl6b* (B-cell CLL/lymphoma 6, member B) and *Lhx1* (LIM homeobox 1; Oatley et al. 2006). GDNF also stimulates the expression of fibroblast growth factor (FGF) receptor-2 (Hofmann et al. 2005), which might explain why FGFs can work in synergy with GDNF to promote SSC self-renewal (Kubota et al. 2004). Additionally, FGFs and GDNF share *Erm* as a transcriptional target in SSCs (Chen et al. 2005). Interestingly, there are two known SSC-intrinsic factors which are required for spermatogenesis but are not regulated by GDNF: PLZF (Buaas et al. 2004, Costoya et al. 2004) and TAF4B (TATA box-binding protein associated factor 4B; Falender et al. 2005). Besides FSH, *Gdnf* expression in Sertoli cells seems to be dependent on locally produced FGF2 (fibroblast

growth factor 2), Il-1β (interleukin-1beta) and TNFα (tumour necrosis factor alpha) *in vitro* (Simon et al. 2007).

SCF/c-KIT signalling plays an important role at different stages of testis development and function. Already during embryonic development SCF/c-KIT guides the migration of gonocytes from the hindgut to the bipotential gonad (McCoshen & McCallion 1975). It is also needed in the early postnatal migration of gonocytes from the centre of seminiferous tubules to the basement membrane (Orth et al. 1997) and disturbing this event has an adverse effect on the formation of the spermatogonial stem cell pool (Nurmio et al. 2007). The significance of SCF/c-KIT signalling in the adult is emphasized by the lack of spermatogenesis in the absence of either gene's function (Besmer et al. 1993).

SCF expressed in the Sertoli cells is either secreted or bound to the cell membrane (Huang et al. 1992). The membrane-bound form is thought to mediate the signal towards neighbouring germ cells whereas the soluble form carries the message to the interstitium. The steady state levels of *Scf* mRNA in Sertoli cells shows the highest values in stages II-VI of the rat seminiferous epithelial cycle (Hakovirta et al. 1999). Stages II-VI are also responsive to FSH-induced activation of *Scf* transcription (Yan et al. 1999). SCF receptor c-KIT is expressed by a wide range of cells in the testis. Binding of SCF to c-KIT on germ cells results in up-regulation of pro-survival genes and down-regulation of pro-apoptotic genes (Yan et al. 2000B). These findings might explain why SCF has been shown to protect germ cells from apoptosis in a number of studies (Yoshinaga et al. 1991, Pesce et al. 1993, Yan et al. 2000C).

Androgen receptors (AR) located in Leydig, Sertoli and peritubular myoid cells bind testosterone (Bremner et al. 1994). Testosterone is a relative small fat-soluble molecule that reaches its target cells by simple diffusion in the testis. Having bound the ligand AR dimerizes, translocates from the cytoplasm to the nucleus and directly affects the transcription of androgen-responsive genes. It can either induce or repress the transcription of AR target genes depending on the factors that associate with the ligand-receptor complex (Hirawat et al. 2003). This mode of androgen action is known as the classical testosterone signalling. However, there is an increasing amount of evidence that cellular effects of testosterone can be mediated via AR-dependent interaction with cell signalling pathways, including the MAPK pathway (Walker 2009). This is called the non-classical testosterone signalling and it does not involve AR binding to DNA. Over the decades many different models have been employed to study the role of androgens in spermatogenesis (Dym & Raj 1977, Russell & Clermont 1977, Russell et al. 1981, Mason et al. 1986, Awoniyi et al. 1990, Chandolia et al. 1991, Meng et al. 2005, Petrusz et al. 2005, Denolet et al. 2006, Eacker et al. 2007). Based on the findings from these reports and those of others it can be concluded that testosterone has many functions in testis, including development and maintenance of testicular architecture, supporting germ cell differentiation (McLachlan et al. 1994, O'Donnell et al. 1994, O'Donnell et al. 1996) and meiotic cell survival (Henriksén et al. 1995, Erkkilä et al. 1997). In the adult withdrawal of testosterone leads to incomplete spermatogenesis and infertility demonstrating that testosterone is probably the most important single factor that is needed to maintain qualitatively normal spermatogenesis.

Cell-selective ablation of AR has elucidated the role of androgen signalling in the mouse testis. Mice lacking AR in germ cells, Sertoli cells, peritubular myoid cells, Leydig cells and universally in every cell type have been generated (Wang et al. 2009, Walters et al. 2010). Global AR knockout mice display external male-to-female sex reversal, small, immature and intra-abdominal testes, hypotrophy of accessory sex glands and spermatogenic arrest at the pachytene stage. Sertoli

cell-specific AR knockout mice have small testes and normal-sized accessory sex glands but spermatogenesis is arrested at meiosis. Germ cells fail to enter postmeiotic development in mice lacking AR in Leydig cells. In these mice the size of the testes and epididymides is small. Cell-specific ablation of AR in peritubular myoid cells results in small testis and accessory sex gland size, and low number of germ cells and sperm in the testis and epididymis, respectively. Germ cell-specific AR knockout mice are overtly normal (Wang et al. 2009, Walters et al. 2010). It can be concluded that disturbance in androgen action in the somatic cells of the testis severely affects fertility.

2.3 Hedgehog signalling pathway

According to the canonical pathway (Figure 5), Hedgehog (HH) ligand binds to Patched (PTCH) which in the absence of HH acts catalytically to suppress the activity of 7-transmembrane protein Smoothened (SMO) (Taipale et al. 2002). Inactivation of PTCH by HH binding releases SMO to activate GLI (glioma-associated oncogene homolog) proteins, a group of latent cytoplasmic transcription factors. *Gli1* and *Ptch* are among the first genes whose transcription is triggered in response to the activation of Hh signalling pathway (Hooper & Scott 1989, Lee et al. 1997). If there is no ligand available GLI is sequestered in the cytoplasm to a complex with Suppressor of Fused (SUFU), the negative regulator of the Hh pathway (Kogerman et al. 1999). SUFU is also able to repress GLI-mediated transcription (Cheng & Bishop 2002). On the other hand activation of Hh signalling induces turnover of SUFU through the ubiquitin–proteasome pathway (Yue et al. 2009). Hedgehog signalling has a crucial role in the development of many different tissues and organs (McMahon et al. 2003). In adults, it is needed to form and/or maintain stem and progenitor cell populations (Baron 2003, Machold et al. 2003, Adolphe et al. 2004, Maye et al. 2004, Palma & Ruiz i Altaba 2004). Hh signalling has been shown to play a crucial role in the development of mammalian testis but its significance in adult mammalian testis is largely unknown.

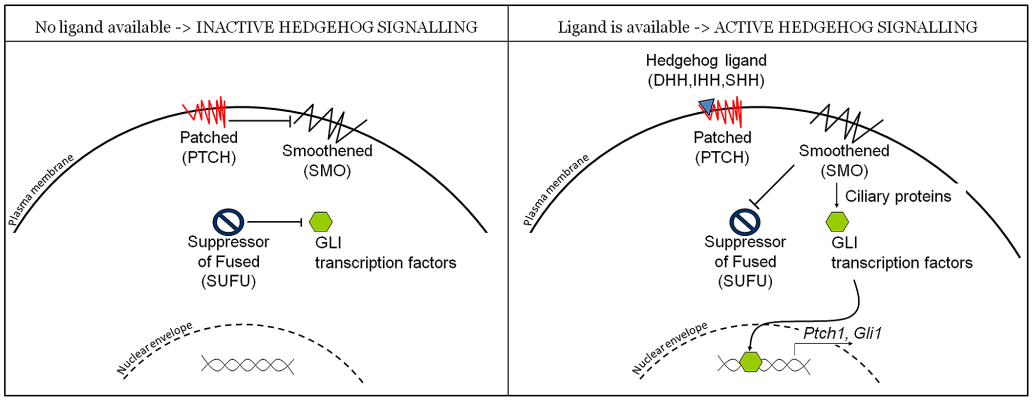

Figure 5. *Canonical Hedgehog signalling pathway. In the absence of Hedgehog ligand Patched (PTCH) inhibits Smoothened (SMO), and GLI transcription factors are sequestered in the cytoplasm by Suppressor of Fused (SuFu). Hedgehog ligand (DHH, IHH or SHH) binding relieves the inhibition of SMO by PTCH. This results in activation and nuclear translocation of GLI transcription factors and suppression of SUFU.*

There are three genes encoding three different Hedgehog proteins in mammals. Desert hedgehog (DHH) is the testis-specific member of the mammalian Hedgehog protein family. In

male gonadal primordium *Dhh* is one of the first genes to be expressed (on embryonic day (ED) 11.5) just approximately one day after *Sry* (sex-determining region of the Y chromosome), the ultimate initiator of testis development (Bitgood et al. 1996). *Dhh* deficient mice lack mature sperm and have obvious defects in testis histology but the severity of the phenotype largely depends on the genetic background of the mouse carrying the mutation (Bitgood et al. 1996, Clark et al. 2000, Pierucci-Alves et al. 2001). Chemical inhibition of Hh signalling by cyclopamine and forskolin in cultured early gonads repeats the disruptive effects of *Dhh* -/- on seminiferous tubule formation (Yao & Capel 2002). Hh signalling has a crucial role in the differentiation of fetal-type Leydig cells, too (Yao et al. 2002, Barsoum et al. 2009). Also adult-type Leydig cell differentiation is defective in *Dhh* KO mice (Clark et al., 2000). These findings imply that mice lacking *Dhh* are hypoandrogenic which probably contributes to the spermatogenic defect and provides an explanation for the feminized external genitalia phenotype of *Dhh* -/- mice (Clark et al. 2000). In patients, features of gonadal dysgenesis syndrome, including XY male-to-female sex reversal, has been seen to take place when *DHH* gene is mutated (Umehara et al. 2000, Canto et al. 2004, Canto et al. 2005, Das et al. 2011).

The regulation of Hh signalling in the testis is poorly characterized. However, there is evidence that environmental factors may impinge on it. Fowler et al. (2008) were able to find a connection between maternal smoking during pregnancy and a decreased *DHH* expression during testis development *in utero*. Brokken et al. (2009) demonstrated that the relationship of Hh signalling and androgen production in rat fetal testis is quite complex. Cyclopamine-induced inhibition of Hh signalling compromised testosterone synthesis, and the transcription of Hh pathway genes and steroidogenic enzymes was inhibited by the antiandrogen flutamide.

The expression of Hh pathway genes and proteins in the mouse testis has been studied. Bitgood et al. (1996), Szczepny et al. (2006) and Morales et al. (2009) demonstrated that Sertoli cells are the source of DHH in the mouse testis. Carpenter et al. (1998) indicated that *Ptch2* is strongly expressed in the seminiferous epithelium, whereas only a weak signal for *Ptch1* can be observed in the testicular interstitium. Szczepny et al. (2006) discovered that the mRNAs of *Gli1, Gli2, Gli3, Ptch2, Smo* and *Fused* are expressed by a wide variety of germ cells, whereas SUFU is restricted to postmeiotic cells. Morales et al. (2009) localized PTCH1 and SMO to late meiotic and postmeiotic cells. In summary, even though these findings are somewhat contradictory it seems that Hh signalling might work at many different levels during the spermatogenic differentiation. The processes that it participates, however, are not known. Cyclopamine-induced inhibition of Hh signalling in short segments of seminiferous tubule cultured in hanging drops did not affect the number proliferative or apoptotic cells but suggested that Hh pathway might regulate SCF/c-Kit signalling in spermatogenesis (Szczepny et al. 2009). Thus Hh signalling inhibition might secondarily affect the survival of differentiating germ cells (Yoshinaga et al. 1991; Hakovirta et al. 1999; Yan et al. 2000A, 2000B, 2000C). There is only one report about Hh signalling in the adult rat testis which indicates that DHH, PTCH1 and SMO are expressed in corresponding cells in the rat and mouse (Morales et al. 2009).

2.4 Cancerous inhibitor of PP2A (CIP2A)

CIP2A (cancerous inhibitor of protein phosphatase 2A (PP2A)) is a recently discovered novel PP2A-interacting protein that was shown to inhibit PP2A activity towards oncoprotein MYC (Junttila et al. 2007). CIP2A target protein PP2A plays an essential role in dephosphorylation

of serine/threonine residues of proteins and is thus regarded as a tumour suppressor protein (Eichhorn et al. 2009). Inhibition of CIP2A leads to dephosphorylation of serine-62 residue on MYC, a known target of PP2A, and MYC degradation (Junttila et al. 2007). Besides MYC-mediated signalling, CIP2A also takes part in regulating PP2A activity towards other signalling proteins, like PKB (protein kinase B) and DAPK (death-associated protein kinase) (Chen et al. 2010, Guenebeaud et al. 2010). CIP2A has a functional role in tumour growth promotion and it is overexpressed in many human cancers (Junttila et al. 2007, Li et al. 2008, Come et al. 2009, Khanna et al. 2009, Qu et al. 2010, Vaarala et al. 2010, Dong et al. 2011, Ma et al. 2011, Wang et al. 2011). This makes CIP2A an interesting target candidate for pharmacological cancer therapy. Besides tumours CIP2A is highly expressed in the testis but remarkably less in other tissues and fills thus the criteria to be classified as a cancer/testis (C/T) gene (Caballero & Chen 2009). Nowadays more than one hundred C/T genes have been reported in the literature. Due to the fact that the proteins encoded by C/T genes are expressed at a low level in normal tissues other than the testis suggests that inhibition of their function at systemic level might not have severe side effects (Caballero & Chen 2009). The physiological role of CIP2A has not been characterized and therefore the question remains whether CIP2A-targeting therapies would have profound systemic side effects. In neural progenitor cells CIP2A has been shown to increase self-renewal in a MYC-dependent manner (Kerosuo et al. 2010).

2.5 Stem cells

A stem cell has the unique potential to either multiply itself by self-renewal division or go through differentiation division and produce progeny that are destined to start the differentiation. Existence of stem cells is a prerequisite for the life-long physiological function of a tissue. Therefore adult stem cells (also known as tissue-specific stem cells) can be found in various tissues. In many tissues self-renewal of stem cells guarantees that the pool of differentiated cells can be replenished if they are lost through normal wear and tear or damage caused by disease or injury. Stem cells can be classified based on their differentiation potential: a totipotent stem cell (i.e., the zygote) is able to give rise to any cell type of the organism, including the extraembryonic tissue, pluripotent stem cells have the potential to differentiate into the cells of the three germ layers (ecto-, meso- and endoderm), multipotent stem cells can differentiate into cells from many but a limited number of lineages, for oligopotent cells the chances are more restricted, and unipotent stem cells can only give rise to a single differentiated cell type. Embryonic stem cells (ESCs) that can be isolated from the inner cell mass of a blastocyst and inner cell mass cells are examples of pluripotent stem cells. Haematopoietic stem cells are multipotential, whereas their direct descendants can only differentiate into a few cell types (oligopotent). Spermatogonial stem cell is an example of a unipotent stem cell, which can only give rise to one cell type, i.e. sperm cells, *in vivo*.

2.5.1 Spermatogonial stem cells

Primordial germ cells (PGCs) are developmentally the first germ cells and thus the predecessors of SSCs. In the mouse, they appear on ED 7 in a region posterior to the primitive streak, then proliferate and migrate through the hindgut and its mesentery to the genital ridge (McLaren 2003). PGCs populate the bipotential gonad at around the time when *Sry* expression is iniatiated in the somatic cells of the XY gonad on ED 10.5 (Koopman et al. 1990, Gubbay et al. 1990). SRY

induces testis development by orchestrating the differentiation of Sertoli cells (Jamieson et al. 1998, Wilhelm et al. 2005). In the early male mouse gonad PGCs proliferate until ED 14.5 and then enter mitotic arrest. These cells, now called gonocytes, are quiescent and only after birth simultaneously with relocating from the adluminal areas of the testis cords to the peritubulus they resume proliferation and are known from then on as type A spermatogonia. They probably consist of two different populations: one that directly enters the differentiation pathway to complete the first round of spermatogenesis without undergoing self-renewal; and another that transforms into SSCs that are ultimately responsible for the life-long ability of sperm production (de Rooij & Russell 2000, Yoshida et al. 2006).

In the adult mouse testis spermatogonial stem cells are located on the basement membrane of the seminiferous epithelium. A-single (A_s) spermatogonium is considered as the *in vivo* counterpart of SSC and in theory it is the only germ cell type that is able to go through either self-renewal or differentiation (de Rooij 1998). An A_s cell can thus divide into either two new A_s cells or two type A-paired (A_{pr}) spermatogonia that are committed to the differentiation pathway. A_{pr} cells connected by an intercellular bridge then give rise to A-aligned (A_{al}) spermatogonia that form chains of 4, 8 or 16 aligned and connected cells. A_s, A_{pr} and A_{al} cells are commonly referred to as undifferentiated spermatogonia. A_{al} spermatogonia differentiate into A_1 spermatogonia that go through six (in the mouse; species-specific) mitotic divisions to become primary spermatocytes that enter meiotic differentiation. A_1 cells and their premeiotic descendants (A_2, A_3, A_4, intermediate and B spermatogonia) are known as differentiating spermatogonia (Huckins 1971, Oakberg 1971). Even though A_s cells are probably the only true stem cells in theory they can be substituted by A_{pr} and A_{al} cells if a stem cell niche is depleted of A_s cells (Nakagawa et al. 2007, Nakagawa et al. 2010). Barroca et al. (2009) demonstrated that even differentiating progenitor spermatogonia are able to populate unoccupied stem cell niches and reconstitute spermatogenesis to experimentally induced infertile recipient. These results elucidate that there is fair deal of plasticity in the spermatogonial cell population and spermatogonia can dedifferentiate many steps backwards to become A_s cells.

In vivo SSC is defined by its ability to reconstitute spermatogenesis to congenitally or experimentally-induced infertile recipient (Brinster & Avarbock 1994, Brinster & Zimmermann 1994). In this procedure, known as transplantation test, germ cells are introduced into the adluminal compartment of the seminiferous tubules. Following attachment to the Sertoli cells SSCs migrate through the inter-Sertoli tight junctions (the blood-testis barrier) to the basal compartment of the seminiferous epithelium. This event, called homing, takes place within a few days after injection of the cells and is, based on the findings of Takashi Shinohara´s laboratory, dependent on β1-integrin and Rac1 (Ras-related C3 botulinum toxin substrate 1) protein function (Kanatsu-Shinohara et al. 2008B, Takashima et al. 2011). SSCs then proliferate and produce differentiating progeny and eventually mature spermatozoa by 2–3 months after transplantation (Nagano et al. 1999). Development of this technique as a functional bioassay of spermatogenic potential made it possible to evaluate whether an experimental cell population possesses SSC activity. However, despite its excellence in functionally defining SSCs, the transplantation test is a laborious assay and can only detect SSCs retrospectively. To address this problem Yeh et al. (2007) developed an *in vitro* test for SSCs. They noted that the number of clusters SSCs form *in vitro* is directly proportional to the number of spermatogenic colonies produced *in vivo* following transplantation.

Only 0.03% of all germ cells are thought to be SSCs in the adult mouse testis (Tegelenbosch & de Rooij 1993). They have been dispersed quite uniformly although not totally randomly along

the seminiferous tubule. Understandably, despite an ever-increasing knowledge about the cell surface markers (reviewed in He et al. 2009) that are preferably expressed by SSCs, their unequivocal isolation has not been achieved. These antigens include α6-integrin, β1-integrin, CD9 and Thy-1 (thymus cell antigen 1) that are known to be expressed by other stem cells, as well. Selection of germ cells positive for these proteins can be used to increase spermatogenic potential in the sorted fraction (Shinohara et al. 1999, Shinohara et al. 2000, Kanatsu-Shinohara et al. 2004A, Kubota et al. 2004). To date, no unique marker for SSCs is known. Recently, however, Arnold et al. (2011) found that *Sox2* (sex determining region Y box 2) is expressed at mRNA level only in solitary spermatogonia although they were not able to detect the protein. They also reported that Sox2 positive cells lack pluripotent capacity and are phenotypically spermatogonia due to their inability to form teratoma *in vivo* and ability to reconstruct spermatogenesis to congenitally infertile recipient mice, respectively (Arnold et al. 2011).

A unique property of undifferentiated spermatogonia is that they can spontaneously give rise to embryonic stem (ES) cell-like cells *in vitro* (Kanatsu-Shinohara et al. 2004B, Guan et al. 2006, Seandel et al. 2007). Interestingly, the four genes (*Klf4* (Krueppel-like factor 4)*, Myc, Oct4* (octamer-binding transcription factor 4) and *Sox2*; Takahashi & Yamanaka 2006) needed to transform a differentiated somatic cell into an ES cell (also known as reprogramming that results in the formation of induced pluripotent stem (iPS) cells) are expressed in SSCs (Kanatsu-Shinohara et al. 2008A). Dependence on GDNF is a hallmark of SSCs and accordingly transformation of SSCs into ES cell-like cells makes them independent of GDNF (Kanatsu-Shinohara et al. 2004B, Guan et al. 2006). Interestingly, in an alien environment lacking GDNF SSCs are able to adopt a fate dictated by the surroundings and differentiate into many different cell types (Boulanger et al. 2007, Guan et al. 2007, Glaser et al. 2008, Simon et al. 2009, Streckfuss-Bömeke et al. 2009, Ning et al. 2010). This transdifferentiation process does not include a prior transformation into ES cell-like cells but happens directly from SSCs demonstrating the plasticity of SSCs. The transformation of SSCs into ES cell-like cells makes them pluripotent and thus able to differentiate into derivatives of all three germ layers. These characteristics of SSCs put them in focus of many different fields of biomedical science, including regenerative medicine.

2.5.1.1 Testicular stem cell niche

Stem cell niche is defined as a microenvironment where stem cells reside, which interacts with stem cells by supporting their self-renewal (Xie & Spradling 2000). By definition, all characteristics of stem cells cannot exist anywhere else. Cells that are potentially responsive to the stem cell niche can exist outside the niche both naturally and after experimental procedure, such as stem cell transplantation. A definitive sign of having exited the niche is after undergoing a cell division the progeny of which is committed to differentiation. Thus the definition of the stem cell niche is more of functional and molecular than of anatomical nature. However, there are certain anatomical premises that specify where A_s cells are preferably located. Chiarini-Garcia and colleagues have demonstrated that the number of undifferentiated spermatogonia is higher in areas where a seminiferous tubule is neighboured by a patch of interstitium than by another tubule (Chiarini-Garcia et al. 2001, Chiarini-Garcia & Russell 2001, Chiarini-Garcia et al. 2003). Yoshida et al. (2007) refined this idea by showing that undifferentiated spermatogonia preferably reside in areas close to the vasculature. Based on what we know about molecular level regulation of SSC fate decision this makes perfect sense: in tubular areas where both vasculature and testicular interstitium are in close proximity the cells are exposed to the highest levels of

Leydig cell-derived CSF-1 (colony-stimulating factor) and FSH that stimulates GDNF production in Sertoli cells.

The major constituent of the testis stem cell niche is the Sertoli cells that dictate the number of stem cell niches and thus limit the population size of SSCs that they can support (Oatley et al. 2011). At molecular level Sertoli cell-derived GDNF (Meng et al. 2000) and CSF-1 originating from Leydig and myoid cells (Oatley et al. 2009) are probably the most important regulators of the testis stem cell niche. Receptors for these cytokines, GFR1A and CSF1R, respectively, are expressed by the undifferentiated spermatogonia (Meng et al. 2000, Hofmann et al. 2005, Kanatsu-Shinohara et al. 2003, Kubota et al. 2004, Kokkinaki et al. 2009). However, there are conflicting data whether GFR1A is a true SSC marker (Buageaw et al. 2005, Ebata et al. 2005, Grisanti et al. 2009).

2.5.1.2 Culture of undifferentiated spermatogonia

Despite the lack of success in isolating a pure population of SSCs, long-term culture of SSCs has been available for more than 10 years (Nagano et al. 1998). Recently the minimal requirements of SSC survival and ample propagation *in vitro* have been defined in greater detail (Kubota et al. 2004, Kanatsu-Shinohara et al. 2011). Using modern methods spermatogonial stem cells can be cultured virtually indefinitely *in vitro* in an environment deprived of serum and feeder cells but dependent of GDNF (Kanatsu-Shinohara et al. 2011). Even though fibroblasts and bone marrow stromal cells better support SSC activity in cultured spermatogonia when compared to Sertoli cell lines (Nagano et al. 2003) not all of the alleged SSCs *in vitro* are able to function like SSCs *in vivo*. In the presence of GDNF SSCs form clusters that are composed of heterogeneous cells, not solely SSCs (Ogawa et al. 2004). This is confirmed by transplantation assay showing that after long-term culture just a small fraction of the cells possesses SSC activity *in vivo* (Kanatsu-Shinohara et al. 2005A, Oatley & Brinster 2008, Lee et al. 2009). The percentage of true SSCs was found to be only 0.02% in one instance (Kanatsu-Shinohara et al. 2005B), which makes it comparable to the estimated proportion of SSCs within the whole germ cell population in adult mouse testis (0.03 %; Tegelenbosch & de Rooij 1993). Pure culture of SSCs has not been achieved and according to a recent article this is probably not even possible because SSCs seem to have an intrinsic program that stochastically self-directs their fate *in vitro* (Wu et al. 2009). This leads to a conclusion that completely homogeneous SSCs culture can never be achieved since some cells always produce differentiating progeny.

Many different groups have succeeded in establishing SSC culture *in vitro* by using a wide range of methods to isolate the cells and set-up the environment. This suggests that the purity of the original cell population is not of crucial importance and SSCs are actually quite flexible in the requirements about the environment. However, GDNF is indispensable for long term propagation of SSCs and basic fibroblast growth factor (bFGF) acts synergistically with it to support *in vitro* expansion of SSCs (Kubota et al. 2004). Long-term culture of undifferentiated spermatogonia has been achieved by plating the selected population of germ cells on laminin or on different kind of feeder cells. However, to date there are no reports available about long-term culturing of undifferentiated spermatogonia with the constituents of the testis stem cell niche, Sertoli and myoid cells even though this is probably the most physiologically relevant environment to study testis stem cell biology.

2.6 p53 signalling pathway

2.6.1 X-irradiation-induced activation of p53 signalling pathway

Exposure of a cell to ionizing radiation can result in DNA damage, such as double-strand breaks (DSBs). The affected cell can either try to repair the damage or enter apoptosis. Genetic alterations inflicted by DNA damage are especially problematic in germ cells since incomplete repair may impinge on fertility of the carrier and predispose its offspring to a wide range of disorders, including cancer. It has been shown that in spermatogonia and in many different cell types activation of the apoptotic pathway after irradiation-induced DSBs is a p53-dependent process (Odorisio et al. 1998). In adult rodent testis p53 is expressed in spermatocytes where its expression is also enhanced by irradiation (Almon et al. 1993, Sjöblom & Lähdetie 1996). X-irradiation strongly and promptly induces p53 in spermatogonia, as well, even though in undisturbed testis they lack p53 (Beumer et al. 1998). These data suggest that p53 is recruited to resolve the DNA damage inflicted by ionizing radiation. In p53 knock-out mice entry of germ cells into apoptosis is compromised after X-ray treatment (Beumer et al. 1998).

The effects of X-irradiation on testicular cells have been well characterized and documented (Meistrich et al. 1978). The adverse impact of irradiation is exerted on the mitotically active cells, i.e. spermatogonia, whereas the meiotic, postmeiotic and somatic testicular cells display radioresistance (Dym & Clermont 1970, Meistrich & Kangasniemi 1997). Within the spermatogonial population the radiosensitivity varies considerably and correlates with the level of differentiation making type A1 to B spermatogonia especially vulnerable to irradiation-induced apoptosis (van der Meer et al. 1992).

2.6.2 Nutlin-induced activation of p53 signalling pathway

Tumour suppressor p53 is mutated in more than half of human tumours emphasizing the importance of malfunctional p53 signalling for proliferative capacity of cancer cells (Hainaut & Hollstein 2000). In response to p53 pathway activation p21 is induced. p21 arrests cell cycle by inhibiting cyclin-cyclin dependent kinase complexes (reviewed in Gartel & Radhakrishnan 2005). Among all other stem cell systems spermatogenesis seems to be especially sensitive to changes in p53/p21 signalling (Liu et al. 2007). The role of p21 signalling in undifferentiated spermatogonia and testis in general is intriguing. Takubo et al. (2008) demonstrated that in Atm-deficient (ataxia telangiectasia mutated) undifferentiated spermatogonia p21 is upregulated which leads to cell cycle arrest and prejudices their SSC capacity. This defect was partially rescued by suppressing p21 in Atm mutant mice. Kanatsu-Shinohara et al. (2010) confirmed the adverse effect of p21 activation on SSC proliferation and showed that p21 expression can be suppressed by cytokines (GDNF, EGF (epidermal growth factor) and bFGF). Lack of p21, on the other hand, does not affect SSC number but might provide more differentiated germ cells a growth advantage over wild-type (WT) cells (Kanatsu-Shinohara et al. 2010). Nutlin-3 is a small molecule that inhibits the interaction of MDM2 (murine double minute 2) and p53. This leads to activation of p53 and p21 signalling pathways, and thus cell cycle arrest and growth inhibition in tumours (Vassilev et al. 2004). Nutlin-3 is known to induce differentiation of human ES cells (Maimets et al. 2008), but the effect of nutlin-3 on testicular cells has not been previously studied.

3 AIMS OF THE PRESENT STUDY

Different steps of spermatogenesis and their regulation are challenging targets for *in vivo* studies. Use of established *in vitro* methods and development of new ones are needed to gain a deeper understanding about the cellular events, and molecular interactions behind them, that are needed to drive the life-long process of spermatogenesis. The present study aims at clarifying the biology of germ-line stem and progenitor cells, and finding out what is the role of Hedgehog signalling in spermatogenesis. To do this we have used a wide range of different methods, both *in vivo* and *in vitro*, to approach these questions from different perspectives.

The specific aims were:

1) To elucidate the role of Hedgehog signalling in rat spermatogenesis and find out how the Hedgehog signalling pathway is regulated (I).

2) To gain an insight into the physiological function of CIP2A in mouse spermatogenesis and evaluate whether systemic inhibition of it would have adverse effects (II).

3) To find out what are the most primitive cells in the adult mammalian testis like. Are they unipotent or is their unipotent nature dictated by the surrounding somatic cells? Do they possess pluripotent capacity or is the spontaneous induction of pluripotency an in-built property of these cells? How are these cells regulated and do they have a physiologically relevant role in spermatogenesis (III)?

4) To develop a method that would allow Nanog-expressing spermatogonia to be studied *in vitro* for an extended period of time (IV).

4 MATERIALS AND METHODS

4.1 Experimental animals and treatments

Male C57BL/6J and transgenic mice universally expressing eGFP (enhanced green fluorescent protein; Okabe et al. 1997; purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) and maintained in NMRI strain in Turku Center for Disease Modeling (TCDM, Turku Finland)), and Sprague-Dawley rats were housed in plastic cages (Tecniplast, Buguggiate, Italy) in a climate-controlled room at 21 ± 3 °C with relative humidity of 55 ± 15% at the Animal Centre of Turku University (Turku, Finland). Aspen chips (Tapvei Co., Kaavi, Finland) were used as bedding material. Animals were maintained on a 12 h light/12 h dark cycle (lighted from 07 to 19 h) and they had free access to tap water and standard laboratory animal feed (Commercial RM3 (E) SQC, Special Diet Service, Witham, UK). All animal experiments were approved by the Turku University Committee on the Ethics of Animal Experimentation.

4.1.1 Generation and characterization of CIP2A transgenic mouse line

The gene-trap Ola129 ES cell clone CD0252, from the Sanger Institute (Cambridge, UK), presents insertion of the pGT0Lxf vector, containing β-galactosidase–neomycin-resistance fusion protein (β-geo) and the engrailed 2 gene, in the first intron of murine CIP2A gene (original communication II, Fig. 1A). The cassette is transcribed into a gene whose product is a truncated CIP2A protein, limited to exon 1 that is fused to the β-geo protein. Expression of the CIP2A exon 1 - β-geo mRNA was confirmed using RT-PCR (reverse transcription polymerase chain reaction; II, Supplementary figure 1). Chimeras were generated by using an embryonic stem cell - embryo co-culture method and were then mated with C57BL/6J mice. The strain was subsequently maintained on a C57BL/6J genetic background.

Genotyping. Genomic DNA was isolated from mouse ear. One microgram of it was added to the 50 µl of PCR mix, which contained 75 mM Tris-HCl (pH 9.0), 50 mM KCl, 2 mM $MgCl_2$, 20 mM ammoniumsulfate, 0.2 µM of each primer (II, Supplementary table 2), 0.2 mM dNTPs and 2.5 U DNA polymerase (Biotools Native DNA Polymerase; Biotools, Madrid, Spain). Thermal cycling protocol was, as follows: initial DNA denaturation at 96°C for 4 min, followed by 96°C for 45 sec, 57°C for 30 sec, and 72°C for 1 min for 25 cycles. The protocol was completed by a final elongation step at 72°C for 10 min. PCR products were analysed by agarose gel electrophoresis and the fragments were visualized with ethidium bromide and UV light exposure.

Counting the epididymal sperm count. The capacity of sperm production was done by counting the number of sperm in the cauda epididymis. First the cauda epididymis was dissected and moved in a well of a 24-well cell culture plate in 1 ml of pre-warmed Dulbecco's PBS (phosphate-buffered saline). Then using iris scissors two transverse and one longitudinal cut was made to disrupt the tissue architecture and allow the movement of sperm from the epididymal duct to the medium. After 15 min incubation (37°C, 5% CO_2, humidified atmosphere) the suspension was mixed by manual pipetting, diluted and counted for sperm concentration under a light microscope using the Bürker chamber (Fortuna, Germany).

Blood counts and hormonal analyses. Mice were anaesthetized with 2.5 % Avertin (Aldrich Chemical Co., Milwaukee, WI, USA) s.c. to collect whole blood in EDTA-coated (ethylenediaminetetraacetic acid) haematocrit tubes by heart punch. Advia120® instrument (Bayer, Siemens Medical Solutions Diagnostics, Erlangen, Germany) was used to determine differential leukocyte counts and complete blood counts. Immunoflurometric assays (Delfia; Wallac, Turku, Finland) were used to measure serum FSH as described previously (van Casteren et al. 2000). Serum samples were extracted two times with diethyl ether and thereafter a RIA protocol described by Huhtaniemi and colleagues (Huhtaniemi et al. 1985) was used to determine serum testosterone levels.

4.1.2 *In vivo* X-irradiation

Two-month-old mice were used for the X-irradiation experiment. The mice were anaesthetised with 2.5% Avertin (Aldrich Chemical Co., Milwaukee, WI, USA) i.p. and locally irradiated (with a water-equivalent build-up layer, focus-target distance 100 cm, field size 4 x 10 cm, dose rate 3 Gy/min) by 3-4 Gy using 6 MV X-rays produced by a Clinac 600C linear accelerator (Varian, Palo Alto, CA, USA). The mice were sacrificed 6, 17, 24, 72, 96 or 144 hours after X-irradiation and their testes were dissected and used in protein and mRNA analyses. Radiation dose in the testis was determined mathematically using a computer tomography based Eclipse planning system (Varian, Palo Alto, CA, USA). Control mice were subjected to the same treatment omitting the X-irradiation.

4.1.3 Experimental cryptorchidism

Two-month old mice were rendered cryptorchid by unilaterally suturing the testis to the abdominal wall. The mice were killed 4-6 months post-operatively and the testes were adopted for future analyses. The un-operated testis served as a control.

4.1.3 Imatinib mesylate treatment

Five-day-old male rats were injected with imatinib mesylate (STI571, Glivec®; Novartis Pharma AG, Basel, Switzerland) intracavitally into the stomach. The dose (50 or 150 mg/kg) was applied either once or on three consecutive days. Control rats were injected with the solvent for imatinib (water). Rats were sacrificed by CO_2 asphyxiation and cervical dislocation on postnatal days 6, 7 and 8. The testes were dissected, decapsulated and snap-frozen in liquid nitrogen (Nurmio et al. 2007).

4.2 Transillumination-assisted microdissection of seminiferous tubules

Mice and rats were killed by CO_2 asphyxiation and cervical dislocation. The testes were dissected and decapsulated. Segments of seminiferous tubule were dissected on a Petri dish containing PBS or culture medium (described below) under a stereomicroscope using transillumination-assisted microdissection method (Parvinen & Vanha-Perttula 1972, Toppari & Parvinen 1985).

4.3 Hormones and growth factors

Following hormones, growth factors and small molecule inhibitors were used in the *in vitro* experiments: recombinant human follicle-stimulating hormone (rhFSH; Gonal F, Serono, Geneva, Switzerland), recombinant mouse glial cell-line derived neurotrophic factor (rmGDNF; PeproTech, London, UK), cyclopamine (LC Laboratories, Woburn, MA, USA) and nutlin-3 (Cayman Chemical, Ann Arbor, MI, USA).

4.4 Tissue culture and stimulation

Mouse and rat testes were decapsulated and moved on a Petri dish containing Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (1:1 DMEM/F12; GIBCO BRL, Paisley, UK) supplemented with 0.1% (w/v) BSA (bovine serum albumin), Penicillin-Streptomycin (50 U and 50 µg/ml, respectively; Gibco 15140; Gaithersburg, MD, USA) and gentamicin sulfate (50 mg/ml; Biowhittaker; Lonza, Walkersville, MD, USA). Five 3-mm-long unstaged segments or ten 1-mm-long segments representing stages XII of the mouse seminiferous epithelial cycle were cultured in 1 ml of DMEM/F12 medium supplemented with 0.1% (w/v) BSA, Penicillin-Streptomycin (50 U and 50 µg/ml, respectively; Gibco 15140; Gaithersburg, MD, USA), gentamicin sulfate (50 mg/ml; Biowhittaker; Lonza, Walkersville, MD, USA) in the presence or absence of 10 µM nutlin-3. After 24, 48 and 72h incubation (34°C, 5% CO2, humidified atmosphere) the cultured segments of mouse seminiferous tubules were collected and snap-frozen in liquid nitrogen.

Six 2-mm-long segments of stages II-VI, VII-VIII and IX-I from three rats were cultured in 1 ml of DMEM/F12 medium supplemented with 0.1% (w/v) BSA, Penicillin-Streptomycin (50 U and 50 µg/ml, respectively; Gibco 15140; Gaithersburg, MD, USA), gentamicin sulfate (50 mg/ml; Biowhittaker; Lonza, Walkersville, MD, USA) and MIX (1-methyl 3-isobutyl xanthine, 0.2 mM; Aldrich Chemie, Steinheim, Germany) in the presence or absence of recombinant human FSH (1, 10, 25, 50 and 200 ng/ml). After 8h and 30h incubation (34°C, 5% CO2, humidified atmosphere) the cultured segments of rat seminiferous tubules were collected and snap-frozen in liquid nitrogen. Within individual experiments each dose was applied on at least three parallel samples.

4.5 Isolation and culture of spermatogonial stem cells

Six (7-10-week-old) male mice were sacrificed to get 20 segments of seminiferous tubule representing stages I-VI, VII-VIII and IX-XII. The segments were treated with 0.05% trypsin for 10 minutes and centrifuged thereafter. Testicular cells were then cultured for 7 days on 0.1% gelatin-coated culture dishes in medium that contained 4 ng/ml GDNF (Peprotech), 15% fetal calf serum (FCS). The cells were then removed on a feeder layer of mitomycin C-inactivated mouse embryonic fibroblasts with basic ESC medium [(Knock out DMEM (Gibco; cat. code 10829), 15% fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 0.12% 2-mercaptoethanol, and 50 U/ml penicillin + 50 µl/ml streptomycin) containing 103 units/ml LIF (leukaemia inhibitory factor; ESGRO)] for 4-5 weeks with 3 passages. The medium was exchanged on a daily basis. Thereafter, colony formation was observed and the colonies were counted. At specific time points cells were fixed for immunocytochemical staining or collected for RT-PCR analysis.

4.6 Establishment of a co-culture system for mouse seminiferous tubule cells

Mice (aged from less than 10 days to 17 months) testes were decapsulated and moved on a Petri dish containing sterile PBS (pH 7.4). Thirty to forty 4-mm-long segments of seminiferous tubule were dissected and moved onto a gelatin-coated cultivation flask with small amount of medium (15% iFCS (inactivated fetal calf serum) in DMEM). The segments were allowed to attach on the bottom of the flask for 6-10 hours (37°C, 5% CO2, humidified atmosphere). Thereafter normal amount of medium (15% iFCS in DMEM) was added and exchanged every two to three days. Seminiferous tubule cells start to proliferate and migrate out from segments of seminiferous tubules *in vitro* (Eddy & Kahri 1976). We observed this event for seven days by phase-contrast microscopy and monitored 80% confluence in the end of the period. Samples were collected for RNA and protein expression analysis. The system was monitored for additional five weeks and sampled weekly or used as a feeder layer for allogeneic seminiferous tubule cells.

4.6.1 Plating of allogeneic cells on one-week-old co-culture

For this purpose 30-40 segments of C57BL/6J or eGFP-expressing mouse seminiferous tubules were dissected and trypsinized (0.05% trypsin, 37°C, 10 min, manual pipetting). After centrifugation (1200 rpm, 5 min) the cell suspension was plated onto a one-week-old culture. Samples were collected for analysis 7, 10, 14, 21 and 28 days after this procedure.

4.6.2 Experimental manipulation of the one-week-old co-culture

The one-week-old co-culture was subjected to rhFSH (10 ng/ml), rmGDNF (10 ng/ml) or nutlin-3 (10 µM). The culture medium (15% iFCS in DMEM) was supplemented with MIX (1-methyl 3-isobutyl xanthine, 0.2 mM; Aldrich Chemie, Steinheim, Germany) in the FSH experiment. Samples were collected 6, 12, 24, 48 and 72 hours after treatment by scraping off the cells, pelleting them by centrifugation (1200 rpm, RT, 5 min) and snap-freezing them in liquid nitrogen. Additionally, in the FSH experiment the medium was exchanged after 72 hours and samples were collected 24 hours later. In the nutlin-3 experiment samples were harvested 24, 48 and 72 hours after the exposure. Within individual experiments each treatment was applied on three parallel samples.

4.7 Introduction of short-interfering RNAs (siRNAs)

Isolated spermatogonial cells or immortalized mouse spermatogonia (GCIspg) were adapted to 50 - 250 nM concentration of CIP2A or scramble (SCR) siRNA (II, Supplementary table 2) or medium (negative control). siRNA was transfected with Oligofectamine reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer´s instructions.

4.8 RNA isolation and processing

Total RNA from freshly collected or cultured segments of mouse and rat seminiferous tubules, cell pellets and cell homogenates was isolated using Trisure reagent (Bioline, London, UK) according to

the manufacturer's instructions. Tissue samples were homogenized with a disperser (UltraTurrax; IKA® Werke GmbH & Co. KG, Staufen, Germany) in 600 µl RLT buffer (from the Qiagen RNeasy Mini Kit; Qiagen, Hilden, Germany) supplemented with 6 µl β-mercaptoethanol. Thereafter total RNA was extracted using Qiagen RNeasy Mini Kit according to the manufacturer's instructions. After isolation, RNA concentration was measured using NanoDrop device (ND-1000; NanoDrop Technologies, Wilmington, DE, USA). RNA quality was assessed visually by confirmation of intact 28S and 18S ribosomal bands following agarose gel electrophoresis and ethidium bromide staining. Before cDNA synthesis traces of contaminating genomic DNA were removed by treating the samples with DNase I (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using DyNAmo cDNA synthesis Kit (F-470; Finnzymes, Espoo, Finland). 500 ng of template RNA was reverse-transcribed in a 20 µl reaction with oligo(dT) primers. DNase I-treated samples were split to two and one half was used for cDNA synthesis while the other served as a template in RT- reaction.

4.9 Quantitative RT-PCR (qRT-PCR)

To avoid amplification of genomic DNA, if feasible, primers (Tables 1-2) were designed to be located to different exonic sequences with the help of Primer 3 software (http://frodo.wi.mit.edu/) and mRNA sequence data available at Ensembl (www.ensembl.org/) and NCBI (http://www.ncbi.nlm.nih.gov/) databases. Amplification of target cDNAs was done using CFX96 real-time PCR detection system device (Bio-Rad Laboratories Inc., Hercules, CA, USA) and the DyNAmo Flash SYBR green qPCR kit (F-415L; Finnzymes, Espoo, Finland) according to the manufacturers' instructions. Quantitative real-time PCR was performed under the following conditions: 95°C for 7 min followed by 40 cycles of 94°C for 1 s and 54-64°C (depending on the primer pair; see Tables 1-2) for 15s. Relative gene expression data was normalized to expression level of endogenous house-keeping genes (Ppia (cyclophilin A) and L19 (L19 ribosomal protein) for mouse cDNAs; Gapdh (glyceraldehyde-3-phosphate dehydrogenase), Hprt1 (hypoxanthine phosphoribosyltransferase 1) and S26 (S26 ribosomal protein) for rat cDNAs) using 2^-ΔΔC(t) method (Livak & Schmittgen 2001). PCR specificity was verified by gel electrophoresis and melting curve analysis. One band of the expected size and a single peak, respectively, were required.

Table 1. *Design, annealing temperatures and PCR product lengths of the mouse primers.*

Mouse Gene	Accession number	Annealing temperature	Primers	Product length
Acta2	NM_007392	62°C	5´-TGCTGTCCCTCTATGCCTCT-3´ 5´-GAAGGAATAGCCACGCTCAG-3´	184 bp
CD9	NM_007657	56°C	5´-TGCAGTGCTTGCTATTGGAC-3´ 5´-GGCGAATATCACCAAGAGGA-3´	219 bp
CIP2A	NM_172616	57°C	5´-GCGCCATGTACTCAGTCAGA-3´ 5´-AGGAAGCAGAAGGGTCACAA-3´	234 bp
c-Kit	NM_021099	56°C	5´-ATCCCGACTTTGTCAGATGG-3´ 5´-AAGGCCAACCAGGAAAAGTT-3´	192 bp
Cyp17a1	NM_007809	60°C	5´-GATACAAGCCAAGATGAATGCAG-3´ 5´-CAGCACAGAGCTAGTTGTCT-3´	136 bp
Dppa4	NM_028610	62°C	5´-AGGGCTTTCCCAGAACAAAT-3´ 5´-GCAGGTATCTGCTCCTCTGG-3´	163 bp
Epcam	NM_008532	58,5 °C	5´-GACGACGTGGACATAGCTGA-3´ 5´-GACACCACCACAATGACAGC-3´	217 bp
Erm	NM_023794	57°C	5´-CAAGAGCCCCGAGATTACTG-3´ 5´-CTCGGGTACCACGCAAGTAT-3´	146 bp
Gdnf	NM_010275	61°C	5´-CGGACGGGACTCTAAGATGA-3´ 5´-CGTCATCAAACTGGTCAGGA-3´	209 bp
Gfr1α	NM_010279	63°C	5´-TGTACTTCGTGCTGCCACTC-3´ 5´-GCTGAAGTTGGTTTCCTTGC-3´	166 bp
Gpr125	XM_991709	59°C	5´-GACCTGACGAACAACCGAAT-3´ 5´-CTGGTGTCTCGCACAGTGAT-3´	235 bp
Itga6	NM_008397	55°C	5´-TGAAGATGGGCCCTATGAAG-3´ 5´-CTCTTGGAGCACCAGACACA-3´	152 bp
ki-67	XM_001000692	56°C	5´-TTGGAAAGGAACCATCAAGG-3´ 5´-TTTCTGCCAGTGTGCTGTTC-3´	214 bp
Klf4	NM_010637	59°C	5´- CTGAACAGCAGGGACTGTCA -3´ 5´-GTGTGGGTGGCTGTTCTTTT-3´	218 bp
L19	NM_009078	55°C	5´-GGACAGAGTCTTGATGATCTC-3´ 5´-CTGAAGGTCAAAGGGAATGTG-3´	195 bp
Lin28	NM_145833	57°C	5´-CAGAAGCGAAGATCCAAAGG-3´ 5´-CAGGCTTTCCCTGAGAACTG-3´	180 bp
Nanog	NM_028016	64°C	5´-CCAGTGGAGTATCCCAGCAT -3´ 5´-GAAGTTATGGAGCGGAGCAG -3´	236 bp
Oct4	NM_013633	64°C	5´-CACGAGTGGAAAGCAACTCA-3´ 5´-AGATGGTGGTCTGGCTGAAC-3´	245 bp
PLZF	NM_001033324	59°C	5´-AACGGTTCCTGGACAGTTG-3´ 5´-CCCACACAGCAGACAGAAGA-3´	172 bp
Ppia	NM_008907	63°C	5´-CATCCTAAAGCATACAGGTCCTG-3´ 5´-TCCATGGCTTCCACAATGTT-3´	164 bp
p21	NM_007669	60°C	5´-TGTCCAATCCTGGTGATGTCC-3´ 5´-GTCAAAGTTCCACCGTTCTCG-3´	151 bp
Sox2	NM_011443	61°C	5´-CACAACTCGGAGATCAGCAA-3´ 5´-CTCCGGGAAGCGTGTACTTA-3´	189 bp
Stra8	NM_009292	57°C	5´-ATGCAATGTTGCTGAAGTGC-3´ 5´-GGAAGCAGCCTTTCTCAATG-3´	161 bp
Sycp3	NM_011517	60°C	5´-AGCCAGTAACCAGAAAATTGAGC-3´ 5´-CCACTGCTGCAACACATTCATA-3´	106 bp
Thy-1	NM_009382	61°C	5´-GGGCGACTACTTTTGTGAGC-3´ 5´-GGAGGAGGGAGAGGGAAAG-3´	163 bp

Table 2. *Design, annealing temperatures and PCR product lengths of the rat primers.*

Rat Gene	Accession number	Annealing temperature	Primers	Product length
Dhh	AF148226	64°C	5´-GACCTCGTCCCCAACTACAA-3´ 5´-AACCTTCAGTCACGCGTAGG-3´	159 bp
Fshr	NM_199237	56°C	5´-CCTCTGGGCCAGTCATTTTA-3´ 5´-GTTCAGAGATTTGCCGCTTC-3´	213 bp
Gapdh	NM_017008	55°C	5´-AGACAGCCGCATCTTCTTGT-3´ 5´-CTTGCCGTGGGTAGAGTCAT-3´	207 bp
Gdnf	NM_019139	61°C	5´-CGGACGGGACTCTAAGATGA-3´ 5´-CGTCATCAAACTGGTCAGGA-3´	205 bp
Gli1	XM_345832	63°C	5´-GGTTATGGGTCTGCCAGAGA-3´ 5´-GCTGGGTGAGGTACGGATTA-3´	159 bp
Gli2	NM_ 01107169	60°C	5´-TACGAGACCAACTGCCACTG-3´ 5´-CCTTTGAGCAGCCTTCAAAC-3´	235 bp
Gli3	XM_225411	59°C	5´-TTCTGAACCCTGTCCAGGTC-3´ 5´-TCTTTGTCCCCTTCCTCCTT-3´	207 bp
Hprt1	NM_012583	54°C	5´-AAGCTTGCTGGTGAAAAGGA-3´ 5´-CCGCTGTCTTTTAGGCTTTG-3´	185 bp
Ihh	NM_053384	60°C	5´-GACCGCGACCGAAATAAGTA-3´ 5´-ACGCTCCCCAGTTTCTAGGT-3´	182 bp
Ptch1	NM_053566	58°C	5´-GGCTGAGAGCGAAGTTTCAA-3´ 5´-GCCTCTGTGGTCAGGACATT-3´	271 bp
Ptch2	NM_ 01108975	63°C	5´-CCAGGAACCTCTGGATTTCA-3´ 5´-GATGCGAAGGTTCTCTCCAG-3´	207 bp
S26	XM_001066146	57°C	5´-AAGGAGAAACAACGGTCGTG-3´ 5´-GCAGGTCTGAATCGTGGTG-3´	300 bp
Shh	NM_017221	60°C	5´-GGAACTCACCCCCAATTACA-3´ 5´-TCACTCGAAGCTTCACTCCA-3´	151 bp
SuFu	NM_ 01024899	60°C	5´-GGTCCCTGGCTGATAACTGA-3´ 5´-GTCTTTGCCAGACAGCCTTC-3´	224 bp

4.10 Squash preparations

Four-mm-long segments representing stages II-VI of the rat seminiferous epithelial cycle were incubated 8 and 16h in the medium and conditions mentioned above in the presence of 100 µM cyclopamine or vehicle only. After the incubation the segments were transferred onto a Petri dish containing PBS and cut to 2mm of length under a stereomicroscope by taking approximately 1mm off from both ends. Squash preparations were made as described by Toppari et al. (1985). Briefly, the segments were taken in 15µl of PBS onto a microscope slide. The tubule segments were carefully squashed between microscope slides and cover slips and the formation of a cellular monolayer was monitored by phase-contrast microscopy. Then the squash preparations were snap-frozen in liquid nitrogen and the cover slips were removed. The specimens were incubated in ice-cold 96% (v/v) ethanol for 3 minutes, allowed to air-dry overnight and stored at -80°C.

4.11 Immunohistochemistry (IHC)

4.11.1 Immunohistochemical stainings

Mouse and human testes were fixed in formalin and rat testes in 4% (v/v) paraformaldehyde (PFA) at +4°C overnight. The fixed samples were dehydrated by using graded ethanol solutions

and stored in 70% (v/v) ethanol at +4°C. After paraffin embedding 5-µm-thick sections were cut and mounted onto poly-lysine-coated slides. Following dewaxing and rehydration with xylene and serial ethanol the sections were washed once in buffer (PBS or TBS (Tris-buffered saline); see Table 3) and twice in buffer+0.2% (v/v) Triton X-100 for 5 min each. Antigen retrieval was performed by microwaving (300 W) the sections for 15 min in sodium citrate buffer (10 mM, pH 6.0). From this step on the staining was performed using Novocastra Novolink kit (Leica Microsystems Inc., Bannockburn, IL, USA) or the following protocol. After washing for 5 minutes in buffer+0.2% (v/v) Triton X-100 two times, autofluorescence was quenched by treating the sections with 100 mM NHCl4 for 3 minutes. Following washes twice with buffer+0.2% (v/v) Triton X-100 and once with buffer alone, blocking solution (20% (v/v) normal goat serum (NGS) or 20% (v/v) BSA in buffer+0.2% (v/v) Triton X-100) was applied to each section and incubated 1 h at RT. After blocking, sections were incubated overnight at +4°C in the presence of primary antibody (see dilution in Table 3, in buffer+0.2% (v/v) Triton X-100 + 1.5% (v/v) NGS or BSA). Primary antibody was omitted in negative controls.

On the following day the sections were washed three times 5 minutes in the buffer. Then a solution with a proper secondary antibody was applied onto a section. Secondary antibodies used were conjugated to fluorescent dyes (Table 4). After 1 hour secondary antibody incubation the sections were washed three times with the buffer and mounted with UltraCruz mounting medium (sc-24941, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Images were captured using a Leica DFC320 camera (Leica, Wetzlar, Germany) installed on a DMRBE microscope from the same manufacturer. At least four sections from at least 3 different animals were included in all IHC analyses.

Human testicular samples for immunohistochemical analyses were obtained from patients who had underwent orchiectomy due to a diagnosis of a testicular neoplasm. Fetal testicular material was collected from routinely autopsied male fetuses after abortion at 14-22 weeks of gestation. The use of human tissue material was approved by the Finnish national authority for medicolegal affairs (Dnro 889/04/047/08) and University of Turku ethical committee (Dnro 146/2007). According to approvals from these authorities an informed consent was not obtained from the tissue donors of this study.

4.11.2 Immunocytochemical stainings

4.11.2.1 On a cell monolayer on a coverslip

Co-culture of seminiferous tubule cells was established on a gelatin-coated coverslip. The specimen was fixed in cold 4% PFA for 10 minutes and stored in cold PBS. Fixed cells were washed three times 5 min in PBS, and then permeabilized with 0.5% Triton X-100 in PBS for 10 min followed by two washes with PBS and PBS+0.1% gelatin for 5 min. Before overnight primary antibody treatment the specimens were incubated (1 h, RT) in 20% NGS or BSA. Negative controls were incubated with normal non-immunized appropriate animal serum. After two washes with PBS and PBS+0.1% gelatin 5 min each, the specimens were incubated with appropriate secondary antibody solution (1 h, RT). The specimens were washed three times with PBS and mounted with UltraCruz mounting medium (sc-24941, Santa Cruz Biotechnology Inc.) Images were captured using a Leica DFC320 camera (Leica, Wetzlar, Germany) installed on a DMRBE microscope from the same manufacturer.

Table 3. *List of primary antibodies used in immunohistochemical and immunocytochemical stainings.*

Antigen	Antibody code	Origin, clonality	Dilution	Buffer	Manufacturer
CIP2A	-	rabbit, polyclonal	1:10000	PBS (pH 7.4)	non-commercial (Soo Hoo et al. 2002)
GLI1	sc-20687	rabbit, polyclonal	1:100	TBS (pH 7.55)	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
KI-67	M7240	mouse, monoclonal	1:5000	PBS (pH 7.4)	Dako Corporation, Santa Barbara, CA, USA
Nanog	sc-33760	rabbit, polyclonal	1:200	PBS (pH 7.4)	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
Oct4	sc-9081	rabbit, polyclonal	1:200	PBS (pH 7.4)	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
PLZF	AF2944	goat, polyclonal	1:200	PBS (pH 7.4)	R&D Systems, Minneapolis, MN, USA
PTCH1	sc-6149	goat, polyclonal	1:100	PBS (pH 7.4)	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
Stra8	ab49602	rabbit, polyclonal	1:200	PBS (pH 7.4)	Abcam Inc., Cambridge, MA, USA
SUFU	sc-10933	goat, polyclonal	1:100	PBS (pH 7.4)	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
WT1	sc-846	rabbit, polyclonal	1:200	PBS (pH 7.4)	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA

Table 4. *List of fluorescent secondary antibodies used in immunohistochemical and immunocytochemical stainings.*

Conjugated to	Antibody code	Origin	Target	Dilution	Manufacturer
AlexaFluor594	A11037	donkey	goat IgGs	1:200	Invitrogen, Carlsbad, CA, USA
FITC	711-095-152	donkey	rabbit IgGs	1:200	Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA
Texas Red	sc-2783	goat	rabbit IgGs	1:200	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA

4.11.2.2 On squash preparation for cleaved Caspase-3

Air-dried squash preparations were stored at -80°C and fixed in 4% (v/v) PFA for 10 minutes. After fixation the specimens were washed twice with TBS (pH 7.55) for 5 minutes and antibody penetration was improved by incubating the specimens 10 min in TBS + 0.2% (v/v) Triton X-100. From this step on cleaved Caspase-3 staining was performed using Novocastra Novolink Polymer Detection Systems (Leica Microsystems Inc., Bannockburn, IL, USA) according to the manufacturer's instructions. Cleaved Caspase-3 primary antibody was purchased from Cell Signalling Technology (Asp175, Beverly, MA, USA) and was used at a dilution of 1:200.

4.12 Western blotting

The samples were first lysed in RIPA buffer (150 mM NaCl, 10 mM Tris (pH 7.2), 0.1% sodium dodecyl sulphate, 1.0% Triton X-100, 1% deoxycholate and 5 mM EDTA) and the protein

concentration was then measured using the Bradford procedure (Bio-Rad Laboratories Inc., Hercules, CA, USA). Tissue samples were analysed by Western blotting using mouse monoclonal actin (Santa Cruz Biotechnology Inc.), rabbit polyclonal CIP2A antibody (Soo Hoo et al. 2002) and rabbit polyclonal Oct4 antibody (sc-9081; Santa Cruz Biotechnology Inc.) and the corresponding horseradish peroxidase-conjugated (HRP) IgG secondary antibodies (purchased from Santa Cruz Biotechnology Inc.).

4.13 Collection, presentation and statistical analysis of the data

Each experiment was repeated at least three times independently. Parametric data is represented as mean ± standard error of the mean (SEM). The results were analysed for statistically significant differences using one-way analysis of variance (ANOVA), followed by Tukey´s test for multiple comparisons of independent groups of samples. Student's t-test was used for pairwise comparison of independent groups of samples. The p values less than 0.05 were considered statistically significant.

5 RESULTS

5.1 Age-dependent changes in mRNA levels in the mouse and rat testis (I, II, III)

5.1.1 In the mouse testis

As analysed by qRT-PCR the highest relative levels of *Nanog*, *Oct4* and *ki-67* transcripts were observed in newborn mouse testis but their levels dramatically decreased thereafter (original communication III, Fig. 3C; II, Supplementary figure 6A). *CIP2A, PLZF* and *Stra8* were at their highest level in 10-day-old mouse testis, whereas transcript of *Sycp3* was found accumulated in 40-120-day-old mouse testis (II, Supplementary figure 6A; III, Fig. 3C). It is noteworthy that the transcript levels of *Stra8* and *Sycp3* are almost undetectably low in the newborn mouse testis.

5.1.2 Hedgehog pathway mRNAs in the rat testis

The highest relative levels of *Gli1* mRNA were recorded in newborn rat testes and they steadily decreased as the rat grew older (I, Fig. 1B). *Dhh, Ptch1, Gli2* and *Gli3* mRNAs reached the highest values prepubertally in 5-20-day-old rats (I, Fig. 1A-B), whereas only very low levels of *SuFu* transcript were recorded until day 30 (I, Fig. 1B). *Ptch2* mRNA levels did not vary significantly between the studied time points (I, Fig. 1A). When compared to the level of *Fshr* transcript as a measure of Sertoli cell number (Heckert & Griswold 1991) *Dhh* level stayed quite constant over time the only significant difference being between days 5 and 30 (I, Fig. 1C).

5.2 Stage-dependent differences in the abundance of mRNAs in the mouse and rat seminiferous epithelial cycle (I, III)

5.2.1 Germ cell and somatic cell markers during mouse spermatogenesis

The abundance of most of the studied mRNAs was found to be stage-dependent. *Nanog* mRNA was at its highest level in stages IX-XII of the mouse seminiferous epithelial cycle, whereas the steady state levels of *Oct4* mRNAs were higher in stages VII-XII than I-VI (III, Fig. 2A). Cell cycle regulator p21 displayed the opposite (III, Fig. 5A). The highest transcript levels of *CD9* and *Stra8* were recorded in stages VII-VIII, whereas there were no statistically significant differences in the relative abundance of *c-Kit*, *Gfr1α, Gpr125* and *PLZF* mRNAs in different stages of the mouse seminiferous epithelial cycle (III, Supporting figure 2A-C). The mRNA levels of *Erm* were at their highest in stages IX-VI and those of *Gdnf* in stages I-VI (III, Supporting figure 2D). A more detailed analysis revealed that within stages I-VI the highest Gdnf values were seen in stages V-VI in which *Nanog* transcript was at the lowest level (III, Fig. 4A-B). The stage-specific expression patterns reflect the cellular origin of the messages, i.e. stage-specific localization of the cells that express these mRNAs.

5.2.2 Hedgehog pathway mRNAs during rat spermatogenesis

The steady state levels of *Dhh* and *Gli1* mRNAs were at their highest in stages II-VI of the rat seminiferous epithelial cycle (I, Fig. 4A-B). *Ptch1* was statistically more abundant in stages II-VIII

than IX-I (I, Fig. 4C). *Ptch2* mRNA levels were lower in stages XIII-I than IX-XII (I, Fig. 4D). The relative level of *SuFu* transcript was significantly higher in stages VII-XII than XIII-VI (I, Fig. 4E).

5.3 Protein localization in the mouse, rat and human testis (I, II, III)

5.3.1 Developmental expression of GLI1 in the rat testis

GLI1 was first localized to the spermatogonia of five-day-old rats (I, Fig. 5A). These cells also expressed PTCH1 (I, Fig. 5B). Basal spermatogonial expression of GLI1 was also observed 10, 20, 30 and 40-day old rats (I, Fig. 5C-F). GLI1 also localized to more adluminally situated spermatocytes and cytoplasms of elongating spermatids in 40-day-old rats.

5.3.2 Expression of SUFU, GLI1 and PTCH1 in the rat testis

SUFU was first observed in the cytoplasms of elongating spermatids in 40-day-old rats (I, Fig. 2A). In the adult rat, SUFU protein was first seen in step 9 spermatids and the strongest signal was observed in step 10-13 spermatids. Thereafter the protein levels were down-regulated and step 15-18 ceased to express SUFU (I, Fig. 2B-C). GLI1 was observed in spermatogonia, spermatocytes and step 15-18 spermatids (I, Fig. 2D-E). The fluorescent signal from stage II-VI spermatocytes was stronger than in other spermatocytes. PTCH1 localized first to leptotene/zygotene spermatocytes and the protein levels markedly increased towards stage VIII pachytene spermatocytes, as estimated by the fluorescent signal intensity (I, Fig. 2F). Other germ cells did not stain specifically for PTCH1. The localization of GLI1, PTCH1 and SUFU in the meiotic and post-meiotic cell types of the rat seminiferous epithelium is summarized in Figure 6.

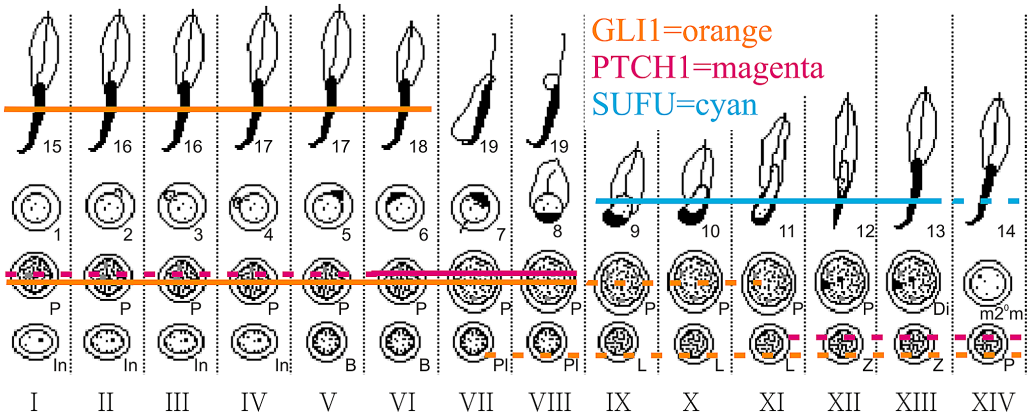

Figure 6. *Schematic illustration of the SUFU, GLI1 and PTCH1 protein localization in the meiotic and post-meiotic cells of the rat seminiferous epithelium. SUFU expression is restricted to step 9-14 condensing spermatids. PTCH1 is first seen in leptotene spermatocytes and its expression is lost in stage IX pachytene spermatocytes. GLI1 is expressed in most spermatocyte types and it reappears in step 15-18 spermatids. The strongest signal in immunohistochemical staining is indicated with a solid line, whereas the dotted lines show the cell types where the protein is present at a lower level. The specific cell associations in the vertical columns represent specific stages (Roman numerals) of the rat seminiferous epithelial cycle. Arabic numerals refer to different steps of postmeiotic germ cell differentiation. In, intermediate spermatogonia; B, type B spermatogonia; Pl, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; Di, diplotene spermatocyte; m2°m, meiotic divisions.*

5.3.3 Expression of CIP2A, PLZF, KI-67, Nanog and Stra8 in the mouse testis

Spermatogonia of the adult mouse stained most intensively for CIP2A but a weak staining was also observed in meiotic cells (II, Fig. 1E, 2A, Supplementary figure 3). Most of the CIP2A positive cells co-expressed KI-67 (II, Fig. 2A-B). To confirm this result we used hypomorphic CIP2A^{HOZ} mice that express β-galactosidase under CIP2A promoter to study in which cell types *CIP2A* is transcribed. Only spermatogonial cells displayed an intensive X-gal staining (II, Fig. 2C).

PLZF expression was observed in a small subset of spermatogonia in the adult mouse testis (II, Fig. 2A-B, 4B; III, Fig. 2D), whereas Stra8 localized most intensively to seminiferous epithelial stage VI-VIII B spermatogonia/preleptotene spermatocytes (III, Fig. 2D). CIP2A was found to be co-expressed with PLZF and KI-67 (II, Fig. 2B). Nanog protein was specifically localized to rare solitary cells in stage XII of the mouse seminiferous epithelial cycle (III, Fig. 2B, 2D). Oct4 was found co-expressed with Nanog in these cells (III, Fig. 2C). Quantitative analysis of the number of cells expressing these three proteins showed that Nanog is expressed in very few cells and the number of PLZF and Stra8 positive cells is 18 and 42 times higher, respectively (III, Fig. 2E).

5.3.4 CIP2A, PLZF and KI-67 expression in human testis ontogenesis

Proliferatively active gonocytes of the fetal human testis expressed CIP2A, PLZF and KI-67, whereas the proteins were not present in the quiescent germ cells of prepubertal boys. These three proteins reappeared in the pubertal testis where cells resume cell cycle progression and start to proliferate (II, Fig. 2D, Table 1).

5.4 Characterization of a two-dimensional co-culture method for mouse undifferentiated spermatogonia on a physiological feeder layer (IV)

Eddy and Kahri (1976) noticed that seminiferous tubule cells start to spontaneously proliferate and migrate out from small fragments of rat seminiferous tubule *in vitro* (Eddy & Kahri 1976). We wanted to test whether this phenomenon is repeatable when using mouse as the tissue donor and whether it could be used to set up a stable mixed culture of seminiferous tubule cells. We were especially interested in what kind of spermatogonia could be maintained in this environment and we therefore incubated the culture at 37°C which impedes germ cell differentiation. After decapsulation of mouse testes, 4-mm-long segments of seminiferous tubule were dissected and transferred to cultivation flasks with small amount of culture medium for 6-10 hours. During this incubation the small fragments of seminiferous tubules attached to the bottom of the cultivation flasks. Thereafter the volume of the culture medium was increased and after 1 week the cells had spread quite homogenously over the bottom of the culture flask and approximately 80 % confluence was achieved (IV, Fig. 1A). We originally established the culture from less than 10-day-old mice but noticed later that seminiferous tubule cells from adult mice (the oldest one being 17 months old) can be used to set up the culture.

Most of the cells of one-week-old culture were identified as Sertoli cells based on morphologic criteria (Aponte et al. 2006) and expression of WT1 (Wilm's tumour antigen 1; Armstrong et

al. 1993) (IV, Fig. 1B). Among these cells there were small round cells that expressed PLZF and Stra8 and were therefore regarded as spermatogonia (IV, Fig. 3C-D). The culture was maintained for additional five weeks and samples were collected weekly for RT-PCR analysis. During the incubation morula-appearing clumps of cells that strongly expressed PLZF were observed (II, Fig. 2A). To monitor how germ cells at different phases of their differentiation survive in this environment, we established the culture from adult mice for these experiments (IV, Fig. 2B). The relative level of *Stra8* transcript decreased sharply during the second week, which suggests that the environment does not support meiotic differentiation of germ cells (Giuili et al. 2002, Zhou et al. 2008). Transcript of meiotic cell marker *Sycp3* (Synaptonemal complex protein 3; La Salle et al. 2009) was lost after the first week of the culture and it never appeared again. The relative transcript levels of *CD9, Gpr125* and *PLZF* were quite constant during the whole six weeks, whereas *c-Kit* transcript was lost after 5 weeks. *Acta2* (smooth muscle actin alpha 2) transcript level was relatively constant during the whole experiment suggesting that peritubular myoid cells thrive for extended periods of time *in vitro* (Tung & Fritz 1990). Transcript of Sertoli and spermatogonial cell marker *Erm* (Chen et al. 2005, Oatley et al. 2007) was observed in all time points and that of Sertoli cell specific gene *Gdnf* (Meng et al. 2000) until 5 weeks. Lack of Leydig cell specific transcript *Cyp17a1* (P450C17) in all time points suggests that the culture was established by cells from the seminiferous tubules and the interstitial cells did not contribute. Interestingly and importantly, a transcript for *Nanog* was detectable until five weeks.

5.5 Plating of allogeneic germ cells on a one-week-old co-culture (IV)

Approximately 120-160 mm of seminiferous tubule from mice was dissected, trypsinized and applied onto a one-week-old co-culture (IV, Fig. 3A). Three days after plating the cells the culture medium was exchanged and it was noticed that the number of spermatogonia had increased markedly and it continued to increase during the next 1-2 weeks (IV, Fig. 3B). To ensure that the proliferating cells were of allogeneic origin we next placed seminiferous tubule cells from eGFP-expressing mice on one-week-old co-culture established from WT mouse. Most of the spermatogonial cell colonies formed strongly expressed GFP as assessed by UV light exposure (IV, Fig. 3E). Spermatogonial marker mRNAs were detected one week after the transplantation and their levels were monitored by qRT-PCR for additional three weeks (IV, Fig. 3G). No statistically significant changes occurred in the mRNA levels of *CD9, PLZF, Stra8, c-Kit* or *Gpr125* although the steady state levels of *Stra8* mRNA tended to decrease during the 18-day period.

The original transplanted cell suspension contained a substantial number of Sertoli cells. However, we could only see very rare eGFP-expressing Sertoli cells 1-2 weeks after the transplantation (IV, Fig. 3F). This finding suggests that Sertoli cells fail to attach to nearly confluent monolayer composed mainly of alike but allogeneic cells. Taken together these data demonstrate that seminiferous tubule cells after migrating out from small segments of seminiferous tubule *in vitro* and forming a nearly confluent co-culture are able to provide allogeneic spermatogonia, but not Sertoli cells, a niche where they can proliferate and survive for many weeks.

5.6 Regulation of gene expression by FSH in the mouse and rat testis (I, IV)

5.6.1 FSH effect on steady state levels of mRNAs in co-cultures

5.6.1.1 Co-cultures established from juvenile mice

Gdnf and *Gfr1*α mRNA levels were acutely elevated by FSH in one-week-old co-cultures established from less than 10-day-old mice (IV, Fig. 4A-B). This phenomenon could be repeated by changing the culture medium at 72 hours. *Stra8* levels were uniformly elevated by FSH (IV, Fig. 4C), whereas the transcript levels of *CD9, CIP2A, c-Kit, c-Myc, Gpr125, Nanog* and *PLZF* were not affected by the treatment (data not shown).

5.6.1.2 Co-cultures established from adult mice

Gdnf and *Gfr1α* mRNA levels were elevated by FSH in one-week-old co-culture established from seminiferous tubules of adult mouse (IV, Fig. 4D-E). *Stra8* mRNA levels were only elevated at 72 hours by FSH (IV, Fig. 4F). Additionally, the steady state levels of *c-Kit, Gpr125* and *PLZF* mRNAs were increased at some data points (IV, Fig. 4G-I). *Nanog* mRNA level was not affected by FSH (data not shown).

5.6.2 Regulation of *Dhh*, *Scf* and *Gdnf* mRNA levels in cultured rat seminiferous tubules

FSH was found to down-regulate *Dhh* mRNA in all three pooled stages of the rat seminiferous epithelial cycle 30 hours after the treatment in a dose-dependent manner (I, Fig. 6B). The steady-state levels of *Scf* mRNA were elevated in all stages at 8 hours, and the stimulation continued in stages IX-VI at 30 hours (I, Fig. 6A). *Gdnf* mRNA levels were specifically down-regulated by FSH in stages II-VI of the rat seminiferous epithelial cycle (I, Fig. 6C).

5.7 Cyclopamine treatment increases the number of cleaved caspase-3 positive cells *in vitro* (I)

To study whether inhibition of Hedgehog signalling affects germ cell survival, four-mm-long segments representing stages II-VI of the rat seminiferous epithelial cycle were cultured with Hedgehog pathway inhibitor cyclopamine (100 μM) for 8 and 16 h. Squash preparations were made and stained for cleaved caspase-3 positive cells. Caspase-3 is a critical executioner of apoptosis. It is activated by proteolytic processing (cleaving) and is either totally or partially responsible for the proteolytic cleavage of many different proteins (Fernandes-Alnemri et al. 1994). Cyclopamine increased the number of cleaved caspase-3 positive cells at both time points and the difference to the control at 16 hours was statistically significant (I, Fig. 7).

5.8 Imatinib mesylate suppresses Hedgehog signalling in immature rat testis (I)

Imatinib mesylate is a specific inhibitor of (receptor) tyrosine kinases (Druker et al. 1996, Nishimura et al. 2003). It has been shown to have an adverse effect on postnatal testis development (Nurmio et al. 2007). To study whether imatinib affects Hedgehog signalling in the early postnatal testis we treated 5-day-old rats with either a single dose of imatinib (50 or 150 mg/kg) and the rats were sacrificed 24, 48 or 72 hours post-operatively, or on three consecutive days 5, 6 and 7 (three times 50 or 150 mg/kg) and the rats were sacrificed 24 hours after the last injection. Imatinib was found to suppress *Dhh* levels at every studied time point (I, Fig. 8A). As a result from decreased ligand production, the transcript levels of Hh pathway genes, *Gli1* and *Ptch1* we found down-regulated on postnatal day 8 (I, Fig. 8B).

5.9 Experimental cryptorchidism induces testis degeneration (III)

Two-month-old mouse testes were unilaterally sutured to the abdominal wall to induce heat-activated degeneration of testicular tissue. The samples were collected four months postoperatively. The size of the operated testis had decreased significantly and the diameter of the seminiferous tubules had dramatically diminished (III, Fig. 3A). To analyse what kind of cells remain in these conditions we studied the gene expression profile of the operated testis by RT-PCR and compared it to the unoperated control from the same mouse (III, Fig. 3B). Among all the studied genes (*Oct4, PLZF, Stra8, c-Kit, Gpr125, ki-67, Nanog, Dppa4, Sox2, Gdnf* and *Erm*) only *Nanog* transcript levels were not significantly down-regulated by the treatment.

5.10 Local 3-4 Gy X-irradiation acutely elevates *Gdnf* mRNA levels in the mouse testis (III)

Adult mice testes were locally irradiated with 3-4 Gy and the acute molecular level changes were monitored 6-144 hours postoperatively by qRT-PCR. The steady state levels of *Gdnf* mRNA were acutely elevated by the treatment (III, Fig. 3D). *PLZF* levels were also elevated 96 and 144 hours postoperatively, whereas *Stra8* transcript was barely detectable from 96 hours on. No changes were recorded in *Nanog* mRNA levels (III, Fig. 3D).

The mRNA level findings were corroborated by counting Nanog, PLZF and Stra8 positive spermatogonia in untreated mice and X-irradiated mice (144 h after the treatment). In the treated mice no Stra8 positive cells we detected, the number PLZF positive cells had increased and the number of Nanog positive cells was unchanged (III, Fig. 3E).

5.11 H-Ras activation in mouse SSCs leads to accumulation of PLZF positive spermatogonia after transplantation (III)

GDNF has been demonstrated to activate Ras signalling pathway in spermatogonial stem cells and consequently induce SSC proliferation (He et al. 2009). Overproduction of GDNF (Meng et al. 2001) and overexpression of H-Ras in spermatogonia (Lee et al. 2009) both result into

similar testicular phenotypes *in vivo* with accumulation of spermatogonia. To find out which spermatogonial markers are associated with the overactivation of GDNF/Ras signalling pathway we used an *in vivo* model where H-Ras overexpressing and WT stem spermatogonia (GS cells) were transplanted into busulfan-treated germ cell-depleted testis (Lee et al. 2009). Transplantation of WT cells led to normal-looking re-arrangement of the seminiferous epithelium, whereas H-Ras overexpressing cells were found to accumulate and not be able to start to differentiate (III, Fig. 4C-D). PLZF was strongly expressed by the accumulated cells, whereas in the testis transplanted with WT cells only a small fraction of cells expressed PLZF (III, Fig. 4E). Quantitative RT-PCR analysis demonstrated that the relative transcript levels of *CD9* and especially *PLZF* were elevated in the H-Ras GS transplanted testis when compared to the WT GS transplanted testis (III, Fig. 4F). There were no differences in *Oct4, Gpr125* or *c-Kit* levels but *Nanog* levels were lower in the H-Ras GS transplanted testis (III, Fig. 4F).

5.12 Nutlin-3 induces p21 signalling in mouse spermatogonia and ES cells (III, IV)

Nutlin-3 is a small molecule activator of p53 signalling. In response to p53 action p21 transcription and signalling are activated. We applied nutlin-3 on one-week-old co-cultures established from short segments of seminiferous tubules. Nutlin induced a proliferation arrest in the cultured stem/progenitor spermatogonia (IV, Fig. 5B) when compared to the control (IV, Fig. 5A). Quantitative RT-PCR analysis revealed that the treatment had elevated the steady state levels of *p21* mRNA indicating that it had worked (III, Fig. 5D, 6A; IV, Fig. 5C). Among all the studied genes only *CIP2A, Nanog* and *Stra8* mRNA levels were down-regulated by the nutlin-3 treatment (III, Fig. 5E; IV, Fig. 5D-E). *PLZF* levels were either unchanged or slightly upregulated by nutlin-3 (III, Fig. 6B). Nutlin-3 did not have an effect on *Gdnf* levels (IV, Fig. 5F).

We also examined the effect of nutlin-3 on cultured segments of mouse seminiferous tubules. Either unstaged or seminiferous tubules representing stage XII of the mouse seminiferous cycle were used. Nutlin3 dramatically down-regulated *Nanog* and *Oct4* levels already after 48 hours when stage XII seminiferous tubules were used (III, Fig. 5C), whereas a similar effect was recorded after 72 hours for the unstaged segments (III, Fig. 5B). *Gfr1*α levels were not affected by the nutlin-3 treatment (III, Fig. 5B-C).

To assess whether activation of p21 signalling acts similarly on *Nanog* and *Oct4* levels in mouse ES cells as on testicular stem cells we next exposed mouse ES cells to nutlin-3. As studied by RT-PCR, nutlin-3 elevated *p21* levels significantly 1-4 days after the treatment. Markedly lower *Nanog* and *Oct4* transcript levels were observed at 3-4 days after the nutlin-3 treatment and simultaneous *p21* upregulation was recorded (III, Fig. 5F).

5.13 Early molecular level events during differentiation of mouse ES cell line ZHBTc4 (III)

To look for putative similarities between testicular and embryonic stem cells we decided to analyse the early events of mouse ZHBTc4 ES cell line differentiation. ZHBTc4 ES cells lack functional endogenous Oct4 but possess a regulatable *Oct4* transgene (Niwa et al. 2000). In the absence of doxycycline ZHBTc4 cells stay in an undifferentiated state. Their differentiation can

be induced by administering doxycycline which represses *Oct4* expression and thus interferes with ES cell self-renewal machinery. In our experiment Oct4 was dramatically down-regulated at 6 and 24 hours after adding doxycycline to the culture medium at mRNA and protein levels, respectively (III, Fig. 6D, 6F). This was followed by a drop in *Nanog* mRNA levels from 48 hours on (III, Fig. 6G). The steady state levels of *PLZF* and *Gfr1*α were acutely upregulated by doxycycline (III, Fig.6H-I). Later on *Gfr1*α returned to control levels or below but *PLZF* levels were found upregulated. An independent model where ES cells were allowed to spontaneously differentiate supported these findings: steady state levels of *PLZF* mRNA were upregulated during the early phase of ES cell differentiation (III, Fig. 6C).

5.14 Stage-dependent ES cell-like cell colony formation (III)

SSCs are known to form colonies in the presence of GDNF *in vitro*. To test if seminiferous tubule cells from different stages of the mouse seminiferous epithelial cycle vary in their colony formation ability we plated cells from three pooled stages of the mouse seminiferous epithelial cycle on gelatin-coated flasks in the presence of GDNF for one week. Thereafter the cells were moved to ES cell culture conditions on a feeder layer of mitomycin C-inactivated fibroblasts. After 4-5 weeks of culturing, cells from stages IX-XII were found to have given rise to the highest number of cell colonies (III, Fig. 1A-B). Stage VII-VIII cells were still able to give rise to some colonies (III, Fig. 1A-B), whereas there were only very few colonies from stage I-VI cells (III, Fig. 1B). The diameter of the colonies that had formed from stage IX-XII cells was markedly higher in stages IX-XII than VII-VIII (III, Fig. 1C). The cell colonies were found to express relatively high levels of pluripotency-related markers Nanog and Oct4 in immunocytochemical staining (III, Fig. 1D). RT-PCR analysis further revealed that transcript levels of *Nanog* and *Oct4* were highest in the cell colonies derived from stage IX-XII cells (III, Fig. 1E).

5.15 Characterization of hypomorphic CIP2A^{HOZ} mice (II)

To evaluate the normal physiological role of novel cancer-associated protein CIP2A (Junttila et al. 2007), we created a CIP2A hypomorphic mouse line. Gene-trap vector was inserted into the first intron of *CIP2A* (II, Fig. 1A). The progenies of mice heterozygous for the mutated allele followed a normal Mendelian distribution of genotypes at birth (data not shown). Homozygous mutant mice (CIP2A^{HOZ}) were viable, displayed no obvious malformations in anatomy or tissue histology and lived as long as their WT siblings (II, Fig. 1B). Adult male CIP2A^{HOZ} mice were moderately lighter than their WT brothers but no difference in female weights was measured (data not shown). No changes were recorded in different blood parameters between CIP2A^{HOZ} and WT mice which suggests that depletion of normal CIP2A allele did not affect haematopoietic stem cell function (data not shown). Serum testosterone and FSH levels were unaltered (II, Supplementary table 1). However, the sperm counts of CIP2A^{HOZ} mice were markedly lower than those of their WT littermates (II, Fig. 3A). They also had smaller and lighter epididymides (II, Fig. 3B-C). In CIP2A^{HOZ} mice the CIP2A mRNA and protein levels were found to be dramatically down-regulated in all CIP2A-expressing tissues that were examined (II, Fig. 1C-E). The residual expression of *CIP2A* mRNA was probably due to low-level leakage of the gene-trap cassette. In summary, these data demonstrate that systemic inhibition of CIP2A impinges on fertility but does not affect viability or development of mice.

5.15.1 Association of CIP2A with spermatogonial progenitor cell proliferation defect

In adult WT mice CIP2A protein was partially found to colocalize with PLZF and KI-67 in spermatogonial progenitor cells (II, Fig. 2A-B). It also shared with PLZF the even mRNA distribution between the different stages of the mouse seminiferous epithelial cycle (II, Supplementary figure 2). Interestingly, we noticed that *PLZF* mRNA levels were suppressed in the CIP2A^{HOZ} mouse seminiferous tubules (II, Fig. 4A). A similar decrease was recorded for self-renewal-associated markers *Nanog* and *Oct4*. However, *Gpr125* levels were maintained and *Stra8* levels highly upregulated in the seminiferous tubules of CIP2A^{HOZ} mice (II, Fig. 4A). CIP2A^{HOZ} mice testes also contained less PLZF positive spermatogonia than WT testes (II, Fig. 4B-C). We also noticed that juvenile mice lacking PLZF had a normal number of CIP2A positive germ cells (II, Fig. 4D-E). Taken together, these results suggest that CIP2A might act upstream of PLZF in the maintenance of spermatogonial progenitor cells and spermatogenesis.

5.15.2 Recovery of the CIP2A^{HOZ} testis phenotype

In the subsequent generations of CIP2A^{HOZ} mice the CIP2A mRNA and protein levels nearly returned to the WT levels in the testis due to progressive increase in the leakage of the gene-trap cassette (II, Fig. 5A-B). This phenomenon took place specifically in the testis while in other tissues 90-95% reduction in CIP2A levels was still observed (II, Supplementary figure 4). In these mice (Post-CIP2A^{HOZ}) *PLZF, Nanog* and *Oct4* returned to the WT levels (II, Fig. 5D) and sperm production potential also recovered. The level of gene-trap cassette leakage was found to be changing a bit from mouse to mouse. Therefore it was studied if *CIP2A* mRNA steady state levels still correlate with spermatogenic capacity in Post-CIP2A^{HOZ} mice. We found out that in all three studied cohorts a correlation could be established (II, Fig. 5C and Supplementary figure 5).

5.16 CIP2A is a novel proliferation and self-renewal-associated protein (II)

To study if CIP2A is involved in normal cell proliferation as it is in cancer cells, we down-regulated *CIP2A* mRNA levels in seminiferous tubule-derived spermatogonia (II, Supplementary figure 6B) *in vitro* by introducing a *CIP2A*-specific siRNA construct. The treatment effectively suppressed *CIP2A* mRNA levels and dramatically reduced the size of the spermatogonial cell colonies without affecting their number (II, Fig. 6A-D). *CIP2A* silencing also had an anti-proliferative effect on an immortalized mouse spermatogonial cell line GC1spg (II, Fig. 6E-F). Taken together these data indicate that CIP2A promotes cellular proliferation and supports self-renewal of progenitor cells.

6 DISCUSSION

6.1 Hedgehog signalling promotes germ cell survival and is needed for meiosis

In the course of rat postnatal testis development the expression of most of the Hh pathway genes was tightly regulated. The highest mRNA levels for *Dhh, Ptch1, Gli2* and *Gli3* were recorded in 5-20-day-old rat testes coinciding with the formation of the spermatogonial cell pool and the first meiotic germ cells (Clermont & Perey 1957, Malkov et al. 1998). These data suggest that Hedgehog signalling might be needed in these processes. However, the steady decrease that was seen in *Gli1* transcript levels indicates that Hedgehog signalling has an important role in the rat testis even before the onset of germ cell differentiation in spermatogenesis. Indeed, it is needed to drive steroidogenesis of fetal Leydig cells (Barsoum et al. 2009, Brokken et al. 2009). GLI1 localized to spermatogonia at all ages starting from spermatogonial stem cells of 5-day-old rats till a subset of spermatogonia in the adult rat testis. Spermatogonia of 5-day-old rats also expressed PTCH1. Developmental expression of GLI1 in spermatocytes of immature rats, in preleptotene to pachytene spermatocytes of adult rats and PTCH1 localization in leptotene to stage VIII pachytene spermatocytes justifies the assumption that Hedgehog signalling is active in these cell types, too.

The mRNA level of *SuFu* remained at a very low level until day 40 and stayed high thereafter suggesting that the cells that express *SuFu* appear very late during the first wave of spermatogenesis. Protein level findings support this assumption: SUFU was first seen in the cytoplasms of 40-day-old rat elongating spermatids. In the adult protein expression was confined to step 9-14 spermatids which was corroborated by stage-specific abundance of *SuFu* mRNA in stages VII-XII of the rat seminiferous epithelial cycle. SUFU has an established role in the cytoplasm where it sequesters GLI1 and thus acts as an inhibitor of Hedgehog signalling (Kogerman et al. 1999). SUFU can also function in the nucleus where it directly represses GLI-mediated transcription (Cheng & Bishop 2002). The fact that SUFU is expressed within a very narrow window during rat spermatogenesis and that GLI1 reappears in step 15-18 spermatids that cease to express SUFU is very intriguing. These results suggest that during the early phase of spermatid elongation Hedgehog signalling needs to be shut down for the process to succeed.

Interestingly, the steady state levels of *Ptch2* mRNA did not change significantly over time. *Ptch2* mRNA levels did not either share the pattern of other Hedgehog pathway mRNAs in different stages of the rat seminiferous epithelial cycle. Unfortunately we never managed to get PTCH2 antibody working for histological sections. PTCH2 was originally regarded as the most important Hedgehog receptor in spermatogenesis (Carpenter et al. 1998, Szczepny et al. 2006). Lack of fertility problems in *Ptch2* deficient mice (Nieuwenhuis et al. 2006) and strong immunostaining of PTCH1 in differentiating germ cells in the mouse and rat (Morales et al. 2009) have refined this understanding. PTCH1 probably has a more important role in spermatogenesis than PTCH2. Reactivation of GLI1 in late elongating spermatids is surprising since these cells are not known to carry any Hedgehog receptor. Recently more evidence on non-canonical mechanisms of GLI transcription factor activation has emerged (reviewed in Lauth & Toftgård 2007). At least PI3-K, MAPK and TGF-β signalling have been shown to converge on activation of GLI transcription

factors (Riobo et al. 2006C, Dennler et al. 2007). PI3-K and PKB can stimulate Hh signalling by inhibiting PKA-mediated GLI inactivation (Riobo et al. 2006B). Furthermore, ERK (extracellular signal-regulated kinase) and Hh signalling can be integrated on the level of GLI proteins (Riobo et al. 2006A). Finding out the function of GLI1 in step 15-18 condensing spermatids calls for further studies since the chromatin of these cells is so tightly packed that GLI1 can hardly act as a transcription factor in these circumstances.

We normalized *Dhh* levels to *Fshr* levels to find out if the decrease of *Dhh* mRNA levels after 10 days of age is due to dilution of Sertoli cell mRNA by germ cell mRNA. This might be one possible explanation since Sertoli cells cease to proliferate on day 15 (Bortolussi et al. 1990, Yang et al. 1990), whereas most of the germ cell types of the adult testis have not yet appeared at this time. However, when normalized to Sertoli cell number (*Fshr* transcript level) *Dhh* stayed quite constant as the rat grew older. Only the *Dhh* levels at 5 days of age were higher when compared to 30 days of age. These data also support that Hh signalling is actively involved in testicular function at all ages.

Dhh mutation on 129/Sv inbred background or overexpression of human GLI1 in mice leads to infertility due to a block in spermatogenic differentiation at pachytene spermatocyte stage (Bitgood et al. 1996, Kroft et al. 2001). These findings suggest that Hedgehog signalling is needed in meiosis but it needs to be down-regulated before the meiotic division. Our data on the stage-specific abundance of Hedgehog pathway transcripts strengthens this idea: all of the studied mRNAs are at a low level in stages XIII-I.

If Hedgehog signalling is needed to support differentiation of germ cells then inhibiting Hedgehog signalling should result in germ cell apoptosis. Introduction of cyclopamine, a specific inhibitor of the Hedgehog pathway, markedly increased the number of apoptotic germ cells *in vitro*. This finding indicates that Hedgehog signalling promotes germ cell survival. Similar task has been previously assigned to SCF/c-KIT signalling (Hakovirta et al. 1999, Yan et al. 2000C). Interestingly, the steady state levels of *Scf* mRNA also reach the highest value in stages II-VI of the rat seminiferous epithelial and are regulated by FSH (Hakovirta et al. 1999, Yan et al. 1999). However, FSH effect on *Scf* levels is acute and stimulatory, whereas the suppressive effect on *Dhh* levels can only be seen as late as 30 hours after the treatment. There are at least two explanations for the different time course: firstly, activated transcription can rapidly elevate mRNA levels of a specific gene, whereas the time after which suppression of gene transcription can be seen is largely dependent on the mRNA half-life; secondly, FSH might act only secondarily on *Dhh*. Anyhow, the fact that two pathways that seem to have a similar function in spermatogenesis are regulated by FSH in an opposite way is surprising. It might be noteworthy that *Dhh* levels were most significantly suppressed in stages VII-VIII whereas elevation of *Scf* levels was most remarkable in stages II-VI. It is possible that some other stage II-VI specific factor maintains *Dhh* levels and works together with FSH. Then down-modulation of Hedgehog signalling in stages VII-VIII takes place in a FSH-dependent manner.

Besides pituitary-derived FSH testicular *Dhh* levels can be affected by locally produced factors. Imatinib mesylate-induced inhibition of RTKs (receptor tyrosine kinases) impinged on *Dhh* mRNA levels and Hedgehog signalling in the immature rat testis. This was an unexpected finding since tyrosine kinase-dependent pathways and Hedgehog signalling are not known to be functionally coupled. It might also be an indication of a more generalized phenomenon in the testis, namely intricate interaction of different paracrine signalling pathways. Therefore targeted disturbance of a specific pathway might secondarily affect the whole network of

paracrine signalling. This is also supported by findings of Szczepny et al. (2009) who showed that cyclopamine suppresses *Scf* and *c-Kit* transcript levels *in vitro*. This might provide an alternative explanation for why cyclopamine increased the number of apoptotic cells in our experiments.

6.2 CIP2A is needed for quantitatively normal spermatogenesis

Many genes that are found to be up-regulated in tumours have a physiological function in the testis. These genes are called cancer-testis (C/T) genes and their number is on the rise (Simpson et al. 2005). Based on our findings *CIP2A* can now be fully considered a justified member of this gene family. The only consistent and apparent defect in the CIP2A transgenic mouse line was the compromised capacity for sperm production. Transgenic male mice that retained some 10 % of *CIP2A* expression when compared to the WT littermates displayed 50 % reduction in spermatogenic output. It is therefore easy to conclude that CIP2A is needed for quantitatively normal spermatogenesis.

Introduction of siRNA construct against *CIP2A* mRNA lead to decreased cell proliferation suggesting that CIP2A normally promotes proliferation. This is an interesting observation because overexpression of CIP2A in tumours (Junttila et al. 2007, Li et al. 2008, Come et al. 2009, Khanna et al. 2009, Qu et al. 2010, Vaarala et al. 2010, Dong et al. 2011, Ma et al. 2011, Wang et al. 2011) might provide the malignant cells with higher proliferation capacity. Nutlin-3, a small molecule inhibitor of the interaction between MDM2 and p53, also induces growth arrest. At the molecular level nutlin-3 indirectly activates p53 signalling and thus induces p21 (Vassilev et al. 2004). Cell cycle is arrested in response to p21 activation due to inhibition of cyclin-cyclin dependent kinase complexes (Gartel & Radhakrishnan 2005). At the cellular level introduction of nutlin-3 leads to growth inhibition in cancer (Vassilev et al. 2004). The effect of nutlin-3 on germ cells that highly express the cancer-testis genes has not been previously studied. In our hands nutlin-3 induced a proliferation/differentiation arrest in spermatogonia. The treatment impinged on *CIP2A* and *Stra8* mRNA levels, but did not uniformly affect the steady state levels of other SSC or progenitor spermatogonia marker mRNAs. These data demonstrate that in most of the spermatogonial populations p53/p21 pathway activation does not induce apoptosis but their proliferation and differentiation might be halted as suggested by smaller cell number and lower *Stra8* level after nutlin-3 treatment, respectively. Different mouse models demonstrate that the effects of indirectly upregulated p21 signalling are quite similar (Barlow et al. 1997, Takubo et al. 2006, Liu et al. 2007). It is interesting that once again CIP2A was found to be associated with low proliferative activity, though inversely. Additionally, CIP2A is also expressed in neural stem cells where it is found to promote their self-renewal (Kerosuo et al. 2010).

CIP2A is overexpressed in many malignancies, which makes it an attractive target for pharmacological cancer therapy. A prerequisite for a good drug target candidate is that disturbing its function should not have too severe side effects. According to our data systemic inhibition of CIP2A did not have any adverse effects on the development, growth or function of any tissues or organs, except for the testis. The studied transgenic mice retained some 10 % of *CIP2A* mRNA expression in many tissues when compared to WT mice. This probably makes this mouse model more suitable for side effect evaluation than using mice that completely lack CIP2A. This is because not even 90% systemic inhibition of protein function due to drug administration is usually achieved. Our data suggest that CIP2A is a putative target for cancer therapy.

6.3 Reconstruction of testis stem cell niche *in vitro*

Research on SSCs *in vivo* is difficult due to their scarcity and distribution along the whole length of the seminiferous tubule. Therefore many different methods have been developed over the decades to promote SSC survival *ex vivo*. Rodent SSCs can be maintained and propagated *in vitro* over half a year (Kanatsu-Shinohara et al. 2003, Kubota et al. 2004, Ryu et al. 2005). Recently, the minimal requirements for their ample propagation have been defined in detail (Kanatsu-Shinohara et al. 2011).

Only short-term co-cultures of Sertoli, peritubular myoid and germ cells exist. We developed a method for long-term co-culturing of these cells. Germ cells and somatic cells from small fragments of seminiferous tubules of different aged mice proliferate *in vitro* and form a stable mixed culture that can be maintained up to six weeks. Advantages of this method are that no special techniques and devices are required, no cell sorting is necessary, no exogenous growth factor stimulation is needed and it can be established in basic culture medium. During the six week culture spermatogonia express a wide range of spermatogonial markers including the SSC marker PLZF. The steady state levels of *Gdnf*, which is crucial for long-term maintenance of SSCs *in vitro* (Kubota et al. 2004), can be elevated by FSH showing that Sertoli cells respond to physiological stimuli (Tadokoro et al. 2002).

Since spermatogonia are able to propagate for weeks and their life span is likely limited by the well-being of Sertoli cells and not by the spermatogonia-intrinsic factors we suppose that stem cell niche has been reconstructed *in vitro*. If it was not so and no self-renewal divisions took place, spermatogonia would be exhausted. The major constituent of the testis stem cell niche are the Sertoli cells that dictate the number of stem cell niches (Oatley et al. 2010) and thus limit the population size of SSCs that they can support. At molecular level Sertoli cell-derived GDNF (Meng et al. 2000) and probably CSF-1 originating from Leydig and myoid cells (Oatley et al. 2009) are the most important regulators of the SSC niche. To see whether our culture system allows allogeneic somatic and germ cells of different ages to be co-cultured we plated testicular cells from GFP positive mice on 80% confluent co-culture. GFP positive spermatogonia were able to attach to the cells beneath and proliferate. This might allow the use of this system in studying how allogeneic SSCs home on their niche and how Sertoli cells at different stages of maturation can support germ cells *in vitro*.

6.4 FSH effect on Sertoli and germ cells is age-dependent

FSH affected Sertoli cell and spermatogonial cell marker expression partially differently in one-week-old co-cultures that were established from juvenile and adult seminiferous tubule cells. This probably derives from the fact that the Sertoli cells in juvenile mice are still proliferative *in vivo*. In adult mice Sertoli cells are quiescent *in vivo* and their main function is to support spermatogenic differentiation of germ cells. Accordingly, the dominant function of FSH on Sertoli cells shifts from driving their proliferation to nurturing germ cells via paracrine growth factor production (Meachem et al. 2005 and references therein). Serum FSH levels are 7-fold higher in adult than in juvenile mice, as measured using RIA (Barakat et al. 2008). Therefore it is unexpected that a similar dose of FSH elevated *Gdnf* mRNA level markedly less in juvenile than in adult Sertoli cells. FSH could only acutely elevate *Gdnf* level in juvenile Sertoli cells, whereas in the adult Sertoli cells the stimulation was longer. On the other hand, this may just underline the

fact that the main function of juvenile Sertoli cells is not growth factor production. Interestingly, GDNF receptor *Gfr-1α* was stimulated at the same data points, which supports the findings of Hofmann et al. (2005) about GDNF inducing the proliferation of GFR1α positive cells *in vitro*. Upregulation of spermatogonial *c-Kit*, *Gpr125* and *PLZF* mRNA levels by FSH specifically in adult testicular cells probably just reflects FSH-induced growth factor production by the Sertoli cells. Our finding that FSH has a bigger impact on *Stra8* mRNA level in cultured juvenile testicular cells than in adult cells probably stems from the developmental state of these cells. Premeiotic/meiotic transition of the most advanced germ cells takes place on day 8-10 in mice (Bellvé et al. 1977) and therefore a relatively greater number of spermatogonia are bound to enter meiosis in juvenile than in adult mouse testis.

6.5 Sertoli cells in adult mice are not terminally-differentiated, quiescent cells

Rodent Sertoli cells cease to proliferate at around two weeks of age (Kluin et al. 1984, Bortolussi et al. 1990, Yang et al. 1990). Thereafter they stop expressing some proteins, like NCAM (neural cell adhesion molecule; Orth & Jester 1995, Orth et al. 2000), and start to express other proteins that have been associated with Sertoli cell maturity, like cell cycle inhibitor $p27^{kip1}$ (Beumer et al. 1999). This period of Sertoli cell differentiation is postponed if the thyroid gland is not producing enough hormones (van Haaster et al. 1992) or if the animal lacks testicular gap junction protein GJA1 (Sridharan et al. 2007). Until recently, mammalian Sertoli cells were regarded terminally-differentiated, quiescent cells - except for the Sertoli cells of seasonal breeders, like the Djungarian hamster, *Phodopus sungorus* (Tarulli et al. 2006). However, Ahmed et al. (2009) demonstrated that adult mouse and human Sertoli cells can re-enter cell cycle *in vitro*. Similar results for rat Sertoli cells were reported already 1976 (Eddy & Kahri 1976). We employed the method of Eddy and Kahri for mouse seminiferous tubules and noticed that seminiferous tubule cells start to proliferate rapidly *in vitro* and migrate out from segments of seminiferous tubules. More than half of the cells that formed a monolayer had Sertoli cell morphology and they also expressed Sertoli cell specific antigen, WT1 (Armstrong et al. 1993). Taken together, we and others have showed that adult mammalian Sertoli can no longer be regarded as terminally-differentiated quiescent cells.

6.6 Nanog is a novel and the first unique A_s spermatogonium marker

Despite decades of research effort no unique marker for A-single (A_s) undifferentiated spermatogonia has been reported. Since the estimated number of A_s spermatogonia is so low - only 0.03% of all germ cells are thought to be A_s cells in the adult mouse testis (Tegelenbosch & de Rooij 1993) - the expression level of such a marker has to be low as well and its cell cycle must be very tightly regulated. These criteria are fulfilled by Nanog-expressing solitary spermatogonia that can be immunohistochemically localized only in stage XII of the mouse seminiferous epithelial cycle. We could not increase their number with any experimental manipulation. Induction of massive cell loss by X-irradiation resulted only in modest change in *Nanog* mRNA levels. Previously, testicular Nanog expression has been only reported in the fetal testis (Hoei-Hansen et al. 2005, Yamaguchi et al. 2005) or in differentiating germ cells (mainly pachytene

spermatocytes) of adult mouse, rat, dog, pig and man (Kuijk et al. 2010). In our data *Nanog* transcript levels were rapidly down-regulated after birth, which probably results from the dilution of *Nanog* mRNA in A_s spermatogonia by RNAs of proliferating Sertoli cells and gradually appearing differentiating germ cells. In the adult mouse testis the *Nanog* levels were barely detectable by qRT-PCR. However, when the seminiferous tubules were dissected and staged, enrichment of *Nanog* mRNA was observed in stages IX-XII. The extremely low transcript level of *Nanog* in the adult mouse testis and its temporal disparity from meiotic cell markers (such as Sycp3, which was also studied) makes it highly unlikely that the protein localization of Nanog in differentiating germ cells (Kuijk et al. 2010) could be of specific nature. When optimizing the conditions for Nanog antibody we also ran into a problem of spermatocyte staining but got rid of it by lowering the antibody concentration. In the study of Yamaguchi et al. (2005) lack of stage-oriented approach might explain why no Nanog expression was observed in the adult mouse testis.

Due to their extreme rarity, Nanog-expressing spermatogonia are a challenging object for *in vivo* studies. Therefore *in vitro* methods have to be developed to support the non-targeted *in vivo* approaches in which the treatment is applied on the whole organ. We used two parallel approaches to study the regulation of Nanog-expressing spermatogonia *in vitro*: short-term seminiferous tubule culture and long-term co-culture of seminiferous tubule cells. The data from both of these experimental setups gave similar results. Nutlin-3-induced activation of p21 signalling leads to loss of Nanog. Human and mouse ES cells respond similarly to introduction of nutlin-3 (Maimets et al. 2008). These data suggest that at least this aspect of Nanog regulation is similar between the Nanog-expressing spermatogonia and ES cells.

We hypothesize that Nanog-expressing cells are the most primitive germ cell population within the adult mammalian testis. Therefore these cells represent the predecessors of all other germ cell types. To evaluate this idea we used two alternative means to induce mouse ES cell differentiation. Both these experimental setups showed that loss of pluripotent markers, such as Nanog, Oct4 and Sox2, in the ES cells is followed by upregulation of SSC-associated markers, such as PLZF, Gfr-1α and Gpr125. Therefore it seems possible that Nanog-expressing spermatogonia and ES cells might share gene regulatory networks even more widely.

Recently, Arnold with her colleagues published a highly interesting article about the role of Sox2, another factor needed to maintain cell pluripotency, in many epithelial tissues, including the testis (Arnold et al. 2011). They succeeded in identifying GFP that was under Sox2 promoter immunohistochemically but failed in the localization of Sox2 protein itself. Sox2 was found to be indispensable for spermatogenesis. Their data also indicate that Sox2 was not confined to the stem cell population, and Sox2 positive cells were able to reconstitute spermatogenesis to an infertile recipient mouse. This implies that Sox2-expressing testicular cells are not pluripotent since no teratoma formation was reported.

6.7 Testicular stem cells and regenerative medicine

Regenerative medicine aims at developing means to replace injured, missing or malfunctional cells, tissues or organs. The differentiated cells for these purposes are derived from pluripotent cells which have to be kept in an undifferentiated state to guarantee continuous supply of differentiating cells. However, extended passaging of cells often leads to chromosomal aberrations and might favour cells that have higher capacity to proliferate (Baker et al. 2007,

Närvä et al. 2010). At the moment only ES cells and iPS (induced pluripotent stem) cells can serve as a starting material for regenerative medicine. However, ES cells and their derivatives are immunologically incompetent with the recipient and their derivation is considered ethically unsustainable by some researchers. Induced pluripotent cells do not have these features but their generation through the reprogramming of differentiated cells can induce genetic and epigenetic abnormalities in these cells (Hussein et al. 2011, Lister et al. 2011). This might predispose them to malignant transformation. It is worth mentioning, however, that there is no information available which steps in the reprogramming of cells to a dedifferentiated state predispose the cells to the accumulation of recurrent genetic or epigenetic lesions.

Based on these facts the search for less problematic raw material is on-going. One approach has been to use less differentiated cells for the reprogramming, which enables the use of fewer factors (originally four; Takahashi & Yamanaka 2006) in the process (Kim et al. 2008). This might decrease the chance of obtaining unwanted changes in the reprogrammed cells. Tissue-specific stem and progenitor cells might offer an alternative solution. However, their purification is hampered by the complex architecture of tissues where they dwell and lack information about the specific cell surface markers. The most promising source of tissue-specific stem cells is the testis. Testicular stem/progenitor cells (or probably just a fraction of them) spontaneously gain pluripotency in proper culture conditions. These germ-line-derived pluripotent cells share many features with ESCs, including gene, protein and miRNA expression profiles and epigenetic status (Zovoilis et al. 2008, Dihazi et al. 2009, Zechner et al. 2009). In addition to being able to undergo spontaneous dedifferentiation to pluripotent state, these cells can also transdifferentiate (without dedifferentiating first) to many different cell types when put into proper somatic environment (Boulanger et al. 2007, Guan et al. 2007, Glaser et al. 2008, Simon et al. 2009, Streckfuss-Bömeke et al. 2009, Ning et al. 2010). It is noteworthy, that the precise anatomic origin and molecular nature of the predecessors of these germ cell-derived cells remains to be discovered.

The discovery of a small population of Nanog-expressing spermatogonia in the adult mouse testis is intriguing also in this respect. Since Nanog has always been associated with cell pluripotency and plays a crucial role in the maintenance of ES cells (Chambers et al. 2003) it is likely that adult mammalian testis houses a low number of pluripotent cells. Their physiological significance, in the maintenance of life-long spermatogenic potential, for instance, remains to be elucidated. Developing a method that would allow these cells to be isolated, purified and amply propagated *in vitro* might solve many of the problems met with ES and iPS cells and provide an ideal source of pluripotent cells and autologous transplants for about a half of the human population. The existence of putatively pluripotent Nanog-expressing spermatogonia in the mouse does not automatically mean that similar cells would also be present in other mammals. Despite decades of work on primate spermatogenesis a perfectly valid scheme for spermatogonial physiology and expansion has not been presented (Ehmcke & Schlatt 2006). In reality there are probably more than two (A_{dark}/A_{pale}) functional subpopulations of type A spermatogonia in the primate testis. A_{dark} spermatogonia are the counterpart of murine A_s spermatogonia in primates. It is highly unlikely that the A_{dark} spermatogonial cell population would present a homogenous group of cells. Functional specialization which is based on inter-cellular interaction and cell density is more likely. The aim of future research is to find out whether any of the A_{dark} spermatogonial cell subpopulations express pluripotency-associated proteins.

6.8 Implications of the discovery of Nanog-expressing spermatogonia for testis cancer research

Testis houses a wide range of different tumours (Oosterhuis & Looijenga 2005), the incidence of which is on the rise (Gilbert et al. 2011). Most of the testicular tumours are of germ cell origin and they are thought to derive from PGCs or gonocytes. Initiation of the tumour formation has been considered to take place already *in utero* (Skakkebaek 1972). However, much of the current understanding about their aetiology is based on the similarities of gene expression profiles and imprinting statuses between tumour cells and PGCs or gonocytes. Existence of pluripotent cells or cells that possess an in-built capacity to gain pluripotency (Guan et al. 2006) could provide an alternative cell of origin for testicular germ cell tumours. Men with non-seminomatous testicular tumour are diagnosed on the average at the age of 25 years (Gilbert et al. 2011). This supports the current hypothesis about their pathogenesis since if they originated from the pluripotent cells of the adult such an age-prevalence would not be expected. Further studies are needed to find out whether adult human testes also house Nanog-expressing cells.

6.9 Location of the testicular stem cell niche

Undifferentiated spermatogonia are enriched in areas that have interstitial tissue (Chiarini-Garcia et al. 2001, Chiarini-Garcia & Russell 2001, Chiarini-Garcia et al. 2003) and blood vessels (Yoshida et al. 2007) in close proximity. Even though, the localization of the testicular stem cell niche is thought to be dictated by molecular rather than anatomical factors in reality they must act in concert. Since different factors that affect testicular stem cell niche are either blood-born or locally produced they have to form a gradient within the testicular tissue. It is due to these gradients that there has to exist microenvironments where the summative amount of self-renewal-promoting factor exceeds that of differentiation-driving factors, i.e. the stem cell niches. Otherwise all stem cells would start to differentiate and no stem cell niches would exist. Including stage XII in the coordinates of the testicular stem cell niche adds a molecular definition to its location. Stages of the seminiferous epithelium are of molecular nature and cannot be given any anatomical coordinates since their location is constantly changing as the cycle progresses. Taken together, our data and that of others propose that A_s spermatogonia are enriched in stage XII seminiferous tubules that are under the influence of relatively high concentration of blood-born (such as FSH) or interstitium-derived factors (such as CSF-1), and where the relative concentration of stem cell differentiation-promoting factors (such as SCF) is low.

6.10 The role of GDNF in the testis stem cell niche

The role of GDNF in the maintenance of spermatogonial population was originally described by Meng and colleagues (2000). They noticed that *Gdnf*-deficient mice are infertile due to lack of germ cells in the adult mouse. It was concluded that without GDNF stem cells escape self-renewal and enter differentiation, which explains why only Sertoli cells exist in the adult mouse seminiferous tubules. In transgenic mice that over-express GDNF, accumulation of spermatogonia can be observed in the elderly mice. This is thought to result from excess of self-renewal-promoting factors (GDNF) and authors considered this an animal model of human seminoma (Meng et al. 2000), although the cells appeared rather spermatogonia than seminoma

cells. The beneficial effect of GDNF on survival of undifferentiated spermatogonia *in vitro* has been recognized in numerous studies, including ours. Including GDNF in the SSC culture medium is a standard procedure in Takashi Shinohara's and Ralph Brinster's laboratories, the two leading groups in SSC culture.

In spite of these facts there are many reasons to suspect the role of GDNF as a one of the main regulators of SSC self-renewal. Firstly, selection of GFR1α or RET positive cells (GFR1α and RET constitute the putative GDNF receptor complex) does not result in enrichment of SSC potential in transplantation assays (Ebata et al. 2005, Grisanti et al. 2009). Secondly, there is GDNF-independent SSC-intrinsic signalling that regulates their self-renewal, like the ones where transcription factors PLZF (Buaas et al. 2004, Costoya et al. 2004) and Taf4b are involved (Falender et al. 2005). GDNF does not regulate PLZF of TAF4b expression (Oatley et al. 2006). In our experiments *Nanog* mRNA levels were never affected in the presence of elevated GDNF *in vivo* or *in vitro*.

Gdnf has been considered a FSH-regulated gene in the Sertoli cells (Tadokoro et al. 2002). FSH also elevated the steady state levels of *Gdnf* mRNA in our mouse seminiferous tubule cell co-culture model. However, when rat seminiferous tubules were cultured in the presence of FSH, *Gdnf* levels were suppressed specifically in stages II-VI of the rat seminiferous epithelial cycle. Since *Scf* levels were elevated simultaneously - confirming that the treatment had worked (Yan et al. 1999) – it is hard to consider this data artefactual. What might then explain the opposite results? Could it be the use of two different rodent species? Hardly. Could it be the experimental setting? Probably. Even though all experiments where FSH regulation of *Gdnf* gene expression has been studied are *in vitro* studies, seminiferous tubule culture mimics the *in vivo* situation better than the others in short-term experiments. In our mouse seminiferous tubule cell co-culture model and in the study of Tadokoro et al. (2002) Sertoli cells were exposed to FSH (10, or 20 and 200 ng/ml, respectively) directly, whereas in seminiferous tubule culture FSH (10 ng/ml) in the culture medium has to penetrate many layers of peritubular cells to reach its receptor in Sertoli cells. Therefore the FSH concentration to which Sertoli cells are exposed is different in each of the experimental settings. Further studies are needed to elucidate whether the FSH effect on *Gdnf* steady state levels is dose-dependent.

The results of Kanatsu-Shinohara et al. (2004B) bring an interesting perspective to the discussion about the role of GDNF in the regulation of SSCs. They found that inhibition of GnRH release during postnatal development in mice impairs SSC proliferation, whereas in adult males SSC proliferation is increased when GnRH is suppressed. These data imply that when there is shortage of FSH – and probably also GDNF if FSH induces GDNF production *in vivo* - SSCs are more likely to undergo self-renewal than differentiation in the adult mouse. Taken together, the contemporary understanding about the role of GDNF in testis stem cell niche is probably exaggerated and there has to be factors that act on predecessors of GDNF target cells.

6.11 The role of PLZF in the testis stem cell niche

If GDNF has been considered the most important SSC-extrinsic regulator, PLZF has had a similar status SSC-intrinsically. Therefore it is surprising that no direct link between these two has been established. On the other hand it might just reflect the complexity of the situation *in vivo*. At the same time it raises the question whether the original model of Oakberg (1971) about spermatogonial cell expansion in the mouse can be considered truthful. In numerous *in vitro* and

in vivo experiments spermatogonia have displayed such plasticity that one-way cell commitment-based differentiation pathway can hardly reflect the *in vivo* situation and spermatogonia can adopt a fate that is largely dictated cell-extrinsically.

PLZF is expressed by a relatively large number of spermatogonia. In our studies the number of PLZF positive cells was 18-fold when compared to the number of Nanog-expressing cells. Based on protein and mRNA level data PLZF-expressing cells were divided quite uniformly in different stages of the mouse seminiferous epithelial cycle. X-irradiation seemed to impinge on *PLZF* levels within the first 24 hours but the steady state levels of *PLZF* mRNA were statistically elevated 96 and 144 hours after X-irradiation. Interestingly, *Gdnf* levels were acutely elevated by X-irradiation. These findings suggest that GDNF is involved in inducing cell proliferation to resolve the massive cell loss caused by X-irradiation and support the hypothesis that GDNF acts on predecessors of PLZF positive cells. PLZF has been shown to directly repress transcription of *c-Kit* in spermatogonia thus impeding their entry into differentiation (Filipponi et al. 2007), which might be another indication about the role of PLZF in progenitor rather than stem spermatogonia.

6.12 Future prospects of testis (stem cell) research

Testis is a complicated cellular environment where many different cellular events take place at the same time. All the possible cellular processes seem to be competing furiously: to self-renew or to differentiate, to differentiate or to dedifferentiate, to survive or to die. The complex and often indirect regulation of these events hardly makes it easier to understand the entity. Generally speaking it is probably safe to say that stem cell and cancer aspects will stay in the focus of testis research in the future.

An answer never stops questioning but on the contrary poses many new questions to be answered. The testis continues to stand up for itself as an intriguing environment to study intercellular interaction. The question whether the Nanog-expressing cells truly possess pluripotent capacity remains unsolved. The expression of Nanog and Oct4 alone is not sufficient to state that the cells are pluripotent. On the other, to my knowledge, there are not any examples of cells that would express these pluripotency-associated proteins but would lack pluripotent capacity. Proteins can be regarded as tools or building material which allow the cell to accomplish different kind of tasks. Expression of pluripotency-associated proteins equips the cell with an access to the genetic library that allows the cell to retain its ability to differentiate into a group of different cell types and to provide this aptitude to its progeny.

The rodent model of germ cell propagation and differentiation that is described in Figure 4 in the Review of the literature was originally developed in the early 1970s by Oakberg (Oakberg 1971) and Huckins (Huckins 1971) and underwent slight modifications three decades later (de Rooij & Russell 2000). It is easy to say that this model has stood the test of time surprisingly well and only until the beginning of this decade the static understanding of the cell fate and differentiation that it portrays has been seriously questioned (Nakagawa et al. 2010). It is increasingly difficult to explain some of the scientific findings on the basis of this classical one-way model and more data suggest that spermatogonial cells within the seminiferous epithelium are indeed a dynamic population of cells whose fate is dictated preferably by interaction with the somatic environment instead of a cell-intrinsic program. Based on Review of the literature,

gain of c-Kit, and ability to respond to Sertoli cell-derived SCF signal, that it endows, seems to be a watershed in germ cell development. In this respect, the classical division of spermatogonia into two categories, i.e. undifferentiated and differentiating spermatogonia, is still valid. The undifferentiated spermatogonia possess plasticity which enables them to dedifferentiate or quickly enter differentiation if that is what is required.

Based on the results and conclusions of this thesis and previous reports from others, a new testicular stem cell model is presented in Figure 7. Nanog and Oct4-expressing A-single (A_s) spermatogonia are likely to enter mitosis in stage XII of the mouse seminiferous epithelial cycle. The progeny of this cell division either retain the stem cell capacity or fail to complete cytokinesis. In the latter case A-paired cells are formed. These cells divide to produce a group of 4-16 aligned and interconnected cells, called A-aligned (A_{al}) spermatogonia. Further development of these cell populations is dictated in stages I-VI: In A_s spermatogonia activation of p21 leads to down-regulation of Nanog and Oct4 and cellular quiescence. In A_{al} spermatogonia Sertoli cell-derived GDNF stimulus activates Ras signalling and PLZF expression via Gfr1α binding. These cells and their predecessors are able to undergo self-renewal as well as dedifferentiation under certain circumstances (Nakagawa et al. 2010). A_{al} cells transform into A_1 spermatogonia which can respond to SCF from Sertoli cell owing to the presence of c-KIT in their plasma membrane. A_1 spermatogonia represent the first generation of differentiating spermatogonia that undergo a succession of mitotic divisions to finally produce type B spermatogonia in which retinoic acid (RA) from the Sertoli cells activates the expression Stra8. Stra8 is a prerequisite for the initiation of meiosis (Anderson et al. 2008).

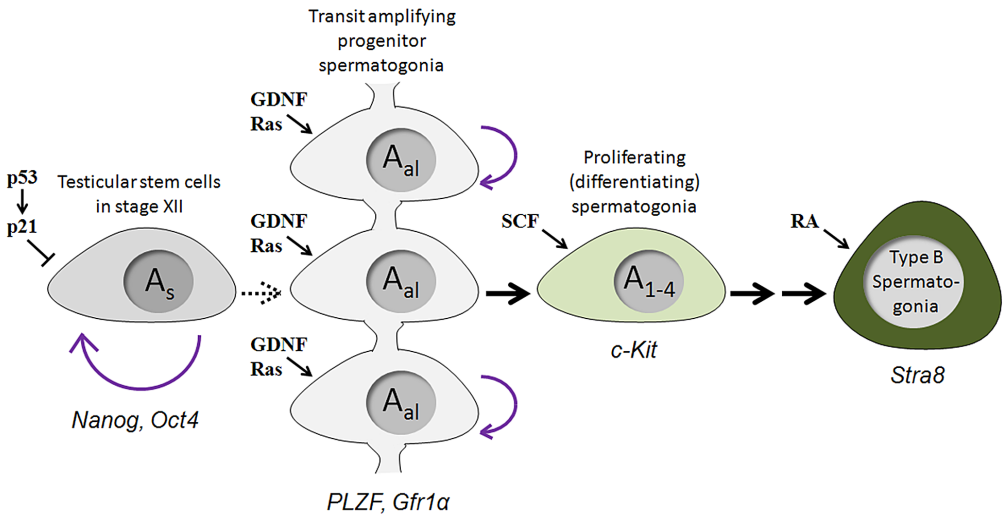

Figure 7. *New testicular stem cell model based on the results and conclusions of this thesis and previous work by others. A-single (A_s) spermatogonia (Nanog and Oct4-expressing cells) divide in stage XII of the mouse seminiferous epithelial cycle and produce either new A_s cells (purple curved arrow) or A_{al} cells (via A_{pr} step that is not included in this figure; black dotted arrow). Upregulation of p21 in stages I-VI results in suppression of Nanog/Oct4 signalling and induction of stem cell quiescence. PLZF and Gfr1α-expressing A-aligned (A_{al}) transit amplifying progenitor spermatogonial cells are stimulated by GDNF in stages I-VI. These cells either self-renew (purple curved arrow) or differentiate into differentiating spermatogonia (A_{1-4}) (black arrow). A_{1-4} spermatogonia undergo a succession of mitoses. They are under SCF regulation due to expression of c-KIT. Finally, type B spermatogonia, the last generation of mitotic cells, are produced. Sertoli cell-derived retinoic acid (RA) activates the expression of Stra8 which is needed in the meiotic entry.*

If our hypothesis holds true and Nanog positive cells in stage XII represent the most primitive and least committed cell type in the adult mammalian testis with putative pluripotent capacity, one question stands out: How to isolate these cells? There are two obvious approaches. Either a surface antigen that is co-expressed with Nanog should be identified and used in cell sorting or alternative means have to be employed to pick the Nanog positive cells. To address this question the stage-dependent steady state levels of *Thy-1, α6-integrin (Itga6), Epcam* (epithelial cell adhesion molecule), *Klf4* and *Lin28* mRNAs were studied. These markers have been successfully used in previous studies to isolate SSCs (Shinohara et al. 2000, Kubota et al. 2004, Ryu et al. 2004) or in the induction of pluripotent cells from somatic cells (Takahashi & Yamanaka 2006, Yu et al. 2007). However, none of these mRNAs shared the pattern of Nanog in the three pooled stages of the mouse seminiferous epithelial cycle (Figure 8A). *Epcam* mRNA levels reached the highest values in stages IX-XII (similar to Nanog) but the differences between the stages were modest and the relative mRNA levels of Epcam grossly exceeded those of Nanog as shown in Figure 8B. In Figure 8B the relative transcript presence of *Stra8, Epcam, CD9, Itga6, Thy-1, Oct4* and *Nanog* in adult mouse seminiferous tubules and mESCs was compared. These data clearly indicate that *Nanog* levels in the seminiferous tubules are low and clearly lower than those of other studied genes. These findings suggest that use of Stra8, Epcam, CD9, Itga6, Thy-1, or Oct4 in the enrichment of Nanog-expressing cells will not be a successful strategy.

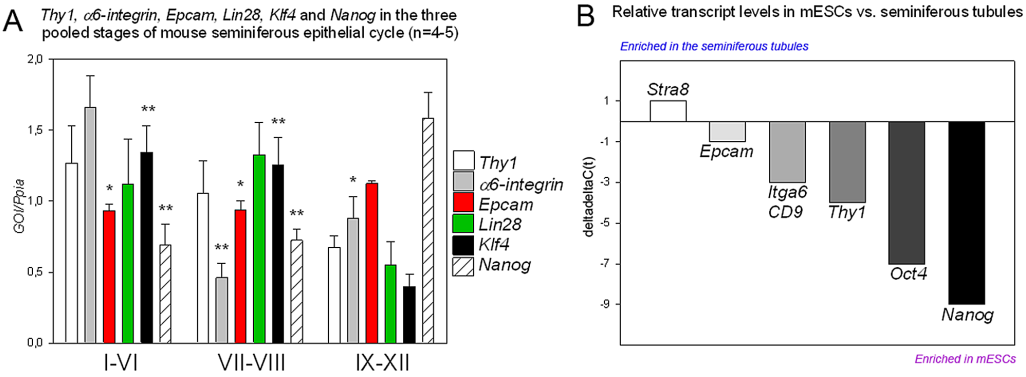

Figure 8. *Testicular Nanog mRNA expression shows two unique features: low steady state levels and stage IX-XII specificity.* ***A)*** *Nanog and Epcam display the highest mRNA levels in stage IX-XII of the mouse seminiferous epithelial cycle. GOI=gene of interest.* ***B)*** *When transcript levels of multiple genes, that have been used in the derivation of ES cell-like cells from germ cells, were studied in adult mouse seminiferous tubule cell homogenate and mESCs, Nanog showed the highest enrichment in mESCs indicating that the Nanog mRNA steady state levels in the seminiferous tubules are remarkably lower that the rest of the studied mRNAs.*

One alternative approach would be the use of transgenic Nanog eGFP-reporter mice (Chambers et al. 2007). Selection of eGFP positive cells might result in enrichment of Nanog-expressing cells. However, there is a cause of concern whether the construct (or similar constructs, as in Arnold et al. 2011) works in a desired manner, as indicated by the report of Kuijk and colleagues (Kuijk et al. 2010). In the testis and germ cells genetic constructs often exhibit undesirable features and fail to follow the wishes of the bioengineer who designed them. This kind of phenomenon was also observed in the CIP2A study. Taken together, stage XII specific isolation of Nanog-expressing cells is the most promising approach for future studies of these cells.

7. SUMMARY AND CONCLUSION

The present study was conducted to better understand the basic biology of spermatogonial cells. Three different approaches were used to gain new insights into the complex process of spermatogenesis with an emphasis on the spermatogonial cells. The main conclusions that can be drawn from the present study are, as follows:

1. Hedgehog signalling functions in the rat testis at three different levels: in mitotic, meiotic and late post-meiotic cells. The pathway is specifically suppressed in early elongating spermatids. One of the physiological functions of testicular Hedgehog signalling is to promote germ cell survival. Hedgehog signalling is regulated in the rat testis by endocrine (FSH) and paracrine (RTK-dependent) factors.

2. CIP2A is a novel C/T gene, the physiological function of which is to support cell proliferation and progenitor cell self-renewal. It is needed in the testis to maintain quantitatively normal spermatogenesis.

3. Adult mammalian testis houses a small population of putatively pluripotent Nanog-expressing spermatogonia.

4. Testicular Nanog-expressing spermatogonia and ES cells share many aspects of their regulatory network. These characteristics make them an attractive source of pluripotent cells for regenerative medicine, and support the hypothesis that Nanog-expressing spermatogonia represent PGC population in the adult.

5. Spontaneous proliferation and migration of mouse seminiferous tubule cells can be exploited to establish a long-term co-culture of seminiferous tubule cells. This system allows all the constituents of the testis stem cell niche to be maintained in an environment that mimics the *in vivo* situation as closely as possible. This method also allows *in vitro* studies on the extremely rare solitary Nanog-expressing spermatogonia.

8. ACKNOWLEDGEMENTS

The thesis work was carried out at the Department of Physiology, Institute of Biomedicine, University of Turku, during the years 2006-2012.

My deepest gratitude goes to my supervisor Professor Jorma Toppari, MD, PhD, Chairman of the Department, for giving me the chance to work at the Department which is technically and scientifically up-to-date. I highly appreciate the freedom that you have given me during the past years and allowed me to find my own way via trial and error and occasional success. Your patience, financial support, encouragement and trust have made this thesis possible. I also want to thank you for the opportunity to be involved in teaching medical students.

My sincerest thanks go to my mentor Sami Ventelä, MD, PhD, for his inspiration, far-sighted scientific approach, and helping me to see the big picture. If our collaboration had never started, these years would have been a solitary march through the desert with the hedgehog. Our collaboration has enormously widened my understanding about what science is all about. I also want to thank you for bringing me into contact with a bunch excellent scientists and collaborators. I sincerely hope that we can continue the fruitful collaboration that we have had this far.

I am thankful to the reviewers of this thesis, Professor Outi Hovatta, MD, PhD, and Professor Stefan Schlatt, PhD, for careful and critical review. Your comments and suggestions improved the quality of the thesis, helped to evaluate the potential clinical relevance of the presented work, and deepened my understanding about mammalian stem cell dynamics.

I wish to sincerely thank the grand-old-man of Finnish testis research Professor Emeritus Martti Parvinen, MD, PhD, for his encouragement, support, technical help, collaboration and co-authorship. I also want express my gratitude to Professor Matti Poutanen, PhD, Professor Ilpo Huhtaniemi, MD, PhD, Professor Emeritus Lauri Pelliniemi, MD, PhD and Matti Vierula, PhD. Your kind words, comments, criticism and support are highly appreciated. I especially wish to acknowledge Jorma Paranko, PhD, for introducing me into scientific research and helping me to take my first steps as a young scientist. I also appreciate your support and willingness to help in various matters. Every story has a beginning. I have had the chance to be a student of many inspiring and supportive teachers of which Päivi Pere-Vieno, MSc, Pirkko Jokisalo, MSc and Janne Liikanen, MSc deserve to be mentioned here.

My special thanks are due to my co-authors. I especially want to thank Sonia Bourguiba-Hachemi, PhD, Vuokko Saario, BM and Professor Jukka Westermarck, MD, PhD. I also wish to acknowledge Leni Kauko, MSc, Christophe Comé, PhD, Mirja Nurmio, PhD, Kirsi Jahnukainen, MD, PhD, Jarmo Kulmala, PhD, Robin M. Hobbs, PhD, Markku Kallajoki, MD, PhD, Professor Edward K. Chan, PhD and Professor Pier Paolo Pandolfi, MD, PhD.

I am thankful to the members of my thesis committee: Noora Kotaja, PhD, Professor Hannu Sariola, MD, PhD and Professor Jussi Taipale, PhD, for comments, criticism and suggestions about my research. During the past five years I have had the privilege to belong to the Turku Doctoral Programme of Biomedical Sciences (TuBS). I wish to thank Professor Olli Lassila, MD, PhD, Mrs.

Nina Widberg, Susanna Rosenberg, MSc, and TuBS organizing committee and members for all the scientific events and social activity that I have been entitled to participate and contribute.

I am thankful to all the friends and colleagues that I have been privileged to teach with: Jaakko Kytölä, MD, Juha Koskenvuo, MD, PhD, Kirsti Ellonen MSc, Susanna Vuorenoja, MD, PhD, Helena Virtanen, MD, PhD, Pia Salo, MD, PhD, Nafis Rahman, MD, PhD, Professor Pekka Kääpä, MD, PhD, Pirjo Pakarinen, PhD, Professor Markku Ahotupa, PhD and Aino Rönnblad, MD, PhD. I am deeply indebted to my friends and colleagues Oliver Meikar, MSc, and Matteo Da Ros, MSc. You are indeed very agreeable gentlemen. Our sometimes a bit prolonged conversations over the lunch have nourished me both physically and mentally. I want to thank the friendly and understanding people at Turun Upseerikerho for providing pleasant atmosphere and the food that has literally made the work possible.

The help that I have received in the lab from Mrs. Hannele Rekola, Mrs. Eila Virransuo, Mrs. Taija Leinonen, Mrs. Taina Kirjonen, Mrs. Jonna Palmu, Ms. Mona Laaksonen and Ms. Erika Nyman is kindly acknowledged. I am grateful to the personnel at the Animal Facility of the University of Turku for their flexibility and good animal care. Mrs. Nina Messner, Mrs. Erja Mäntysalo, Mrs. Heli Niittymäki and Mrs. Katri Hovirinta are acknowledged for their help with the experimental animals. I have been privileged to carry out this thesis work at the Department of Physiology in a friendly and supportive atmosphere that has been created by: Annika Adamsson, PhD, Petteri Ahtiainen, MD, PhD, Ida Björkgren, MSc, Marcin Chrusciel, PhD, Anastasios Damdimopoulos, PhD, Kame El-Darwish, PhD, Antti Ellonen, BM, Heidi Gerke, MSc, Janne Hakkarainen, MSc, Kaisa Huhtinen, PhD, Veera Hämäläinen, MSc, Mrs. Liubov Hännikäinen, Jenni Jalkanen, MD, PhD, Min Jiang, MD, PhD, Heli Jokela, MSc, Päivi Järvensivu, MSc, Mrs. Johanna Järvi, Jenny Kallio, MSc, Mrs. Minna Kangasperko, Tero Karhi, MSc, Jukka Kero, MD, PhD, Matias Knuuttila, MSc, Hanna Korhonen, MSc, Jaakko Koskenniemi, BM, Heidi Lagerbohm, BM, Tarja Lamminen, PhD, Mari Lehti, MSc, Mrs. Merja Leppiaho-Vallinhovi, Heidi Liljenbäck, MSc, Ramin Lindqvist, PhD, Ilkka Martikainen, MD, PhD, Katja Mäkelä, MSc, Antti Perheentupa, MD, PhD, Jenni Paviala, MSc, Ram Prakash Yadav, MSc, Dwi Ari Pujianto, PhD, Pia Rantakari, PhD, Pia Roering, MSc, Sergey Sadov, MD, Anu Salminen, MSc, Taija Saloniemi, PhD, Petra Sipilä, PhD, Anu Sironen, PhD, Leena Strauss, PhD, Ms. Katja Suomi, Ms. Maire Tohv, Ashutosh Trehan, MSc, Heikki Turunen, PhD, Henriette Undeutsch, MSc, Kaarle Vaahtoranta, Fuping Zhang, PhD, Shen Zhongyi, PhD and all the other people that I acknowledge elsewhere or have forgotten to mention. I especially wish to thank Tuula Hämäläinen, PhD, Mrs. Anneli Vesa, Marko Tirri, MSc, Veronika Mamaeva, MD, PhD, Adolfo Rivero-Müller, PhD, Emmi Rotgers, MD, Mrs. Leena Karlsson and Mrs. Marjatta Keistinen for conversations and collaboration over the past years.

My parents, Eila and Antti, I am very thankful to you for the support that you have given me over the years. Without your encouragement and trust I probably would not have strived for higher achievements. Thank you sowing the seed of sound competition and willingness to go for better grades and success in life. Tanja and Jarkko, I want to thank you for the supportive and challenging environment in which we have grown together. My parents-in-law, Anna-Mari and Martti, I want to express my gratitude for accepting me as a part of the family and providing an excellent example of family life. Your contribution to the lives of your children and grandchildren will echo for the generations to come. My aunt and uncle, Marjatta and Matti, I wish to thank you for all the support you have given me and my family. Getting to know you personally has greatly enriched our lives. What would life be without friends? I have been privileged to

spend time with the finest of people and I especially wish to express my gratitude to Pekka and Tiina, and Johannes and Elisa.

My beloved wife, Elli-Maija, words cannot express how deeply I value the support and sacrifices that you have made for me during these years. If it weren´t for your commitment to our family, I would have never been able to complete the work. I admire the way you make things happen. I am indebted to my children Aukust, Vanamo and Aleksis for bringing my life into perspective and helping me realize that the most important things in life are given, not achieved.

To me, and supposedly to many others as well, the beauty in doing science lies in the bittersweet success after multiple rounds of trial and error. Testing new hypotheses is always very exciting. Sometimes it is even too easy to get lost in the jungle of new ideas and thoughts, experiments and approaches. However, it rarely helps the struggle of finding the way out if you decide to stick to the plan. Doctoral thesis is a labyrinth. No matter how attractive some turns and corners might seem, at some point you have to choose the way that most probably leads you out. Science makes a difference. Science makes change possible. And the very science is made by millions of pairs of hands every day. It is crucially important to understand that the progress and future of mankind lies in the hands of those who are trying to unveil the miracles of nature.

This study was financially supported by grants from the Sigrid Jusélius Foundation, Finnish Cultural Foundation and Emil Aaltonen Foundation. SDG.

Turku, May 2012

Juho-Antti Mäkelä

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